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PRINCIPAL INVESTIGATOR: Qing, Chen Ph.D., M.D.

CONTRACTING ORGANIZATION: Health Research Inc
Buffalo NY 14263

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Qing, Chen Ph.D., M.D.

E-Mail: qing.chen@roswellpark.org

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14. ABSTRACT:
The tumor microvasculature is a barrier to immunotherapy because of its failure to express adhesion molecules necessary for recruitment of tumor-reactive lymphocytes. Immune cells are frequently excluded from the intratumoral region of primary tumors including breast cancer. Our studies demonstrate that fever-range thermal therapy increases lymphocyte trafficking selectively in tumor sites and secondary lymphoid organs, but not in extralymphoid organs. Intravital microscopy studies and short term homing assays allow us to observe thermal enhanced lymphocyte-endothelial interactions in vivo. Underlying mechanism was detected focusing on the trafficking molecules which control egress of blood-borne lymphocytes into tissues. Heat treatment enhanced intravascular expression of intercellular adhesion molecule-1 (ICAM-1) in tumor microvessels and high endothelial venules in lymphoid organs. This induced ICAM-1 expression is functional linked with thermal activated lymphocyte-endothelial interaction and lymphocyte homing. Neutralization of proinflammatory cytokines IL-6, but not TNF or IL-1 beta, suppresses thermal induction of ICAM-1-dependent lymphocyte recruitment. Soluble gp130 also prevented ICAM-1 induction, indicating that thermal activities in vascular targets are dependent on an IL-6 trans-signaling mechanism. These results support the hypothesis that IL-6-dependent signaling mechanisms activate tumor immunity through stimulation of heightened trafficking of lymphocyte subsets to tumor sites and lymphoid organs during fever-range thermal therapy.

15. SUBJECT TERMS
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INTRODUCTION

Clinical studies have documented the presence of dense accumulations of immune inflammatory cells in the peritumoral region immediately surrounding human primary tumors including breast cancer. This phenomenon provides direct evidence of a host immune response at tumor sites. However, immune effector cells such as $CD8^+$ T cells are frequently excluded from intratumoral region and thus, are incapable of initiating contact-dependent lysis of tumor targets. Thus, a major impediment to successful immunotherapy is the failure of the appropriate immune cells to gain access to tumors. A vital challenge is to develop novel approaches to target delivery of tumor-specific immune effector cells to tumor tissues. Our laboratory is exploring the role of fever-range thermal therapy in controlling lymphocyte trafficking. We postulate that stimulation of lymphocyte-endothelial adhesion in tumor vessels will culminate in significant anti-tumor immune activity as a result of increased recruitment of tumor-specific cytotoxic T lymphocytes (CTL) to tumors. The proinflammatory cytokine, interleukin-6 (IL-6) is further hypothesized to be the key regulator of thermally activated adhesion events in breast tumor microvessels. Studies proposed in this grant investigated the hypothesis that IL-6 mediates thermal activation of lymphocyte adhesion to tumor vessels in the complex microenvironment of breast cancer tissues. Studies performed during the funding period of the DOD predoctoral fellowship support the long term goal of using thermal therapy as an adjuvant to T cell-based immunotherapy for the treatment of breast cancer. The aims have not changed from the original proposal.

BODY

AIM 1: To determine if fever-range thermal stress stimulates adhesion of immune effector cells to breast tumor microvessels via a mechanism that depends on IL-6 or other proinflammatory cytokines (TNF, IL-1β, IFN-γ). A major barrier to anti-tumor immunity is the failure of tumor-reactive $CD8^+$ cytolytic lymphocytes to gain access to tumor tissues. This deficiency is associated with a poor prognosis in cancer patients. In studies published during the funding period, we have shown that poor infiltration of tumor tissues in murine models correlates with limited expression of critical gatekeeper adhesion molecules in vascular endothelial cells such as intercellular adhesion molecule-1 (ICAM-1) which are known to control delivery of blood-borne lymphocytes into tissues (1-5).

In follow-up studies we have determined that elevating the core body temperature to the range of naturally occurring fever (39.5-40°C) for 2 to 6 hours by whole body hypethermia (WBH) treatment increases lymphocyte adhesion and trafficking. Three distinct vascular beds were compared in terms of basal levels and thermal regulation of lymphocyte trafficking: 1) specialized high endothelial venules (HEVs) in lymphoid organs (i.e., lymph nodes [LNs] and Peyer's Patches; 2) non-activated vessels in extralymphoid organs (i.e., pancreas, liver, lung, heart, kidney); and 3) tumor microvessels. Compared with HEVs, which efficiently support lymphocyte recruitment, both non-activated vessels and tumor microvessels express low levels

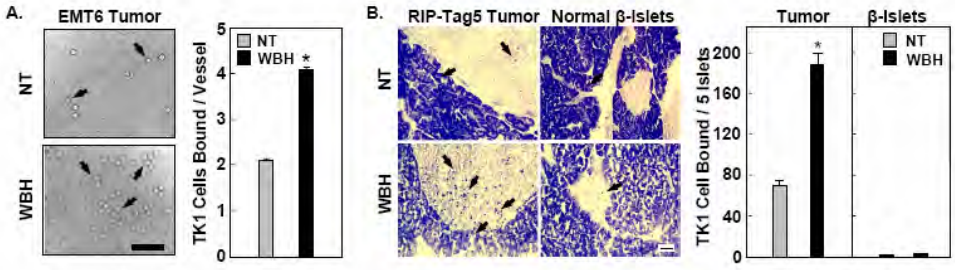


Figure 1. Fever-range thermal stress selectively stimulates lymphocyte adhesion to intratumoral vessels. Adherence of $CD8^+$ murine TK1 T cells to intratumoral vessels was evaluated in tissue cryosections from normothermal control (NT) and whole body hyperthermia (WBH)-treated mice. ***(A),*** The number of adherent $CD8^+$ T cells were quantified and calculated in more than 100 intratumoral vessels of EMT6 tumors. ***(B),*** Adherent $CD8^+$ T cells were quantified in islet tumors or normal islets of RIP-Tag5 pancreas. For consistency in double-blind evaluation, intratumoral vessels were quantified only if they contained ≥ 1 adherent cell. Photomicrographs show typical images. Scale bar, 50 μm. Data are the mean ± SE and are representative of ≥ 3 independent experiments. *P* values represent the difference between normothermal control and WBH treatment (*, $P < 0.001$).

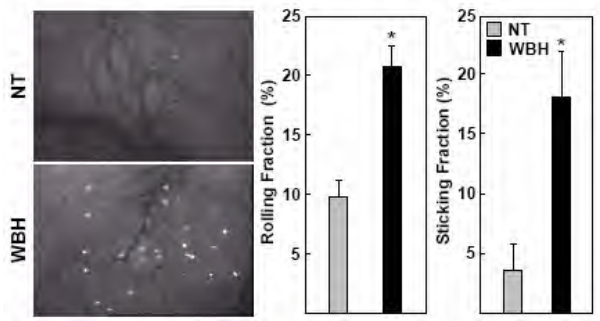


Figure 2. Fever-range thermal therapy enhances interactions between CD8$^+$ T cells and tumor microvessels. Interactions between calcein-labeled CD8$^+$ T cells with tumor vessels were assessed by intravital microscopy in CT26 tumors implanted in dorsal skin-flap window chambers. The rolling fraction was defined as the percentage of total cells passing through the vessel that transiently interact with vascular endothelial cells during the observation period. The sticking fraction was defined as the percentage of rolling cells that adhered to vessel walls for ≥ 30 s. For rolling and sticking fractions, data were generated from 3 NT control mice and 3 WBH-treated mice. Thermal therapy induced a ~2-fold increase in the rolling fraction and a ~5-fold increase in the sticking fraction. Photomicrographs show typical images at the end of observation under each condition. *P* values represent the difference between NT control and WBH treatment (*, $P < 0.03$).

of trafficking molecules and have low levels of lymphocyte trafficking (3, 4). Several approaches were taken to detect thermal effects on vascular endothelial cells to promote lymphocyte-endothelial interactions. Lymphocyte adhesion was first detected in tissues harvested from animals with or without heat treatment using an *in vitro* frozen-section adherence assay. In these studies, WBH treatment enhanced CD8$^+$ lymphocyte adhesion in peripheral LN (PLN) HEVs (6) as well as in tumor vessels in transplantable EMT6 breast tumors (Figure 1A) as well as in R3230 rat mammary tumors (7, 8). Similar enhancement of CD8$^+$ T cell adhesion was observed in additional histological tumor types including RIP-Tag5 transgenic pancreatic tumors (Figure 1B), Colon 26 (CT26) tumors, and B16 melanoma (7-16). In contrast to the pro-adhesive thermal effects on HEVs and tumor microvessels, heat treatment had no effect on lymphocyte adhesion in non-activated vessels in extralymphoid organs (e.g., pancreatic β-islets) (Figure 1B) (6-16). Furthermore, intravital microscopy (IVM) was employed to observe, in real time, the interactions between circulating lymphocytes and endothelial cells. In secondary lymphoid organs, WBH-treatment increased secondary firm adhesion of lymphocytes without changing primary tethering and rolling interactions with HEVs (6). IVM studies also confirmed that thermal therapy has no effect on the adhesive properties of non-activated vessels (6). In tumors, IVM observations indicated that in compared to the efficient lymphocyte adhesion in HEVs under steady state condition, CD8$^+$ T cells interact in frequently with tumor microvessles (6-16). Our most recent data demonstrated that thermal stress activated endothelial cells lining tumor microvessels to enhance both primary tethering and rolling and secondary firm adhesion events (Figure 2) (7-16). Of particular note was was that, compared with lymphoid organ HEVs, a greater increase in CD8$^+$ lymphocyte trafficking (~5 fold) was detected in tumor microvessels after heat treatment (7-16). Short-term (1 hour) *in vivo* homing assays further demonstrated a ~2 fold increase in CD8$^+$ T cell trafficking in lymphoid organs in response to thermal therapy, while heat treatment did not change the low level of lymphocyte trafficking observed in extralymphpoid organs (6). In addition, immunohistochemical analysis established that fever-range thermal stress causes an increase in the infiltration of endogenous CD8$^+$ T cells into intratumoral regions (7-16).

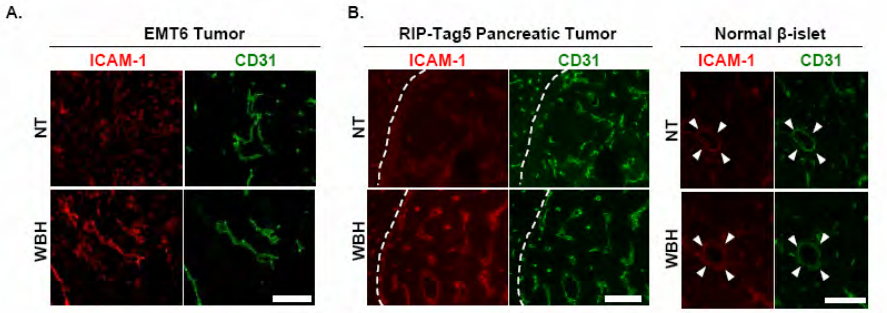


Figure 3. Fever-range thermal stress induces ICAM-1 expression in tumor microvessels. ***(A),*** Microscopic analysis of sections dually stained for ICAM-1 and CD31 revealed that ICAM-1 induction occurred on CD31$^+$ vessels in EMT6 transplanted tumors. ***(B),*** Induction of ICAM-1 expression on the luminal surface of the CD31$^+$ blood vessels of RIP-Tag5 pancreatic tumors was confirmed by intravenous injection of primary ICAM-1 mAb and subsequent staining of tissue sections with TRITC-labeled secondary antibody. Right panels indicate that the thermal stress did not affect ICAM-1 expression in normal islets of RIP-Tag5 pancreas. Photomicrographs show typical images. Scale bar, 50 µm.

We have identified the underlying mechanisms of thermal activation of lymphocyte trafficking at the molecular level. The expression of several trafficking molecules in endothelial cells was determined from the 3 vascular beds enumerated above following heat treatment. Marked induction of ICAM-1 was observed on HEVs of lymphoid organs in response to thermal therapy, whereas no induction of ICAM-1 expression was detected in vessels of non-malignant extralymphoid organs (6). In EMT6 breast tumors, fever-range thermal therapy markedly upregulated expression of ICAM-1 in intratumoral vessels (Figure 3A) (7, 8). Similar ICAM-1 induction was confirmed in all murine tumor models examined including RIP-Tag5 transgenic pancreatic tumors (Figure 3B), CT26-tumors, and B16 melanoma (7-16). Two approaches were taken to establish that ICAM-1 induction occurs specifically on tumor vessels, and not on tumor cells or stromal cells within the tumor microenvironment. Two-color fluorescence confocal microscopy demonstrated that induction of ICAM-1 occurred exclusively on $CD31^+$ vessels within tumor tissues in response to thermal therapy (Figure 3A) (6-16). Moreover, *i.v.* injection of anti-ICAM-1 mAb into WBH-treated mice and subsequent staining of tissue sections with fluorescent-labeled secondary Ab showed that thermal stress upregulates ICAM-1 expression on the lumenal surface of tumor vessels (i.e., at the site necessary to initiate firm adhesion of lymphocytes to vessel walls) (Figure 3B) (6-16).

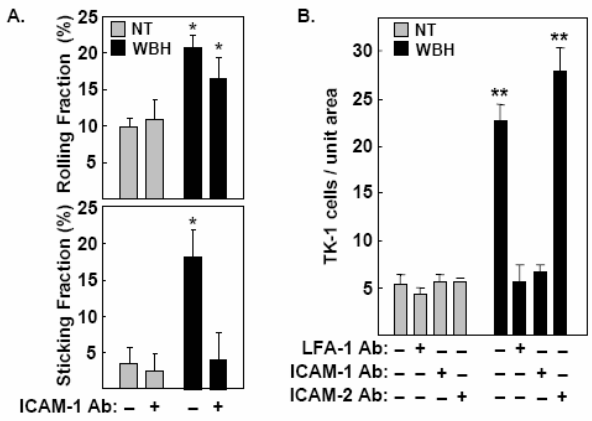


Figure 4. Thermal induction of $CD8^+$ T cell trafficking is ICAM-1 dependent. ***(A),*** Intravital microscopy observations. Functional blocking mAb of ICAM-1 did not selectively inhibited thermally enhanced firm sticking interactions between $CD8^+$ T cell and vessel walls. ***(B),*** Short-term (1 h) homing assays of $CD8^+$ T cell into tumors. WBH treatment induced a ~5-fold increase of $CD8^+$ T cell recruitment into CT26 tumors. The relative contributions of LFA-1 and its cognate receptors, ICAM-1 and ICAM-2, to thermal stimulation of lymphocyte trafficking were examined by either pre-treating $CD8^+$ T cell with LFA-1 function-blocking mAb or the recipient mice with ICAM-1 or ICAM-2 functional blocking mAb prior to adoptive transfer in IVM and short term homing assays. Heat induced $CD8^+$ T cell firm adhesion and homing was inhibited by blocking mAb for LFA-1 ot ICAM-1, but not by ICAM-2 mAb. *P* values represent the difference between NT control and WBH treatment (*. $P < 0.03$: **. $P < 0.0001$).

An important finding is that thermally regulated ICAM-1 is functionally linked to improved lymphocyte-endothelial adhesion and site-specific homing to tumor beds (6-16). Using neutralizing antibody approaches outlined in the application, we demonstrated that thermal induction of HEV adhesion and lymphocytes homing in secondary lymphoid organs were dependent on binding of endothelial ICAM-1 to its lymphocyte counter-receptor, leukocyte function associated antigen-1 (LFA-1) (6-16). ICAM-1 deficient mice were further used to confirm the requirement of ICAM-1 for enhanced lymphocyte trafficking during thermal therapy (6). Our most recent studies have confirmed a role for ICAM-1 in lymphocyte-endothelial interactions and $CD8^+$ lymphocyte trafficking in tumors after WBH treatment. ICAM-1 blocking mAb selectively inhibited enhanced secondary firm adhesion after heat treatment in tumor microvessels (Figure 4A) and also inhibited lymphocyte recruitment into tumor beds induced by thermal stress (Figure 4B) (7-16).

We have performed studies proposed in the application to investigate the role of IL-6 in stimulating the expression of homing molecules. For these studies, experimental mice were injected intravenously with neutralizing antibodies specific for IL-6, TNF, IL-1β or IFN-γ prior to initiation of fever-range WBH treatment. Neutralization of IL-6, but not TNF-α, IL-1β or IFN-γ, fully blocked thermal induction of ICAM-1 expression as well as thermal enhancement of lymphocyte-endothelial adhesion in frozen-section adherence assays (6-16). Thermal enhancement of lymphocyte homing to HEV-bearing organs (LNs, Peyer's patches) was also shown to be dependent on IL-6 while the low-level of trafficking of lymphocytes to extralymphoid organs was not

affected by neutralization of IL-6, TNF, IL-1β or IFN-γ (6-16). The fact that IL-6 deficient (*Il6*$^{-/-}$) mice were refractory to heat treatment confirmed the requirement for IL-6 in thermal regulation of ICAM-1 expression and ICAM-1-dependent lymphocyte trafficking in HEVs of lymphoid organs (6). In recent studies, B16 melanoma tumors in wild type (C57BL/6) and *Il6*$^{-/-}$ mice were used to confirm the non-redundant role of IL-6 in thermal response of tumor microvessels. B16 tumors were used in these studies since they are syngeneic to C57BL/6 background of *Il6*$^{-/-}$ mice. Thermal therapy failed to induce both ICAM-1 expression (Figure 5A) and infiltration of endogenous CD8^{+} T cell into tumors (Figure 5B) were absent in *Il6*$^{-/-}$ mice. To discriminate between the involvements of a membrane (m) versus soluble (s) form of the binding subunit of the IL-6 receptor (IL-6R) in thermal responses, mice were injected with a soluble form of the signaling subunit of the IL-6R, gp130. We and others have established that soluble gp130 functions as a competitive inhibitor of IL-6/sIL-6R trans-signaling (known as trans-signaling), but not of IL-6/mIL-6R signaling *in vitro* and *in vivo*. Blockade of IL-6/sIL-6R trans-signaling with soluble gp130 effectively prevents thermal induction of ICAM-1 expression, lymphocyte-endothelial adhesion, and homing to tumor tissues or to lymphoid organs (6-16). These results provide direct evidence that the gp130 signal transducing subunit of the IL-6R is the critical regulator of tumor microvascular adhesion in response to fever-range thermal stress.

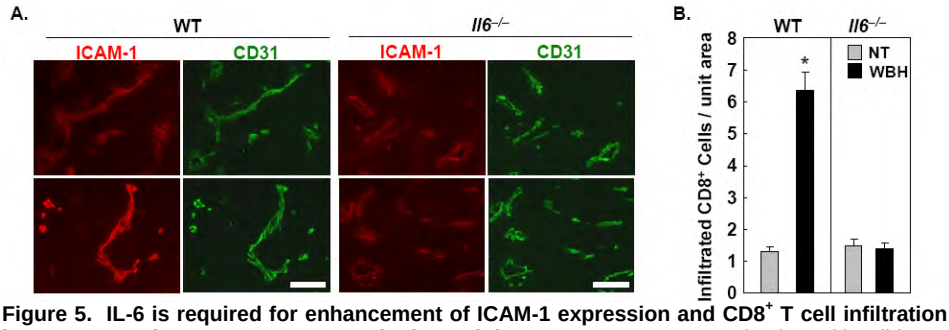


Figure 5. IL-6 is required for enhancement of ICAM-1 expression and CD8^{+} T cell infiltration in B16 tumors in response to systemic thermal therapy. B16 tumors were implanted in wild type (WT) or IL-6 deficient (*Il6*$^{-/-}$) mice. ***(A),*** Photomicrographs show IF staining of ICAM-1 expression in tumor cryosections of NT control and WBH-treated mice. Thermal upregulation of vascular ICAM-1 expression was abolished in IL-6 KO mice. Photomicrographs show typical images. Scale bar, 50 μm. ***(B),*** Tumor infiltration by endogenous CD8^{+} T cells was quantified in tissue sections after immunohistochemical (IHC) staining. Heat induced CD8^{+} T cell infiltration of tumors detected in WT mice was not observed in IL-6 deficient mice. *P* values represent the difference between normothermal control and WBH treatment (*, $P < 0.0001$).

AIM 2: To investigate whether thermal stress increases IL-6 biosynthesis in breast tumor tissue and to identify the cellular source of IL-6 in malignant breast carcinoma in situ. The results from *Aim 1* support our hypothesis that IL-6 is a key regulator of the pro-adhesive effects of fever-range thermal stress on tumor microvessels. Initial studies were done to detect local changes in IL-6 levels in tumor tissues using Luminex assays after heat treatment. Preliminary results suggested that thermal therapy did not alter IL-6 concentrations in whole-tumor lysates or in lymphoid organs at the end of the 6-hour heat treatment (Figure 6) (6, 14). As expected, IL-6 was not detected in tissue lysates from *Il6*$^{-/-}$ mice bearing B16 tumors which express a low level of IL-6 (Figure 7A). In this regard, ICAM-1 expression in B16 tumors from wild type mice is inducible by heat treatment, suggesting that stromal cells in tumor microenvironments are sufficient to support thermal response in tumor microvessels. Larger numbers of samples need to be analyzed due to the variation between samples, especially in the WBH-

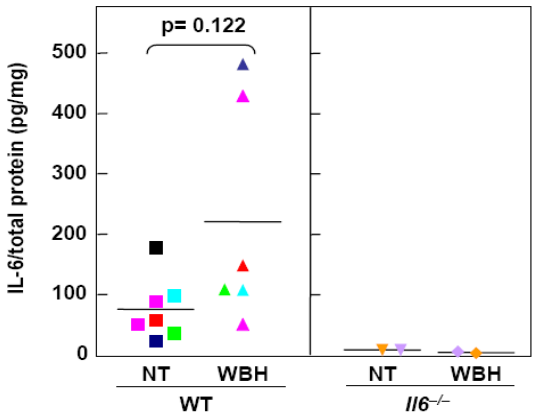


Figure 6. Fever-range WBH treatment does not change IL-6 concentration in tumor tissue lysates. B16 tumors were harvest from wild type (WT) or IL-6 deficient (*Il6*$^{-/-}$) mice after heat treament. IL-6 concentration were detected by Luminex assays and normalized by the total protein concentration of tissue lysate. Data shown represent the amount of IL-6 (pg) in 1 mg of total protein. *P* values represent the difference between normothermal control and WBH treatment. Background level of IL-6 was detected in tumors from IL-6 deficient mice.

treated group. Kinetics studies will also be performed to detect IL-6 levels in tumors at earlier time points during thermal therapy. In follow-up experiments, we aimed to identify the cellular source(s) of IL-6 in tumor microenvironments using approaches outlined in the initial application. Immunohistochemical staining was performed using a commercially available clone of IL-6 mAb (MP5-20F3) that is commonly used in the literature. We selected PLNs as the positive control tissue since we have shown that leukocytes secrete IL-6 under steady-state conditions. IL-6 staining was observed in PLNs, while no staining was detected in isotype-matched Ig controls of the primary IL-6 Ab. A problem arose, however when we set out to confirm these results using PLNs from *Il6*$^{-/-}$ mice. Unexpectedly, we observed immunohistochemical reactivity with our IL-6 Ab in *Il6*$^{-/-}$ mice. Two possible explanations for this result are: 1) the mAb for IL-6 may cross-react with some other molecule sharing the same epitope of IL-6 molecule, and 2) since the *Il6*$^{-/-}$ mice were generated by disrupting exon 2 of IL-6 gene, it is possible that the undisrupted part of the IL-6 transcript is translated and that our IL-6 mAb recognizes some epitope encoded by exon 1 and the initial part of exon 2. To address this issue, we are currently screening a panel of mAb and polyclonal Ab to identify one that reacts positively only in tissues from wild type mice. A complementary approach was used to identify which cellular components in the tumor microenvironment are required for the IL-6-dependent thermal response. We took advantage of the difference in IL-6 production between CT26 cells and B16 cells and compared IL-6 staining in both tumors *in vivo*. In vitro, B16 cells secrete extremely low levels of IL-6 into the culture supernant, in contrast, we detected high levels of IL-6 secreted by CT26 cells in culture (Figure 7A) (14). After immunohistochemical staining of B16 and CT26 tumors for IL-6, we detected positively staining leukocytes and endothelial cells in both tumor systems (Figure 7B). Consistent with IL-6 levels measured in cell culture supernants, CT26 tumor cells stained positive for IL-6 while no staining was observed in B16 tumor cells (Figure 7B) (14). These initial immunohistochemical staining results will be confirmed when a specific Ab that does not react with tissues from *IL6*$^{-/-}$ mice is identified. Since both CT26 (IL-6^{hi}) and B16 (IL-6^{low}) tumors respond to heat to induce vascular ICAM-1 expression (7-16), these data suggest that while tumor cells can be a source of IL-6, stromal cells in tumor microenvironments are sufficient for thermal regulation of ICAM-1. Since one cellular source of IL-6 we observed in tumors was leukocytes, we asked the question whether these cells are required for thermal responses in tumors. We are currently generating bone marrow chimeric mice by transplanting bone marrow cells from *Il6*$^{-/-}$ mice into irradiated wild type mice (and vice-versa) to identify which type(s) of stromal cells (hematopoietic derived, endothelial or fibroblast cells) are the requisite source of IL-6. After all these issues have been resolved using IL-6^{hi} and IL-6^{low} tumors, we will apply the same approaches to analyze cellular source of IL-6 in breast tumor tissues. Concurrently, we are performing pilot experiments using laser-capture microdissection followed by real-time quantitative PCR to evaluate individual cell types for IL-6 production at the mRNA level as an additional approach to detect thermal regulation on IL-6 production in tumor microenvironments.

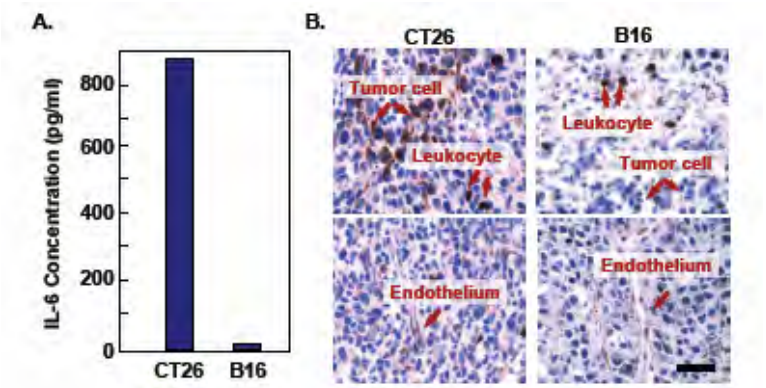


Figure 7. CT26 and B16 tumor models differ in the cellular source(s) of IL-6. *(A),* The amount of IL-6 in culture media of tumor cells determined by Luminex assays. ***(B),*** Immunohistochemical staining of IL-6 in CT26 and B16 tumor tissues harvested from tumor-bearing mice. In CT26 tumor model, IL-6 positive cells (as pointed by arrows) included tumor cells, leukocytes, and endothelial cells. In contrast, in B16 tumor tissues, tumor cells do not produce IL-6; the only IL-6^{+} cells detected were leukocytes and endothelial cells. Photomicrographs show typical images. Scale bar, 50 μm.

KEY RESEARCH ACCOMPLISHMENTS

- Fever-range thermal stress increases adhesion, homing and infiltration of $CD8^+$ T lymphocytes in tumors and HEV-bearing lymphoid tissues.
- Fever-range thermal induction of $CD8^+$ T lymphocyte trafficking is site specific in tumor and secondary lymphoid organs, but not in extralymphoid organs.
- Fever-range thermal therapy induces ICAM-1 expression and function exclusively on tumor vessels and HEV in lymphoid organs.
- These studies provide the first evidence that ICAM-1 is functionally linked with the entry of lymphocytes across tumor microvessels into tumor sites.
- Fever-range thermal stimulation of adhesion in intratumoral vessels is dependent on an IL-6/sIL-6R trans-signaling.

REPORTABLE OUTCOMES

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8. **Chen, Q.,** Fisher D.T., Lei Z., Clancy K.A., Passanese, J., Wang W.C., Unger E., Baumann H., Repasky E.A. and Evans S.S. Role of IL-6 trans-signaling in promoting ICAM-1-dependent T lymphocyte trafficking in the tumor microenvironment during thermal therapy. *(manuscript in preparation)*
9. Chen, Q., Appenheimer, M.M., Muhitch, J.B., Clancy, K.A., Wang W.C. and Evans S.S. Sustained Fever-Range Thermal Stress Is Required for Optimal Induction of ICAM-1-Dependent Lymphocyte Trafficking Across High Endothelial Venules. *(manuscript in preparation)*

Abstracts:

1. **Chen, Q.,** Kucinska, S.A., Wang, W.C., Wallace, P.K., Baumann, H., and Evans, S.S. Fever-range thermal stress stimulates lymphocyte homing receptor function through an interleukin-6-dependent trans-signaling mechanism. 12th International Congress of Immunology. July, 2004; Montreal, Canada.
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***Media Interviews*:**

2006	Work published in Nature Immunol., Dec. 2006, was the subject of > 50 articles in national and international news outlets
2006	H. Ledford, Research highlights – 'Hot-blooded', ***Nature*** 444:126, 2006
2006	J. Kaiser, 'Don't fight the fever', ***ScienceNOW***, Nov. 2006
2006	W. Wells, Research Roundup – 'Fever brings in the lymphocytes', ***Journal of Cell Biology***, vol. 175:678, 2006
2006	A. Coghlan, 'Helpful fevers come in from the cold', New Scientist Nov. 2006
2006	Interview, J. Zak, WBEN Radio, Dec. 2006
2006	Interview, J. Berman, 'Fever may help the body heal itself', Voice of America Radio, Nov. 2006
2007	J. Ruvinsky, Story in ***Discover Magazine***, March 2007
2007	Story: J. Wettlaufer, 'Molecules, crystals, and robots: biosphere Buffalo, ***Buffalo Spree***, Jan. 2007

Awards:

2005	**NIAID Scholarship,** Keystone Symposia, Leukocyte Trafficking: Cellular and Molecular Mechanisms
2005	**Travel Award,** 2005 Annual Meeting of Society for Thermal Medicine
2005	**Best Presentation, Graduate Student Seminar Award**, Department of Immunology, Roswell Park Cancer Institute
2006	**NIAID Scholarship,** Keystone Symposia, Chemokines and Chemokine Receptors
2006	**New Investigator Award,** 2006 Annual Meeting of Society for Thermal Medicine

2006 **Roswell Park Dean's Award,** for outstanding dissertation research in Immunology, Roswell Park Division, State University of New York at Buffalo

2007 **Keystone Symposia Education Fund Scholarship,** Keystone Symposium, Mechanisms Linking Inflammation and Cancer

Training:

02-01-2004 ~ 02-07-2004 Attended training course and participated in collaborative studies involving intravital microscopy techniques in the laboratory of Dr. Mark Dewhirst, Duke U. Medical School, Durham, NC.

04-04-2004 ~ 24-04-2004 Participated in collaborative studies involving intravital microscopy techniques in the laboratory of Dr. Ulrich von Andrian, Harvard University, Medical School, Boston, MA.

Degree:

2000-2006 Ph.D., Immunology, Roswell Park Cancer Institute, State University of New York at Buffalo, Buffalo, NY, USA. Thesis advisor: Dr. Sharon S. Evans. Thesis title: 'Role of proinflammatory cytokines in controlling lymphocyte trafficking during fever-range thermal stress.'

CONCLUSIONS

These studies provide the first evidence that fever-range thermal therapy acts through an IL-6/sIL-6R trans-signaling mechanism to dynamically activate lymphocyte-endothelial adhesion in the tumor microenvironment. These results support the hypothesis that fever-range temperatures can overcome the microvascular barrier to tumor immunity through stimulation of heightened trafficking of $CD8^+$ cytotoxic T lymphocyte subsets to tumor sites and tumor-draining lymph nodes.

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APPENDICES

Papers:

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1. Chen, Q., Kucinska, S.A., Wang, W.C., Wallace, P.K., Baumann, H., and Evans, S.S. Fever-range thermal stress stimulates lymphocyte homing receptor function through an interleukin-6-dependent trans-signaling mechanism. 12th International Congress of Immunology. July, 2004; Montreal, Canada.
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Certificates:

1. Training course of intravital microscopy techniques in the laboratory of Dr. Mark Dewhirst, Duke U. Medical School, Durham, NC. 2004
2. NIAID scholarship, Keystone Symposium, Leukocyte Trafficking: Cellular and Molecular Mechanisms**.** March, 2005.
3. NIAID scholarship, Keystone Symposium, Chemokine and Chemokine Receptors. January, 2006.
4. New Investigator Award, Annual Meeting of Society for Thermal Medicine. April, 2006
5. Roswell Park Dean's Award, for outstanding dissertation research in Immunology, Roswell Park Division, State University of New York at Buffalo. May 2006
6. Keystone Symposia Education Fund Scholarship, Keystone Symposium, Mechanisms Linking Inflammation and Cancer. February, 2007.
7. Ph.D. diploma from University at Buffalo, SUNY. 2006

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Impact of Fever-Range Thermal Stress on Lymphocyte-Endothelial Adhesion and Lymphocyte Trafficking

Michelle M. Appenheimer, Qing Chen, Rachael A. Girard, Wan-Chao Wang, and Sharon S. Evans

Department of Immunology, Roswell Park Cancer Institute, Buffalo, New York, USA

The evolutionarily conserved febrile response has been associated with improved survival during infection in endothermic and ectothermic species although its protective mechanism of action is not fully understood. Temperatures within the range of physiologic fever influence multiple parameters of the immune response including lymphocyte proliferation and cytotoxic activity, neutrophil and dendritic cell migration, and production or bioactivity of proinflammatory cytokines. This review focuses on the emerging role of fever-range thermal stress in promoting lymphocyte trafficking to secondary lymphoid organs that are major sites for launching effective immune responses during infection or inflammation. Specific emphasis will be on the molecular basis of thermal control of lymphocyte-endothelial adhesion, a critical checkpoint controlling lymphocyte extravasation, as well as the contribution of interleukin-6 (IL-6) trans-signaling to thermal activities. New results are presented indicating that thermal stimulation of lymphocyte homing potential is evident in evolutionarily distant endothermic vertebrate species. These observations support the view that the evolutionarily conserved febrile response contributes to immune protection and host survival by amplifying lymphocyte access to peripheral lymphoid organs.

Keywords Fever, Lymphocyte trafficking, IL-6, Adhesion molecules, Endothelium.

Address correspondence to Sharon S. Evans, Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263-0001, USA; E-mail: sharon.evans@roswellpark.org

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INTRODUCTION: RELATIONSHIP BETWEEN FEVER AND THE IMMUNE RESPONSE

Fever is a highly conserved response to infection that evolved hundreds of millions of years ago. Local increases in temperature at sites of inflammation and systemic fever are cardinal features of a host response to pathogenic stimuli. Surprisingly, many of the specific functions of fever, and its influence on disease state and disease resolution, have not been fully defined. Fever is a complex physiologic response to infection or related stimuli (e.g., bacterial endotoxin, inflammation, injury) that is triggered by the local release of endogenous pyrogenic cytokines including tumor necrosis factor-α (TNF-α), IL-1β, and IL-6. In endothermic (warm-blooded) animals these mediators act on the hypothalamus to raise the thermoregulatory setpoint and initiate a cascade of biochemical, physiologic and behavioral responses that result in the elevation of core body temperature (Kluger, 1991). Fever is an actively regulated process that can be inhibited by antipyretic drugs such as aspirin or acetaminophen. In contrast, hyperthermia is an unregulated, passive rise in body temperature resulting from exogenous heating, and is not associated with changes in thermoregulatory setpoint.

Elevated body temperature, due either to natural fever or hyperthermia, has been associated with enhanced survival in numerous infectious disease models in endothermic species. Mice, rabbits, and dogs have increased resistance to bacterial and viral pathogens when their body temperature is artificially increased (Hasday et al., 2000; Kluger, 1991; Mackowiak, 2000). Similarly, antipyretic therapy results in increased mortality during bacterial infections in mice and rabbits (Esposito, 1984; Kurosawa et al., 1987; Vaughn et al., 1980). Clinical data in humans have also demonstrated the benefit of fever and risk of antipyresis in gram negative bacteremia (Bryant et al., 1971), bacterial peritonitis (Weinstein et al., 1978), chicken pox (Doran et al., 1989), and rhinovirus infection (Stanley et al., 1975). In contrast, some studies have shown harmful effects of fever in animal models during bacterial sepsis (Hasday et al., 2000; Jiang et al., 1999a, 2000; Klatersky, 1971). It is thought that the high metabolic burden of maintaining a fever may contribute to the negative outcomes sometimes observed among seriously ill (septic) hosts (Hasday et al., 2000; Manthous et al., 1995; Schumacker et al., 1987). Moreover, some of the activities of fever that may contribute to survival in less severely ill hosts (i.e., enhanced cytokine production/activity, augmented leukocyte recruitment, increased microbial killing mechanisms) may cause collateral tissue damage during life threatening infections (Hasday et al., 2000, 2003; Mackowiak, 1994).

While it is intuitively obvious that endothermic animals are capable of generating fever, febrile responses have also been documented in ectothermic (cold-blooded) animals. Ectotherms cannot regulate their core body

temperature endogenously, but instead engage in heat-seeking behaviors to achieve elevated body temperature, or an *environmental fever*. In one seminal study, the desert iguana *Dipsosaurus dorsalis* was infected with live bacteria (*Aeromonas hydrophila*) and allowed to migrate within a temperature gradient. Infected animals self-select higher temperatures within the gradient (4°C above the normal, afebrile temperature selected by uninfected animals) and have enhanced survival compared to animals housed at afebrile temperatures (Vaughn et al., 1974). This heat-seeking behavior and the survival benefit of elevated body temperature have also been observed in fish and even in insects (Covert and Reynolds, 1977; Kluger, 1991; Kluger et al., 1996; Mackowiak, 2000; Reynolds, 1977). Interestingly, the heat-seeking behavior observed in lizards and fish appears to have a biochemical basis. Infected animals that are given a dose of the antipyretic drug sodium salicylate fail to select a febrile temperature within their microenvironment and have higher mortality than infected animals not given antipyretic therapy (Bernheim and Kluger, 1976; Reynolds, 1977).

A powerful argument for the beneficial nature of fever is its presence throughout the class of vertebrate animals despite its high metabolic cost. In endotherms, maintaining even a 1°C fever corresponds to a 10–12.5% increase in energy requirements (Kluger, 1991). For ectothermic animals, the heat-seeking behaviors associated with fever generation, i.e., moving to warmer environments, require energy and may also expose sick and vulnerable animals to increased risk of predation. The existence of febrile responses in such diverse animals strongly suggests that the act of increasing body temperature during illness has evolved as a host defense mechanism, possibly dating back 300 million years or more.

Throughout much of human history fever has been regarded as a protective response. The ancient Greek physician Parmenides (ca. 500 BCE) said, "Give me the power to induce fever and I will cure all diseases" (Hobohm, 2001), while Hippocratic physicians routinely used heat treatment, including warm baths and burying patients in hot desert sands, to treat many diseases including cancer (Longrigg, 1998). Since the 1860s, physicians have experimented with using bacterial infections and accompanying fevers to treat cancer. In 1891, Coley treated a large cohort of sarcoma patients with a combination of heat-killed bacterial strains to induce high-grade fevers, and achieved a 10-year disease-free survival rate of over 25% (Starnes, 1992). But due to the relative rarity of sarcomas, and the belief, widely held as recently as 50 years ago, that the body has no intrinsic ability to fight cancers, the use of fever as a cancer therapy has not been widely pursued (Hobohm, 2001). Indeed, the general view of fever as a mechanism to fight disease changed once the antipyretic drug aspirin was introduced in 1889. At this point fever and its uncomfortable side-effects became viewed as something that could be controlled. This attitude toward fever has persisted for more than a century and remains current. In

recent years, however, new data detailing specific functions of heat has fueled a resurgence of interest in harnessing fever and thermal stress to enhance immune responses, particularly in the treatment of cancer (Dewhirst et al., 1997; Hobohm, 2001; Li and Dewhirst, 2002; Pritchard et al., 2004).

Fever-Range Thermal Effects on Immune Activation

Numerous reports have documented that febrile-range temperatures are associated with enhancement of the innate and adaptive arms of the immune response. Interestingly, while fever/heat initiates generalized, indiscriminate responses to elevated temperatures (e.g., vasodilatation), fever-range thermal stress also acts in a cell-type and function-specific manner, and not all immune functions are augmented by heat. The effects of fever-range thermal stress on immune parameters are briefly summarized below. More detailed information on these topics is provided in several comprehensive reviews (Blatteis, 2003; Hasday et al., 2000; Kluger, 1991; Kluger et al., 1998) and in other articles in this issue.

Several studies have shown that febrile-range temperatures stimulate cellular mediators of the innate immune response. Fever augments neutrophil migration, motility and chemotaxis, resulting in increased granulocyte infiltration into inflamed or infected areas in mammalian systems (Hasday et al., 2003; Nahas et al., 1971; Roberts, 1991; Roberts and Sandberg, 1979). Similar effects on neutrophil function have been observed in the desert iguana *D. dorsalis* (Bernheim et al., 1978). The bactericidal activity as well as FcR expression of macrophages is also reportedly enhanced by fever-range hyperthermia (Berman and Neva, 1981; van Bruggen et al., 1991). A recent series of studies demonstrate that febrile temperatures regulate migration of dendritic cells in vivo (Ostberg et al., 2000a, 2001, 2003; Yoshioka et al., 1989). In this regard, exposure of mice to fever-range temperatures results in the mobilization of Langerhans cells (i.e., skin dendritic cells) out of the local skin environment and into draining lymph nodes, where these cells are in position to provide a functional link to activation of antigen-specific T cells.

Fever temperatures have also been shown to augment adaptive immunity. T cell activities, including T cell cytotoxicity and proliferative response to mitogens or to cytokines (IL-1 and IL-2), are enhanced by fever-range temperatures (Di et al., 1997; Downing and Taylor, 1987; Duff and Durum, 1982; Lederman et al., 1987). Additionally, heat stimulates T cell helper function, resulting in enhanced antibody synthesis by murine B cells (Jampel et al., 1983). Thermal stress enables some antibodies to irreversibly neutralize specific viruses (i.e., to have viricidal activity) (Boeye et al., 1994; Delaet and Boeye, 1993). Exposure of lymphocytes to fever-range thermal stress alters the intracellular organization, expression, or activation status of cytoskeletal proteins (i.e., spectrin), heat shock proteins (hsp70 family), and signal

transduction molecules (protein kinase C, ERK1/2) (Chen et al., 2004; Di et al., 1997; Hasday and Singh, 2000; Hughes et al., 1987; Ostberg et al., 2002; Wang et al., 1999), events that may be causally linked to lymphocyte activation, motility, and adhesion.

Fever-range temperatures reportedly have complex effects on cytokine production and bioactivity. These responses are highly dependent on the timing and degree of temperature change, as well as the stimulus of cytokine production. In the absence of any pathogenic or inflammatory stimuli, negligible effects on cytokine or chemokine production (IL-1β, IL-6, IL-8, IL-11, IL-12, IL-13, TNF-α, interferon α [IFN], IFN-γ, RANTES, MCP-1) are observed in leukocytes or endothelial cells exposed to thermal stress in vitro (Chen et al., 2004; Hasday et al., 2001; Shah et al., 2002). Similarly, fever-range temperatures have no appreciable effect on the circulating levels of cytokines (IFN-α, IL-6, TNF-α, IL-1β) when administered in vivo (Chen et al., 2004; Downing et al., 1987; Haveman et al., 1996; Ostberg et al., 2000b). This is in contrast to experimental models where fever-range thermal stress, delivered along with a strong inflammatory challenge such as LPS (bacterial endotoxin, a nonreplicating inflammatory agonist), causes a transient increase in pro-inflammatory cytokine levels in vitro or in vivo (IL-6, TNF-α, IL-1β) (Fairchild et al., 2000; Hasday et al., 2000, 2001; Jiang et al., 1999a,b; Ostberg et al., 2000b). Heat treatment has also been shown to augment the antiviral and antiproliferative activities of human and mouse IFNs (Chang and Wu, 1991; Downing and Taylor, 1987; Downing et al., 1988; Heron and Berg, 1978; Roberts, 1991).

Under certain circumstances, fever may have self-limiting effects on immune defenses that may contribute to negative feedback loops. In this regard, febrile temperatures have been demonstrated to attenuate cytokine responses by inhibiting TNF-α expression at the RNA level (Ensor et al., 1995; Hasday et al., 2000; Singh et al., 2000, 2002). Moreover, even moderately elevated temperatures (e.g., 1°C above physiologically normal temperatures) suppress NK cell function in vitro (Azocar et al., 1982; Roberts, 1991) while high temperatures inhibit cytotoxic T lymphocyte (CTL) responses (Harris and Meneses, 1978).

MOLECULAR MECHANISMS UNDERLYING FEVER-RANGE THERMAL CONTROL OF LYMPHOCYTE TRAFFICKING

Until recently, the prevailing paradigm has been that fever influences leukocyte recruitment to tissues primarily by causing vasodilation and subsequent changes in hemodynamic blood flow (Goldsby et al., 2000). According to this scenario, a direct physiologic consequence of enhanced blood flow is that increased numbers of leukocytes have the opportunity to be delivered to

tissues. If appropriate vascular endothelial adhesion molecules and chemokines are expressed within a specific tissue locale, leukocytes could then initiate the cascade of adhesive events that culminate in extravasation and overall improvement in lymphocyte trafficking. Emerging evidence indicates that fever-range temperatures play a more active role in directing cell migration into tissues. Notably, these studies identified novel mechanisms by which thermal stress amplifies lymphocyte trafficking using experimental endpoints where the results cannot be attributed solely to changes in blood flow.

Lymphocyte-Endothelial Adhesion and Trafficking to Lymphoid Organs

An active immune response depends on the recirculation of lymphocytes through peripheral lymphoid organs. All secondary lymphoid organs (i.e., lymph nodes [LN] and Peyer's patches [PP]), except spleen, have specialized high endothelial venules (HEV) that constitutively express adhesion molecules and chemokines on the luminal surface to support the continuous recruitment of circulating lymphocytes (Butcher and Picker, 1996; Girard and Springer, 1995). The process of lymphocyte extravasation across HEV and into lymphoid tissues is well characterized at the molecular level and involves multiple sequential adhesive interactions that include: 1) initial attachment, followed by reversible tethering and rolling of lymphocytes along endothelial surfaces; 2) activation of lymphocyte chemokine receptors by chemokines displayed on HEV; 3) firm, stable adhesion; and 4) transendothelial migration (Butcher and Picker, 1996; Butcher et al., 1999; Springer, 1994; von Andrian and Mempel, 2003).

High expression of the L-selectin homing receptor supports tethering and rolling of naïve and central memory lymphocytes in HEV of peripheral and mesenteric lymph nodes (PLN, MLN) by binding to sialomucin-like endothelial counter-receptors collectively termed peripheral LN addressins (PNAd). PNAd molecules include posttranslationally modified forms of CD34 (human, mouse), GLYCAM-1 (mouse), podocalyxin (human), and endoglycan (human, mouse) (Butcher and Picker, 1996; Butcher et al., 1999; Girard and Springer, 1995; Rosen, 2004; von Andrian and Mempel, 2003). Tethering and rolling of naïve/central memory lymphocytes in MLN and PP is mediated by L-selectin and $\alpha 4\beta 7$ integrin-dependent binding to distinct domains within mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Butcher and Picker, 1996; Butcher et al., 1999; Girard and Springer, 1995; von Andrian and Mempel, 2003). Once the action of L-selectin or $\alpha 4\beta 7$ integrin slows the velocity of blood-borne lymphocytes, chemokine receptors (i.e., CCR7) on circulating lymphocytes interact with chemokines (i.e., CCL21) presented on the surface of HEV. G-protein linked chemokine receptors initiate the activation of downstream

signaling pathways to trigger avid (firm) binding of the integrin, leukocyte function-associated antigen-1 (LFA-1), to its endothelial counter-receptors intercellular cell adhesion molecule-1 and -2 (ICAM-1/2) (Butcher and Picker, 1996; Butcher et al., 1999; Campbell and Butcher, 2000; Girard and Springer, 1995; von Andrian and Mempel, 2003). The specific role of adhesion molecules and chemokines in the final transmigration step across HEV in lymphoid organs is less understood, but is speculated to involve LFA-1/ICAM-1/2, CCL21/CCR7, and CXCL12/CXCR4 interactions (Butcher and Picker, 1996; Butcher et al., 1999; Campbell and Butcher, 2000; Kashiwazaki et al., 2003; Miyasaka and Tanaka, 2004).

In contrast to the specialized cuboidal HEV of lymphoid organs, the majority of vessels throughout the body are lined by squamous vascular endothelial cells that express only limited levels of adhesion molecules or chemokines. However, under inflammatory conditions, the local release of proinflammatory cytokines (i.e., IL-1β, TNF-α, IL-6, lymphotoxin-α/β and IFN-γ) induces expression of endothelial adhesion molecules (i.e., ICAM-1, VCAM-1, E-selectin, PNAd, and MAdCAM-1) and chemokines (IL-8, MCP-1, RANTES, MIP-1α and β) (Butcher and Picker, 1996; Butcher et al., 1999; Choi et al., 2004; Fabbri et al., 1999; Girard and Springer, 1995; Rosen, 2004; Springer, 1994; von Andrian and Mempel, 2003). Through these mechanisms, activated/effector or memory lymphocyte subsets are recruited to sites of inflammation or injury in tertiary organs.

Fever-Range Hyperthermia Promotes Endothelial Adhesion in Lymphoid Organs

Experimental approaches have been employed in which fever-range thermal stress was examined without the addition of exogenous cytokines in order to dissect the effects of temperature elevation on lymphocyte trafficking to lymphoid organs (Chen et al., 2003, 2004; Evans et al., 2001; Wang et al., 1998). These conditions simulate the physiological events of early infectious responses where elevation of core body temperatures can occur in the absence of corresponding increases in circulating cytokine levels (Kluger, 1991). Thus, febrile temperatures have the potential to directly regulate biological responses within tissue milieus that are distal to the initial site of infection. These experimental strategies allowed for the identification of heat as a critical component in regulating the molecular adhesion events that control lymphocyte trafficking. Moreover, because studies were performed under aseptic conditions, in the absence of overt infection and the accompanying complex neuronal, hormonal, and cytokine networks that are associated locally or systemically with natural infections (Hasday et al., 2000; Kluger,

1991; Roberts, 1991), it was possible to identify a novel IL-6-dependent trans-signaling mechanism operative in controlling lymphocyte homing in response to thermal stimuli (Chen et al., 2004).

Exposure of mice to fever-range whole body hyperthermia (WBH) treatment (which raises the core body temperature to febrile levels) results in the mobilization of lymphocytes out of the peripheral blood compartment and into secondary lymphoid organs (i.e., LN and PP) or tumor tissues (Burd et al., 1998; Chen et al., 2003, 2004; Evans et al., 2001; Ostberg et al., 2000a). Notably, thermal stress does not induce lymphocyte accumulation in lymphoid organs such as the spleen that lack HEV, suggesting that thermal control of lymphocyte localization is an organ-specific response and does not merely represent the wholesale exit of cells out of the circulation. Similar mechanisms may be operative during clinical fever-range WBH therapy based on evidence that the numbers of circulating T lymphocytes decrease immediately after therapy in a subset of advanced cancer patients with solid tumors (Kraybill et al., 2002).

Complementary experimental approaches were taken to determine if the thermal effects on lymphocyte accumulation in tissues are due to changes in adhesion in target endothelium. Short-term (1 hour) in vivo homing experiments were performed to track the destination of fluorescently labeled splenocytes introduced via the tail vein into mice pretreated with WBH for 6 hours (Chen et al., 2003; Evans et al., 2001). These experimental conditions principally assess endothelial responses to thermal stress since WBH-treated mice are allowed to revert to their normothermic body temperature prior to adoptive transfer of fluorescent-tagged lymphocytes. Thus, fluorescent-labeled lymphocytes are not exposed to thermal stress in these studies. Heat-treatment causes a 2–5-fold increase in lymphocyte homing to lymphoid organs (i.e., PLN, MLN, PP, but not spleen) relative to normothermic controls (Chen et al., 2003, 2004; Evans et al., 2001; Wang et al., 1998). Moreover, the combined use of mAb-based adhesion-blocking strategies and lymphocyte cell lines expressing defined homing receptors (i.e., L-selectin or $\alpha 4\beta 7$ integrin) demonstrated that the HEV-specific adhesion molecules, PNAd and MAdCAM-1, play a critical role in mediating thermal enhancement of lymphocyte homing to secondary lymphoid organs. Based on estimates that 5×10^6 lymphocytes traverse HEV per second in humans under normothermic conditions (Girard and Springer, 1995), the 2–5-fold increase in homing observed in response to febrile temperatures potentially represents a biologically relevant increase in the number of lymphocytes that gain access to peripheral tissues. Collectively, these results suggest that an important outcome of fever-range thermal stress is to promote recruitment of naïve/central memory lymphocytes into sites where they have the opportunity to productively encounter foreign antigens.

These findings were confirmed by in vitro assays in which HEV adhesion is evaluated by measuring binding of lymphocytes to HEV in cryosections of lymphoid organs from WBH-treated mice (Chen et al., 2003; Evans et al., 2001). Analysis of HEV adhesion by these in vitro assays has an important advantage in that it circumvents contributions of hemodynamic flow that could influence lymphocyte homing during in vivo assays. These studies established that fever-range WBH enhances the ability of HEV in PLN, MLN, and PP to support L-selectin/PNAd or L-selectin/$\alpha 4\beta 7$ integrin/MAdCAM-1-dependent lymphocyte adhesion under shear. Notably, the pro-adhesive changes sustained by HEV cells during WBH are remarkably stable and are not lost during freezing of tissues prior to the adherence assay. Similar increases in HEV adhesion are observed following induction of natural fevers by bacterial LPS, which induces systemic inflammation, or turpentine, which induces local inflamed abscesses (Evans et al., 2001).

Thermal regulation of endothelial adhesion is temporally regulated in selected vascular beds. In this respect, moderate increases in HEV adhesion are detected 2 hours following WBH whereas adhesion is markedly augmented after 6 hours of WBH (Evans et al., 2001). As would be predicted under physiologically relevant conditions associated with resolution of natural fever, the effects of thermal stress on HEV adhesion are transient, returning to normal levels within 12 hours after cessation of WBH. Heat was further shown to regulate endothelial adhesion and lymphocyte trafficking only in organs bearing cuboidal HEV (i.e., PLN, MLN, PP) and not in vessels lined by squamous endothelium in lymphoid organs (LN, PP, spleen) or at extra-lymphoid sites (e.g., pancreas) (Chen et al., 2003; Evans et al., 2001; Wang et al., 1998). These results suggest that only a subset of blood vessels such as differentiated HEV (in lymphoid organs) or HEV-like vessels (at sites of inflammation) respond to thermal stimuli. It is tempting to speculate that selected vascular responses to thermal stress serve to focus the delivery of immune effector cells to sites necessary for a timely and productive immune response to infection while preventing inappropriate trafficking to other tissues during a physiologic febrile episode.

Parallel observations have been made using in vitro models for squamous, non-activated endothelium. Mild fever-range hyperthermia (40°C for 6 h) has no effect on expression of adhesion molecules (ICAM-1, E-selectin, P-selectin, PECAM, VCAM-1, PNAd, or MAdCAM-1) in human macrovascular or microvascular endothelial cells in vitro (human umbilical vein endothelial cells [HUVEC], or human dermal microvascular endothelial cells [HMVEC], respectively) (Shah et al., 2002). Fever-range hyperthermia also does not increase the ability of primary endothelial cells to support lymphocyte adhesion under shear in vitro or to produce cytokines (IL-1β, IL-6, IL-11, IL-12, IL-13, TGF-β1) or chemokines (IL-8, RANTES, MCP-1, MIP-1β, MIG) (Hasday

et al., 2000; Shah et al., 2002). Interestingly, although cultured endothelial cells do not respond to heat with changes in adhesion, conditioned medium from heat-treated HUVEC or HMVEC contain a proadhesive factor that is capable of acting in trans to activate the binding function of L-selectin or $\alpha 4\beta 7$ integrin in lymphocytes (Chen et al., 2004; Shah et al., 2002). These studies raise the possibility that the extensive vascular beds throughout the body could serve as a sentinel during inflammatory responses to promote immune surveillance by stimulating the function of lymphocyte homing receptors.

The mechanisms underlying the highly regulated control of vascular endothelial adhesion by fever-range temperatures remain to be determined. In experiments using cultured lymphoid organ explants (i.e., LN, PP), fever-range hyperthermia treatment in vitro significantly increases HEV adhesive properties, closely paralleling responses of these tissues during WBH treatment in vivo (Evans et al., 2001). Thus, local factors within lymphoid organs are sufficient to regulate adhesion, ruling out an obligate role for both the hypothalamus-pituitary-adrenal axis, which is known to regulate many febrile responses (Kluger, 1991; Roberts, 1991), as well as the afferent lymph, which delivers factors that support the maintenance of HEV structures (Girard and Springer, 1995; Hendriks et al., 1987; Mebius et al., 1991; Springer, 1994; von Andrian and Mempel, 2003). It is likely that the local tissue microenvironment, including extracellular matrix and stromal cells, as well as resident leukocytes or endothelial cells, contribute to activation of HEV adhesion in response to thermal stimuli.

Surprisingly, the pro-adhesive changes observed in endothelial cells occur in the absence of detectable changes in the cell surface density of the vascular addressins PNAd or MAdCAM-1 (Evans et al., 2001). These findings suggest febrile temperatures alter the avidity and/or affinity of HEV-specific adhesion molecules. One possibility is that thermal stress regulates interactions between endothelial adhesion molecules and the structural cytoskeleton, thereby strengthening their ability to support lymphocyte adhesion under hemodynamic shear forces in vivo. A similar role has been proposed for linkages between the actin-based contractile cytoskeleton and the cytoplasmic domains of several adhesion molecules, including E-selectin, ICAM-1, ICAM-2, L-selectin, and LFA-1 (Carpen et al., 1992; Chen et al., 2004; Dwir et al., 2001; Elemer and Edgington, 1994; Evans et al., 1999; Heiska et al., 1996; Pardi et al., 1992; Pavalko and LaRoche, 1993; Pavalko et al., 1995; Wang et al., 2001; Yoshida et al., 1996). In support of this notion, fever-range thermal stress has been shown to augment actin polymerization in primary endothelial cell cultures in vitro (Shah et al., 2002). Moreover, the intracellular domains of all three cloned PNAd transmembrane proteins, podocalyxin, endoglycan, and CD34, contain the amino acid motif DTHL (or related sequence DTEL), which in the case of podocalyxin has been shown to interact with the cytoskeleton

linker protein, ezrin (Clark et al., 2003; Kershaw et al., 1997; Nakamura et al., 1993; Takeda, 2003).

Fever-Range Thermal Stress Stimulates the Function of Lymphocyte Homing Receptors

Multiple lines of evidence indicate that fever-range thermal stress acts directly on lymphocytes to control their binding to HEV. Of particular note, the changes in lymphocyte adhesion induced by thermal stress parallel the dynamic responses reported for endothelial cells (described earlier). Direct exposure of cultured lymphocytes to fever-range thermal stress promotes a 2–5-fold increase in binding to HEV in in vitro frozen tissue-section adherence assays, as well as trafficking to lymphoid organs (PLN, MLN, and PP) in short-term in vivo homing studies (Chen et al., 2003, 2004; Evans et al., 1999, 2000, 2001; Pritchard et al., 2004; Wang et al., 1998). Kinetic studies showed that modest increases in lymphocyte adhesion are detectable as early as 2 hours after heat treatment while maximal induction of adhesion occurs after continuous thermal stimulation for 6–12 hours (Chen et al., 2004; Evans et al., 2001; Wang et al., 1998). Moreover, thermal effects on lymphocyte adhesion are fully reversible, returning to baseline levels within 12 hours of cessation of heat treatment (Wang et al., 1998). Two major lymphocyte homing receptors, L-selectin and $\alpha4\beta7$ integrin, were shown to mediate thermal responses in lymphocytes using function-blocking mAb and murine indicator cell lines that express a defined profile of adhesion molecules (i.e., 300.19 B cell transfectants express full-length human L-selectin and are $\alpha4\beta7$ integrin^{lo} LFA-1^{lo}; TK1 CD8$^+$ T cells are $\alpha4\beta7$ integrin^{hi}/L-selectin^{lo}) (Andrew et al., 1994; Chen et al., 2004; Dwir et al., 2001; Evans et al., 1999, 2000, 2001; Kansas et al., 1993; Steeber et al., 1997). Notably, similar increases in adhesion are detected when lymphocytes experience heat in vivo (Chen et al., 2003, 2004; Evans et al., 2000, 2001; Pritchard et al., 2004). Thus, splenocytes isolated from WBH-treated (6 hr) mice showed marked increases in both L-selectin and $\alpha4\beta7$ integrin-dependent adhesion to HEV when compared with lymphocytes from normal-temperature control mice. Taken together, these studies suggest that a physiologically important outcome of fever is to improve access of lymphocytes to peripheral lymphoid organs.

An example of these results is shown (Figure 1) where human peripheral blood lymphocytes (PBL) were cultured for 6 hours at normothermal temperature (37°C) or fever-range temperature (40°C) and then allowed to adhere to HEV of mouse PLN cryosections under shear. Quantification of lymphocyte binding to HEV demonstrated that thermal treatment causes a significant increase in the level of L-selectin-specific adhesion that could be inhibited by a L-selectin-blocking mAb, DREG-56 (i.e., indicated by brackets in

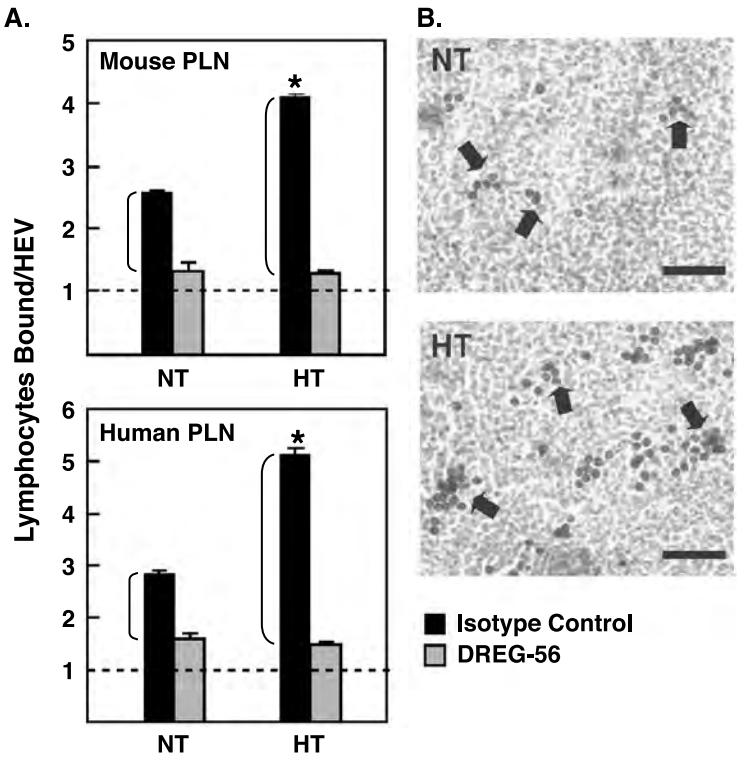


Figure 1: Fever-range thermal stress stimulates adhesion of human lymphocytes under shear to mouse and human PLN HEV. (A) The effect of thermal stress on L-selectin-dependent adhesion of lymphocytes to HEV was examined in an in vitro adherence assay, as described previously (Chen et al., 2004; Evans et al., 1999, 2001; Wang et al., 1998). Human peripheral blood lymphocytes were cultured for 6 hours at normothermic (NT, 37°C) or fever-range hyperthermic temperatures (HT, 40°C). Lymphocytes (5×10^6 cells in 100 μl) were then incubated with L-selectin function-blocking mAb (DREG-56, 20 μg/ml) or isotype-matched negative control Ab and overlaid onto 12 μm-thick PLN cryosections. Mouse PLN (from female BALB/c mice ~ 8 weeks of age) consisted of pooled superficial inguinal, brachial, axillary, popliteal, superficial, and deep cervical nodes. Normal human cervical LN were procured from patients undergoing carotid endarterectomys (kindly provided by Dr. Steven Bernstein, Roswell Park Cancer Institute). The adherence assay was performed under mechanical rotation to simulate in vivo shear forces. Following staining with 0.5% toluidine, the number of adherent cells bound to HEV were quantified by light microscopy. For consistency in double-blind evaluation, HEV were quantified only if they contained ≥ 1 adherent cell (minimal level of adhesion is indicated by dotted lines) and a total of 100 HEV were evaluated per sample. Data are the mean number of lymphocytes bound per HEV ± SE of triplicate samples. The differences between adhesion of normothermic and hyperthermic cells were significant, $p < 0.0001$ (*), by unpaired two-tailed Student's t-test. (B) Photomicrographs of representative fields from frozen section adherence assays using BALB/c mouse PLN. Exogenously added human lymphocytes exhibit a darkly stained, round appearance that is distinguished from histologically distinct tissue lymphocytes and HEV. Note higher numbers of human lymphocytes bound to individual HEV (arrows) in HT-treated samples relative to normothermic control. Bar, 50 μm.

Figure 1A) (Chen et al., 2004; Evans et al., 1999; Wang et al., 1998). Notably, the in vitro adherence assay (employing mouse LN tissues that are readily available for experimental study) is highly predictive of lymphocyte homing potential in vivo (Butcher et al., 1979a; Chen et al., 2004; Evans et al., 2001; Gallatin et al., 1983; Hamann et al., 1994; Rosen, 2004). This cross-species assay relies on the evolutionary conservation of lymphocyte-endothelial adhesion molecules. In this regard, the N-terminal lectin binding domains of human and mouse L-selectin are homologous, sharing 83% amino acid similarity (Tedder et al., 1989) while sulfation-dependent functional determinants of L-selectin ligands (PNAd) of human and mouse HEV are recognized by the same mAb (MECA-79) (Hemmerich et al., 1994; Michie et al., 1993; Rosen, 2004). To gain further insight into the physiological relevance of these findings to humans, the analysis was extended to examine the effect of thermal stress on human lymphocyte binding to human PLN HEV (Figure 1A). These results establish that thermal stress induces similar increases in L-selectin-dependent adhesion of human lymphocytes to mouse and human HEV substrates.

Fever-range thermal stress targets the function of selected homing receptors without globally stimulating adhesion in lymphocytes. In this regard, hyperthermia treatment of lymphocytes does not stimulate the ability of the β2 integrin, LFA-1, to mediate adhesion to ICAM-1 in frozen section in vitro assays (Wang et al., 1998). With respect to the α4β7 integrin, fever-range thermal stress has widely divergent effects on distinct functional domains even within the same adhesion molecule. Thus, while thermal stress strongly amplifies α4β7 integrin-dependent lymphocyte adhesion to the

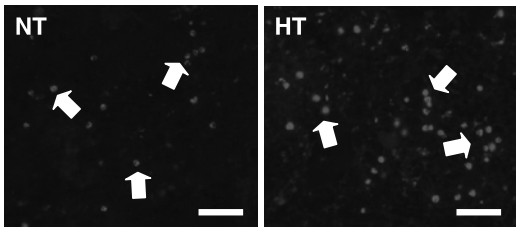


Figure 2: Human $CD3^+$ T cells are stimulated by fever-range thermal stress to bind HEV. Adhesion of fluorochrome-labeled human $CD3^+$ T lymphocytes to HEV in murine LN cryosections was evaluated by fluorescence microscopy, according to previously described methods (Chantakru et al., 2002, 2003; Chen et al., 2004; Frey et al., 1998). The phenotype of adherent human leukocyte subsets was determined by labeling cells with mAb specific for CD3 and by RITC-labeled goat-anti-mouse IgG prior to the frozen section assay. Exposure to fever-range thermal stress (HT, 40°C for 6 h) in vitro markedly stimulated adhesion of $CD3^+$ T cells (arrows) to HEV under shear whereas fewer adherent cells were detected when cells were maintained at normothermic temperature (NT, 37°C). Note that the underlying HEV structures are not visible under fluorescence microscopy. Data are representative of ≥ 3 independent experiments. Bar, 50 μm.

vascular endothelial counter-receptor MAdCAM-1, it suppresses α4β7 integrin-mediated binding to the extracellular matrix protein, fibronectin (Evans et al., 2000, 2001). These results are not entirely unexpected since mAb mapping studies indicate that distinct, albeit partially overlapping epitopes, are involved in α4β7 integrin-mediated binding to MAdCAM-1 and fibronectin (Andrew et al., 1994; Berlin et al., 1993; Kamata et al., 1995; Tidswell et al., 1997). One interpretation of these findings is that the migratory/homing properties of lymphocytes are preferentially enhanced by thermal stress.

The efficacy of an immune response is dictated by the profile of leukocyte subsets that gain entry into tissues. Therefore, the effects of thermal stress on adhesion by leukocyte subpopulations were evaluated in a modified in vitro frozen tissue-section adherence assay (Chantakru et al., 2003; Frey et al., 1998). For these studies, adherent human leukocyte subsets were phenotypically identified by fluorescently labeling cells with mAb specific for leukocyte antigens prior to assay. Multiple lymphocyte subsets were found to be responsive to thermal stress including $CD3^+$ T cells (Figure 2), $CD4^+$ and $CD8^+$ T cell subsets, $CD19^+$ B cells, and $CD56^{bright}$ NK cells, while $CD14^+$ monocytes were refractory to thermal stimulation (Chen et al., 2004). Of particular note, thermal stress enhances L-selectin-dependent adhesion in both naïve ($CD45RA^+$) and memory ($CD45RO^+$) lymphocyte subsets. These findings strongly support the notion that the thermal element of fever amplifies the magnitude of the immune response by mobilizing the egress of both naïve and central memory cells across HEV and into peripheral lymphoid organs.

Fever-range thermal stress activates similar levels of adhesion in vertebrate species (human and mouse) that evolved from a common ancestor 90 million years ago (Chen et al., 2003, 2004; Evans et al., 2000, 2001; Hedges, 2002; Reisz and Muller, 2004; Wang et al., 1998). These observations raised the question of whether febrile temperatures regulate lymphocyte adhesion in species that are even more evolutionarily distant. This question was addressed using splenocytes from an avian species (chicken, *Gallus gallus*) which descended from dinosaurs and diverged from the mammalian lineage over 300 million years ago. Chicken is an important model organism in the fields of immunology, virology, developmental biology and oncogenesis (Hedges, 2002; International Chicken Genome Sequencing Consortium, 2004; Reisz and Muller, 2004). Chicken-derived splenocytes were cultured for 6 hours under normothermic (40.9°C) or fever-range hyperthermic temperatures (42.7°C) (Leshchinsky and Klasing, 2001; Pittman et al., 1976) and then examined for the ability to bind to HEV of mouse MLN. MLN HEV are valuable tools to evaluate adhesive mechanisms governing tethering and rolling events in lymphocytes because they co-express both PNAd (i.e., L-selectin ligands) and MAdCAM-1 (i.e., ligand for both L-selectin and α4β7 integrin). Since cross-reactive Ab reagents are not available for analysis of homing receptors in avian

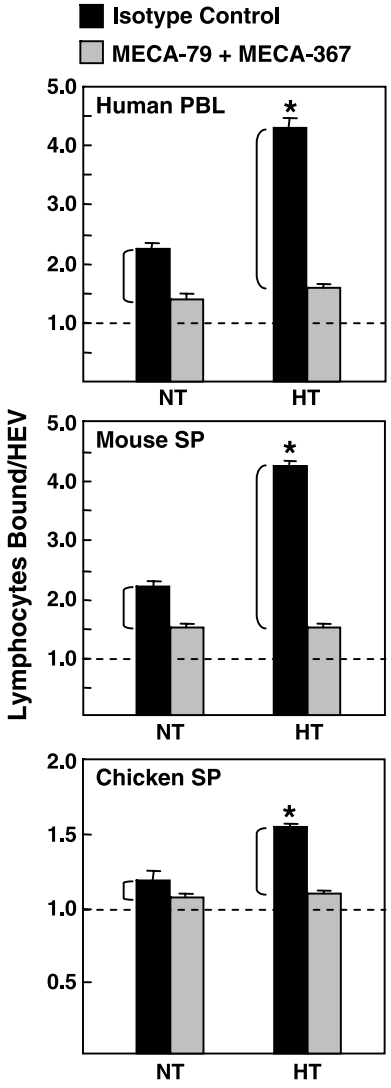


Figure 3: Fever range thermal stress enhances binding of leukocytes from evolutionarily diverse species to HEV of mouse MLN. Human peripheral blood lymphocytes (PBL) or mouse and chicken splenocytes (SP) were incubated for 6 hours at normothermic body temperatures (NT: human, 37°C; mouse, 36.5°C; chicken, 40.9°C) or at fever-range hyperthermic temperatures (HT: human, 40°C; mouse, 39.5°C; chicken, 42.7°C) and evaluated by in vitro adherence assays, using methods described in Figure 1 and in previous reports (Chen et al., 2004; Evans et al., 2001; Wang et al., 1998). Prior to assay, tissue cryosections were preincubated with function-blocking mAb specific for endothelial PNAd and MAdCAM-1 (MECA-79 and MECA-367, respectively), or with isotype-matched negative control Ab. Data are the mean ± SE of triplicate counts and are representative of ≥ 3 independent experiments. The differences between adhesion of normothermic and hyperthermic leukocytes were significant, $p < 0.05$ (*), by unpaired two-tailed Student's *t*-test.

species, HEV-specific adhesion was evaluated using blocking mAb specific for PNAd and MAdCAM-1 (i.e., MECA-79 and MECA-367, respectively). Consistent with prior reports (Butcher et al., 1979a,b), chicken leukocytes bind weakly to mouse MLN under shear although PNAd/MAdCAM-1-specific interactions could be detected (indicated by brackets, Figure 3). Despite the low level of basal adhesion, exposure of chicken leukocytes to fever-range thermal stress causes a significant increase in adhesion to MLN HEV, paralleling observations in human and mouse lymphocytes (Figure 3). These results raise the possibility that thermal control of lymphocyte homing receptor function is a biologically important host response that is conserved throughout the evolution of endothermic vertebrates.

The molecular basis of thermal control of lymphocyte adhesion was addressed in a recent series of studies. These findings excluded several potential mechanisms that have commonly been invoked to explain regulation of adhesion. While the surface density of homing receptors can influence their ability to efficiently support adhesion under shear, fever-range thermal stress does not increase the level of expression of L-selectin or $\alpha 4\beta 7$ integrin (Chen et al., 2004; Evans et al., 1999, 2000; Wang et al., 1998). Febrile temperatures also do not alter topographic localization of L-selectin on microvillous membrane projections (which is required for optimal tethering and rolling) or the lectin-binding activity of the N-terminal domain of L-selectin (although this domain is required for adhesion to HEV in both normothermal control and heat-treated lymphocytes) (Wang et al., 1998). Moreover, thermal activation of lymphocyte adhesion does not appear to depend on global effects of elevated temperatures on plasma membrane fluidity based on evidence that: 1) increased adhesion is detected by in vitro adherence assays performed at 4°C, a temperature that would be expected to reverse any acute effects of heat on membrane dynamics, and 2) conditioned medium derived from heat-treated cells is capable of activating both L-selectin and $\alpha 4\beta 7$ integrin-dependent adhesion in responder cells that are maintained at normothermal temperatures (Chen et al., 2004; Evans et al., 1998; Shah et al., 2002; Wang et al., 1998). These results ruled out a direct effect of heat, per se, on the conformation or organization of adhesion molecules in the plasma membrane and suggested that soluble factors regulate the affinity and/or avidity of lymphocyte homing receptors.

As discussed in the previous section, the flexible cytoskeletal matrix has been proposed to dynamically control the affinity and/or avidity of various adhesion molecules. Thus, it was of interest to examine the effects of fever-range hyperthermia on L-selectin interactions with the structural cytoskeleton. Under normothermal conditions, stable associations between L-selectin and the detergent-insoluble cytoskeletal matrix are only evident after L-selectin becomes engaged by physiologic ligands (GLYCAM-1) or antibodies

that mimic cross-linking by complex carbohydrate receptors (Chen et al., 2004; Evans et al., 1999; Leid et al., 2001, 2002). The kinetics of L-selectin redistribution to the detergent-insoluble subcellular fraction are very rapid (1–5 seconds) (Evans et al., 1999), consistent with the time-frame reported for tethering and rolling of lymphocytes along HEV surfaces (Butcher and Picker, 1996; von Andrian and Mempel, 2003). Notably, fever-range thermal stress causes L-selectin to preassociate with the detergent-insoluble cytoskeletal matrix in the absence of ligation or physical cross-linking (Chen et al., 2004; Evans et al., 1999). Moreover, this interaction is dependent on a C-terminal domain within the L-selectin cytoplasmic tail that contains a binding site for the cytoskeletal linker protein, α-actinin (Chen et al., 2004; Dwir et al., 2001; Evans et al., 1999; Kansas et al., 1993; Pavalko et al., 1995) Based on these findings, it has been proposed that the stable associations induced by thermal stress between L-selectin and the structural cytoskeletal scaffold alter the conformation and/or avidity of L-selectin, thereby enhancing its tensile strength and the efficiency with which it withstands physiologic hemodynamic shear within blood vessels (Chen et al., 2004; Evans et al., 1999).

Recent studies revealed an unexpected mechanism underlying thermal control of L-selectin-cytoskeletal interactions and L-selectin binding activity. These studies are an extension of findings that L-selectin or α4β7 integrin-dependent lymphocyte adhesion could be activated by conditioned medium from heat-treated hematopoietic cells (B and T lymphocytes, monocytes) and stromal cells (endothelium, fibroblast), but not paranchymal cells (breast, lung, melanocytes, hepatocytes, neuroblasts) (Chen et al., 2004; Evans et al., 1998, 1999; Shah et al., 2002; Wang et al., 1998). While soluble factors, namely proinflammatory cytokines such as TNF-α, IL-1β, IFNs, and IL-6, are well established regulators of endothelial adhesion during inflammation (Pober, 2002), these factors are not generally recognized participants in control of L-selectin adhesion. Thus, it was of interest that IL-6 was identified as the central mediator of thermal activation of L-selectin adhesion (Chen et al., 2004). In this regard, IL-6 neutralizing antibodies block thermal stimulation of lymphocyte adhesion during heat treatment in vitro and WBH treatment in vivo while functional blockade of other cytokines (i.e., IL-8, IFN-α, IFN-γ, TNF-α, IL-1β) is ineffective. Moreover, mAb-targeted inhibition of the individual IL-6 receptor components, i.e., the IL-6 receptor α binding subunit (IL-6Rα/CD126) and the transmembrane gp130 signal transducing chain (CD130) (Jones and Rose-John, 2002), fully prevents stimulation of adhesion in response to direct heat or conditioned medium from heat-treated cells (Chen et al., 2004). Control of L-selectin binding function by fever-range thermal stress was found to be exquisitely regulated, not only by IL-6, but also by a soluble form of the IL-6Rα subunit (Chen et al., 2004; Rose-John and Neurath, 2004). Together, these molecules function as a heterodimeric complex to

initiate trans-signaling and control L-selectin adhesion in lymphocytes. This mechanism of action was operationally defined in experiments in which thermal activation of L-selectin adhesion in vitro and in vivo was shown to be blocked by recombinant soluble gp130 (sgp130), a competitive inhibitor of IL-6/sIL-6Rα trans-signaling that is essentially ineffective in blocking signaling via membrane-bound IL-6Rα (Chen et al., 2004; Jones and Rose-John, 2002; Jostock et al., 2001; Muller-Newen et al., 1998; Narazaki et al., 1993; Rose-John and Neurath, 2004).

Combined biochemical and pharmacological methods identified the nature of the IL-6/sIL-6Rα signal transduction pathway responsible for amplifying L-selectin adhesion during thermal stress (Chen et al., 2004). These studies positioned the MEK1/ERK1-2 MAPK pathway upstream of activation of L-selectin-cytoskeletal interactions and L-selectin avidity/affinity. In this regard, Western blot analysis showed that fever-range thermal treatment of human lymphocytes triggered IL-6-dependent activation of ERK1-2, but not other stress-related MAPK (i.e., JNK and p38). The kinetics of this response paralleled the time-course for thermal activation of adhesion. Moreover, pharmacologic inhibitors of MEK/ERK signaling (UO126, PD98059) (Alessi et al., 1995; Favata et al., 1998), but not p38 (SB203580) or JNK (SP600125) (Bennett et al., 2001; Dean et al., 1999), prevented thermal stimulation of L-selectin-cytoskeletal interactions and L-selectin-dependent adhesion. An important question for future investigation is how IL-6-driven MEK-1/ERK1-2 signaling integrates changes in L-selectin/cytoskeletal interactions and lymphocyte adhesion.

The mechanisms by which thermal stress regulates IL-6/sIL-6Rα responses are distinguished at multiple levels from what has been reported for control of IL-6 trans-signaling during infection or inflammation. While elevated local or systemic concentrations of IL-6 and/or sIL-6Rα are detected in patients with pathologic inflammatory disorders or in experimental animal models for infection or inflammatory disease (Jones and Rose-John, 2002), thermal stress appears to increase the bioactivity or bioavailability of IL-6 without changing the apparent concentrations of IL-6, sIL-6Rα, or sgp130 (Chen et al., 2004). Thus, the increase in the proadhesive activity of IL-6 cannot be attributed to an imbalance in the concentrations of IL-6, sIL-6Rα, or sgp130. A potentially confounding finding was that thermal responses are fully maintained in IL-6-deficient mice despite compelling evidence that IL-6 is the principal mediator of thermal activation of L-selectin adhesion under normal physiologic conditions (Chen et al., 2004). These observations are in contrast to reports documenting defective inflammatory responses in IL-$6^{-/-}$ mice with regard to local neutrophil recruitment and chemokine production, liver regeneration, cutaneous wound healing, and post-traumatic tissue repair in the central nervous system (Cressman et al., 1996; Gallucci et al., 2000;

Hurst et al., 2001; Romano et al., 1997; Swartz et al., 2001). An explanation for this apparent paradox is provided by data showing that thermal control of lymphocyte adhesion in IL-6-deficient mice involves gp130-dependent IL-6 family cytokines (oncostatin M, LIF, IL-11) (Chen et al., 2004; Jones and Rose-John, 2002) that substitute for the loss of IL-6 in these mice. Thus, gp130-driven signal transduction is fundamental for mediating thermal control of homing receptor function in lymphocytes. The development of compensatory mechanisms in IL-6-deficient mice may be an indicator of the evolutionary importance of maintaining gp130-dependent signaling events for protection of the host against pathogenic challenges during febrile responses.

CONCLUSIONS AND FUTURE PERSPECTIVES

Studies detailed in this review support the emerging concept that the febrile component of fever initiates an orchestrated series of physiological responses that promote immune surveillance and immune protection during pathogenic challenge (Figure 4). Control of lymphocyte trafficking involves remarkably concerted activities whereby fever-range thermal stress enhances adhesion in both lymphocytes and selected target endothelium. Results indicating that thermal regulation of adhesion is evident in mammalian and nonmammalian vertebrate species (i.e., human, mouse, and chicken), strongly suggest that this mechanism of amplifying lymphocyte trafficking contributes to host survival.

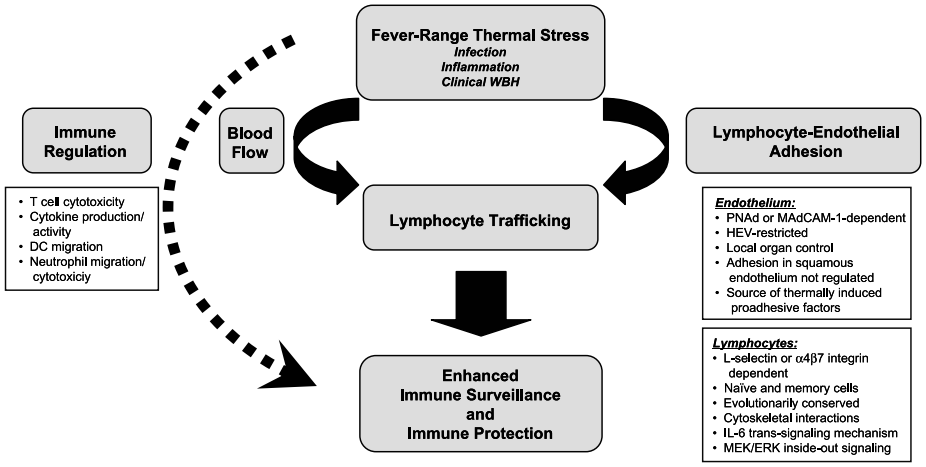


Figure 4: Model for integrated role of fever in promoting immune surveillance and immune protection.

Major questions remain regarding the molecular basis of thermal control of lymphocyte trafficking. In particular, intensive investigation is required to define the precise role of proinflammatory cytokines in controlling the avidity or affinity of adhesion molecules at the lymphocyte-endothelial interface. Given the established role of proinflammatory cytokines (TNF-α, IL-1β, lymphotoxin, IL-6) in regulating endothelial adhesion in inflammation it is tempting to speculate that one or more of these intercellular mediators regulates endothelial adhesion during febrile responses. Another unresolved issue relates to how thermal stress enhances the bioactivity of IL-6 in the absence of altering the protein levels of IL-6 or sIL-6Rα. A particularly compelling area of future investigation relates to the nature of the tightly regulated mechanisms that amplify adhesion in selected vascular beds, i.e., HEV, while sparing the majority of vessels throughout the body. Without this control, febrile temperatures could potentially drive a major exodus of leukocytes into extralymphoid tissues, thereby diminishing the impact of the immune response while promoting inappropriate contact with normal bystander tissues. This line of investigation is especially challenging since in vitro models have yet to be identified in order to biochemically dissect the signaling pathways responsible for transducing changes in endothelial adhesion during thermal stress. Future understanding of how fever-range thermal stress contributes to inflammatory responses has considerable clinical relevance for the development of novel strategies to either promote immune surveillance of peripheral tissues (i.e., during treatment of acute infections or cancer) or interfere with lymphocyte trafficking during pathologic conditions associated with chronic inflammation (e.g., autoimmune disorders).

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Thermal regulation of lymphocyte trafficking: Hot spots of the immune response

QING CHEN & SHARON S. EVANS

Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY, USA

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Abstract
Lymphocytes use extensive vascular networks to traffic to various destinations in the body, including lymphoid organs and extra-lymphoid tissues. This discussion will focus on the emerging evidence that thermal stress regulates the traffic signals that direct the exit of lymphocytes from the vascular freeway. This issue is particularly relevant to T cell-based cancer immunotherapy where delivery of immune effector lymphocytes to neoplastic lesions depends on their extravasation across tumour micro-vessels. Although tumours are frequently highly vascularized by vessels that are competent to support blood flow, the tumour micro-environment has been characterized as non-permissive to lymphocyte extravasation. This may lead to a scenario where limited leukocyte infiltration at tumour sites correlates with a poor prognosis. These observations support the thesis that adjuvant strategies that promote trafficking of tumour-reactive cytolytic leukocytes to tumour sites have the potential to improve the efficacy of immune-based cancer therapy.

Keywords: *Lymphocyte, trafficking, thermal stress, fever, tumour microvessels*

Relationship between endothelial adhesion and lymphocyte trafficking

In considering the mechanisms that potentially limit lymphocyte trafficking to tumour sites, it is instructive to compare and contrast adhesion events at neoplastic sites with the molecularly well-defined mechanisms that control the egress of blood-borne lymphocytes into peripheral lymphoid organs. In lymph nodes and Peyer's patches, lymphocyte extravasation occurs preferentially across specialized vessels termed high endothelial venules (HEV) [1–4]. The efficiency of this physiological process is revealed by estimates that $\sim 5 \times 10^6$ lymphocytes continuously extravasate through HEV per second in humans [1]. HEV are lined by cuboidal endothelial cells that are demarked by high level expression of the CD31 pan-endothelial adhesion molecule (Figure 1(a)). HEV are morphologically and biochemically distinguished from squamous endothelium found in the majority of vessels

Correspondence: Sharon S. Evans, Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263-0001, USA. Tel: 71916-845-3421. E-mail: sharon.evans@roswellpark.org

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in extra-lymphoid organs where limited lymphocyte extravasation occurs under basal conditions [1–3]. Notably, local injury or inflammation induces squamous endothelium to undergo transformation into HEV-like vessels that are a locus for leukocyte recruitment at extra-lymphoid sites [1].

Lymphocyte extravasation across HEV in lymphoid organs involves highly ordered, step-wise adhesive interactions [1–4]. In this context, endothelial adhesion molecules and chemokines function as traffic signals that control cellular transit through vascular roadways. Overall, it is estimated that 25% of lymphocytes that enter HEV successfully complete the full sequence of adhesion events culminating in extravasation [4]. In peripheral lymph nodes, free-flowing lymphocytes (travelling with a 'green light') enter lymph node HEV and convert to slow-rolling cells ('yellow light') through L-selectin-dependent tethering and rolling interactions with sialomucin-like adhesion molecules on vessel walls. In Peyer's patches, a second lymphocyte homing receptor, $\alpha 4\beta 7$ integrin, also contributes to tethering and rolling events in HEV. In both lymph nodes and Peyer's patches, HEV presentation of the CC chemokine ligand (CCL)21 (TCA-4/SLC/6C-kine/exodus-2) to the CCR7 chemokine receptor on apposing naïve and central memory lymphocytes stimulates the transition from rolling cells to firmly adherent cells ('red light'). Firm arrest and subsequent extravasation across the endothelial barrier are mediated by binding of leukocyte function adhesion molecule-1 (LFA-1) to its endothelial receptors inter-cellular adhesion molecule (ICAM)-1 (Figure 1(a)) and ICAM-2.

Tumour vessels of murine tumours or human patients are generally flat-walled, in contrast to the typical cuboidal morphology of HEV in lymphoid organs (Figure 1(a, b)) [5–7]. Consistent with these findings, *in vivo* imaging techniques using intra-vital microscopy indicate that lymphocytes interact poorly with tumour vessels under haemodynamic shear [6, 8, 9]. One explanation for limited lymphocyte interactions in tumour vessels is the comparatively low level of expression of endothelial adhesion molecules (e.g. ICAM-1) and chemokines that are hallmarks of extravasation or inflammation (Figure 1(a, b)) [5, 6]. Notably, the failure of lymphocytes to infiltrate tumour tissues has been correlated with an unfavourable prognosis in melanoma, lung and ovarian cancer patients and in numerous mouse models (Figure 1(b)) [5, 6, 10, 11]. These observations support the notion that novel strategies are needed to improve lymphocyte trafficking to neoplastic tissues. This issue is particularly important in the development of effective vaccine therapies where immunization aims to increase the frequency of tumour-reactive cytotoxic T cells (CTL). A frequently overlooked consideration is that optimal vaccine therapy is equally dependent on tumour-specific CTL gaining access to tumour tissues in order to be effective in killing targets by contact-mediated lysis.

Stimulation of lymphocyte-endothelial cell interactions by thermal stress

Multiple lines of evidence indicate that thermal stress in the form of therapeutic hyperthermia can control lymphocyte-endothelial adhesion. The role of high temperature, short duration heat shock in regulating adhesion has principally been investigated by *in vitro* models for resting, non-activated squamous endothelial cells, i.e. human primary endothelial cell cultures including macro-vascular HUVEC (human umbilical vein endothelial cells) and micro-vascular HMVEC (human micro-vascular endothelial cells). Direct exposure of HUVEC or HMVEC to heat shock (e.g. 43°C for 2 h) increases endothelial cell expression of ICAM-1 molecules [12, 13]. Interestingly, several studies have reported that the major cellular products of heat shock, i.e. heat shock proteins (HSP), can act extra-cellularly to stimulate endothelial adhesion. In this regard, recombinant

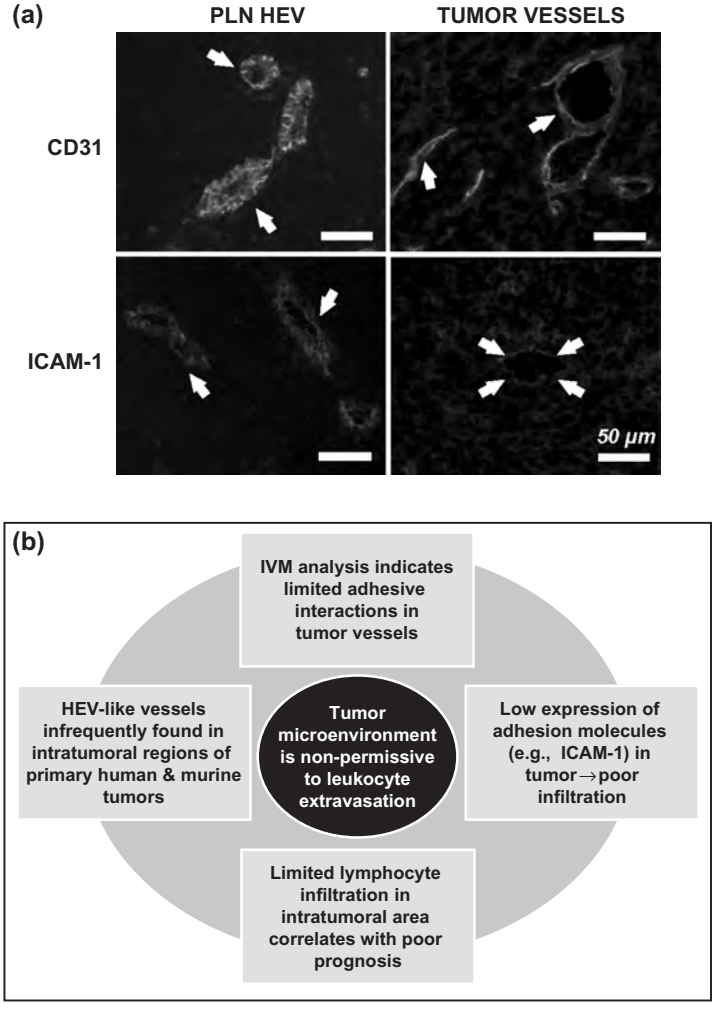


Figure 1. Comparative analysis of lymphoid organ HEV and tumour vessels. (a) Cryosections of peripheral lymph nodes (PLN) and transplanted colon 26 tumours from the same experimental mouse were stained with CD31-specific monoclonal antibody (mAb; 5 μg ml^{-1}) and FITC-labelled secondary antibody. ICAM-1 expression on the luminal surface of vessels was detected by intravenous injection of ICAM-1-specific mAb (500 μg per mouse) into colon 26 tumour-bearing mice. Tissues were harvested after 20 min and cryosetions were stained with TRITC-labelled secondary antibody. Immunofluorescent images were captured using the same exposure time in order to compare the relative expression levels of adhesion molecules by vessels. Arrows indicate cuboidal HEV in PLN or flat-walled squamous vessels in tumour tissues. ICAM-1 staining is low on tumour vessels compared to PLN HEV. Note that staining of ICAM-1 on tumour cells is also evident, which may result from access of ICAM-1 mAb to tissues outside the vascular compartment due to the inherent leakiness of tumour vessels [7]. (b) Schematic shows proposed inter-relationship between the parameters that limit lymphocyte-endothelial adhesion in the tumour micro-environment and clinical outcome.

HSP (HSP60, HSP65 or HSP70) of mammalian or bacterial origin act on HUVEC through an NF-κB signalling pathway to induce ICAM-1 expression and augment the ability to support lymphocyte adhesion [14–16]. One of the interesting findings to emerge is that increased shear forces also stimulate ICAM-1 expression by HUVEC *in vitro* [17].

This may be relevant during hyperthermia responses in tissues when haemodynamic shear is influenced by changes in blood flow [18]. While the effects of heat shock or haemodynamic shear on endothelial adhesion have not been explored *in vivo*, it is tempting to speculate, based on *in vitro* endothelial systems, that high temperature thermal therapy could elicit changes in vascular adhesion and lymphocyte homing if delivered locally to tumour sites.

A recent series of *in vivo* studies have established that fever-range thermal therapy enhances lymphocyte-endothelial adhesion and site-specific trafficking [6, 19, 20]. An intriguing aspect is that fever-range thermal stress promotes homing by inducing independent changes in adhesion in both lymphocytes and target endothelium. In this regard, exposure of lymphocyte sub-sets (including naïve and central memory lymphocytes and $CD4^+$ and $CD8^+$ T cells) to febrile-range temperatures (38–40°C for 6 h) *in vitro* or *in vivo* stimulates the binding activity of lymphocyte homing receptors including L-selectin and $\alpha 4\beta 7$ integrin [19, 21–25]. This results in enhanced trafficking of lymphocytes across HEV in lymphoid organs [21, 26]. Thermal stimulation of lymphocyte adhesion was found to be dependent on the pro-inflammatory cytokine, interleukin-6 (IL-6), through its ability to initiate MEK-1/ERK-1-2 signalling downstream of the gp130 signal transduction molecule [25].

The adhesive properties of endothelial cells in HEV of lymphoid organs have further been shown to be amplified by fever-range whole body thermal therapy [26, 27]. Thus, elevation of mouse core temperatures to the febrile range (39.5–40°C for 6 h), using procedures originally developed by Dr. Repasky's laboratory [27], augments the capacity of HEV to support lymphocyte adhesion under shear and promotes lymphocyte trafficking to lymph nodes and Peyer's patches [19, 20, 26]. Enhanced lymphocyte trafficking to secondary lymphoid organs increases the probability that lymphocytes encounter cognate antigens within a micro-environment conducive to generating optimal adaptive immune responses. Related studies indicate that lymphocyte-endothelial adhesion is similarly enhanced in tumour vessels by fever-range thermal therapy (Q. Chen and S. S. Evans, unpublished observations). Notably, thermal stress does not promote changes in adhesion or trafficking across squamous, resting endothelium of non-malignant tissues *in vivo* [6, 26]. These observations suggest that control of vascular adhesion is influenced by exquisitely regulated molecular events within unique tissue micro-environments of lymphoid organs, tumour tissues and extra-lymphoid organs. Site-specific control of endothelial adhesion serves to focus the delivery of immune effectors cells to physiologically relevant tissues while preventing inappropriate encounters between cytolytic lymphocytes and normal tissues.

Conclusions and future directions

The emerging data support the concept that immune surveillance is promoted by physiological febrile responses that accompany infection and inflammation by virtue of the ability to improve lymphocyte trafficking across lymphoid organ HEV (Figure 2). Optimal immune protection is maintained under non-pathologic conditions since fever-range thermal stress does not indiscriminately amplify lymphocyte migration across squamous endothelium of all tertiary organs. Further study is required to determine if local or whole body thermal therapy can invoke similar adhesive mechanisms within heterogeneous tumour micro-environments, thereby augmenting delivery of tumour-specific $CD8^+$ cytotoxic T cells (Figure 2). Enhanced infiltration by $CD8^+$ T cells in primary and secondary lesions would be predicted to lead to improved tumour growth

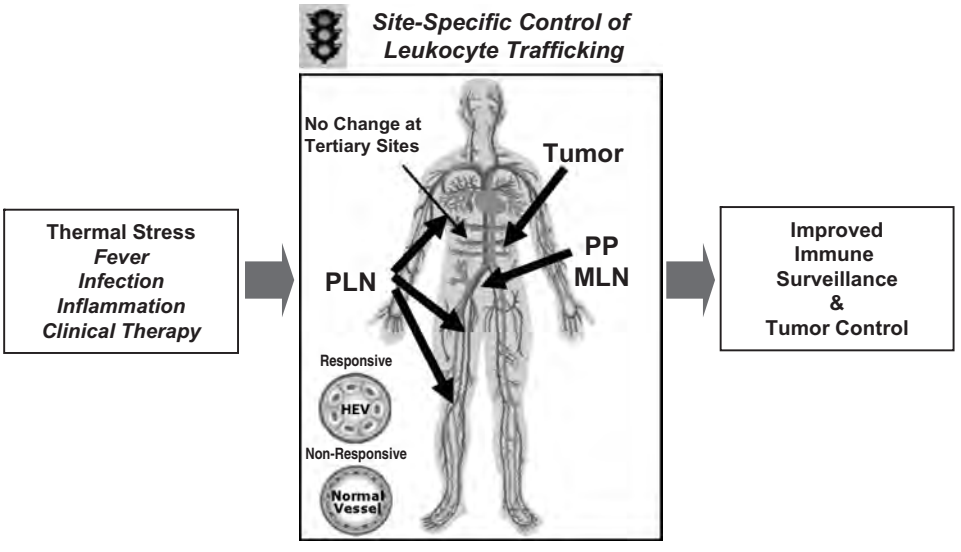


Figure 2. Model for thermal regulation of traffic signals that direct site-specific extravasation of lymphocytes across vascular beds. Thermal stress induced by inflammation or clinical therapy promotes lymphocyte trafficking across cuboidal HEV in lymphoid organs (PLN, mesenteric LN [MLN] and Peyer's Patches [PP]) and potentially in tumour vessels. In contrast, lymphocyte-endothelial adhesion and trafficking across non-activated squamous endothelium of extra-lymphoid organs is not augmented by fever-range thermal stress under non-pathological conditions. Thus, site-specific control of vascular adhesion by thermal stress has the potential to enhance immune surveillance and limit tumour progression.

control in immunologically responsive malignancies. The underlying mechanisms by which thermal stress stimulates endothelial adhesion within a select sub-set of blood vessels remain to be addressed. Likely candidate effector mechanisms are pro-inflammatory cytokines, since these molecules are known to be potent stimulators of endothelial adhesion. Additional investigation is required to determine the mode of thermal regulation of vascular endothelial adhesion. Resolution of this question depends on the identification of the specific traffic signals (i.e. adhesion molecules and chemokines) that are targeted by thermal stress in addition to mapping of the signal transduction pathways responsible for improving the delivery of lymphocytes across vascular roadways.

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Thermal regulation of lymphocyte trafficking: Hot spots of the immune response

Qing Chen [a]; Sharon S. Evans [a]
[a] Department of Immunology, Roswell Park Cancer Institute. Buffalo, NY. USA

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Thermal regulation of lymphocyte trafficking: Hot spots of the immune response

QING CHEN & SHARON S. EVANS

Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY, USA

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Abstract
Lymphocytes use extensive vascular networks to traffic to various destinations in the body, including lymphoid organs and extra-lymphoid tissues. This discussion will focus on the emerging evidence that thermal stress regulates the traffic signals that direct the exit of lymphocytes from the vascular freeway. This issue is particularly relevant to T cell-based cancer immunotherapy where delivery of immune effector lymphocytes to neoplastic lesions depends on their extravasation across tumour micro-vessels. Although tumours are frequently highly vascularized by vessels that are competent to support blood flow, the tumour micro-environment has been characterized as non-permissive to lymphocyte extravasation. This may lead to a scenario where limited leukocyte infiltration at tumour sites correlates with a poor prognosis. These observations support the thesis that adjuvant strategies that promote trafficking of tumour-reactive cytolytic leukocytes to tumour sites have the potential to improve the efficacy of immune-based cancer therapy.

Keywords: *Lymphocyte, trafficking, thermal stress, fever, tumour microvessels*

Relationship between endothelial adhesion and lymphocyte trafficking

In considering the mechanisms that potentially limit lymphocyte trafficking to tumour sites, it is instructive to compare and contrast adhesion events at neoplastic sites with the molecularly well-defined mechanisms that control the egress of blood-borne lymphocytes into peripheral lymphoid organs. In lymph nodes and Peyer's patches, lymphocyte extravasation occurs preferentially across specialized vessels termed high endothelial venules (HEV) [1–4]. The efficiency of this physiological process is revealed by estimates that $\sim 5 \times 10^6$ lymphocytes continuously extravasate through HEV per second in humans [1]. HEV are lined by cuboidal endothelial cells that are demarked by high level expression of the CD31 pan-endothelial adhesion molecule (Figure 1(a)). HEV are morphologically and biochemically distinguished from squamous endothelium found in the majority of vessels

Correspondence: Sharon S. Evans, Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263-0001, USA. Tel: 71916-845-3421. E-mail: sharon.evans@roswellpark.org

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in extra-lymphoid organs where limited lymphocyte extravasation occurs under basal conditions [1–3]. Notably, local injury or inflammation induces squamous endothelium to undergo transformation into HEV-like vessels that are a locus for leukocyte recruitment at extra-lymphoid sites [1].

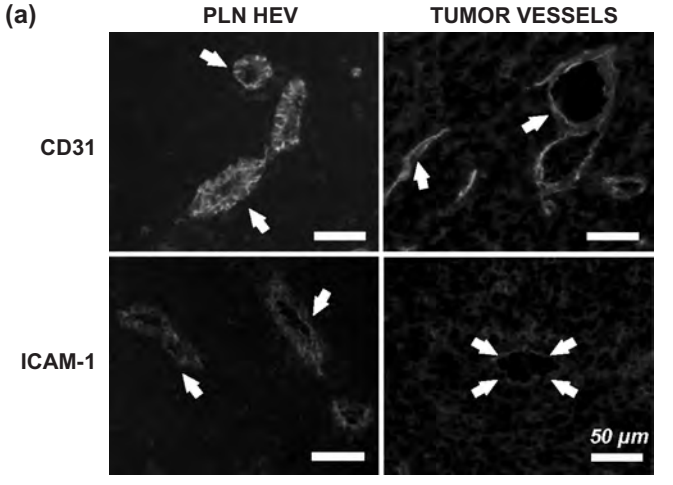
Lymphocyte extravasation across HEV in lymphoid organs involves highly ordered, step-wise adhesive interactions [1–4]. In this context, endothelial adhesion molecules and chemokines function as traffic signals that control cellular transit through vascular roadways. Overall, it is estimated that 25% of lymphocytes that enter HEV successfully complete the full sequence of adhesion events culminating in extravasation [4]. In peripheral lymph nodes, free-flowing lymphocytes (travelling with a 'green light') enter lymph node HEV and convert to slow-rolling cells ('yellow light') through L-selectin-dependent tethering and rolling interactions with sialomucin-like adhesion molecules on vessel walls. In Peyer's patches, a second lymphocyte homing receptor, $\alpha 4\beta 7$ integrin, also contributes to tethering and rolling events in HEV. In both lymph nodes and Peyer's patches, HEV presentation of the CC chemokine ligand (CCL)21 (TCA-4/SLC/6C-kine/exodus-2) to the CCR7 chemokine receptor on apposing naïve and central memory lymphocytes stimulates the transition from rolling cells to firmly adherent cells ('red light'). Firm arrest and subsequent extravasation across the endothelial barrier are mediated by binding of leukocyte function adhesion molecule-1 (LFA-1) to its endothelial receptors inter-cellular adhesion molecule (ICAM)-1 (Figure 1(a)) and ICAM-2.

Tumour vessels of murine tumours or human patients are generally flat-walled, in contrast to the typical cuboidal morphology of HEV in lymphoid organs (Figure 1(a, b)) [5–7]. Consistent with these findings, *in vivo* imaging techniques using intra-vital microscopy indicate that lymphocytes interact poorly with tumour vessels under haemodynamic shear [6, 8, 9]. One explanation for limited lymphocyte interactions in tumour vessels is the comparatively low level of expression of endothelial adhesion molecules (e.g. ICAM-1) and chemokines that are hallmarks of extravasation or inflammation (Figure 1(a, b)) [5, 6]. Notably, the failure of lymphocytes to infiltrate tumour tissues has been correlated with an unfavourable prognosis in melanoma, lung and ovarian cancer patients and in numerous mouse models (Figure 1(b)) [5, 6, 10, 11]. These observations support the notion that novel strategies are needed to improve lymphocyte trafficking to neoplastic tissues. This issue is particularly important in the development of effective vaccine therapies where immunization aims to increase the frequency of tumour-reactive cytotoxic T cells (CTL). A frequently overlooked consideration is that optimal vaccine therapy is equally dependent on tumour-specific CTL gaining access to tumour tissues in order to be effective in killing targets by contact-mediated lysis.

Stimulation of lymphocyte-endothelial cell interactions by thermal stress

Multiple lines of evidence indicate that thermal stress in the form of therapeutic hyperthermia can control lymphocyte-endothelial adhesion. The role of high temperature, short duration heat shock in regulating adhesion has principally been investigated by *in vitro* models for resting, non-activated squamous endothelial cells, i.e. human primary endothelial cell cultures including macro-vascular HUVEC (human umbilical vein endothelial cells) and micro-vascular HMVEC (human micro-vascular endothelial cells). Direct exposure of HUVEC or HMVEC to heat shock (e.g. 43°C for 2 h) increases endothelial cell expression of ICAM-1 molecules [12, 13]. Interestingly, several studies have reported that the major cellular products of heat shock, i.e. heat shock proteins (HSP), can act extra-cellularly to stimulate endothelial adhesion. In this regard, recombinant

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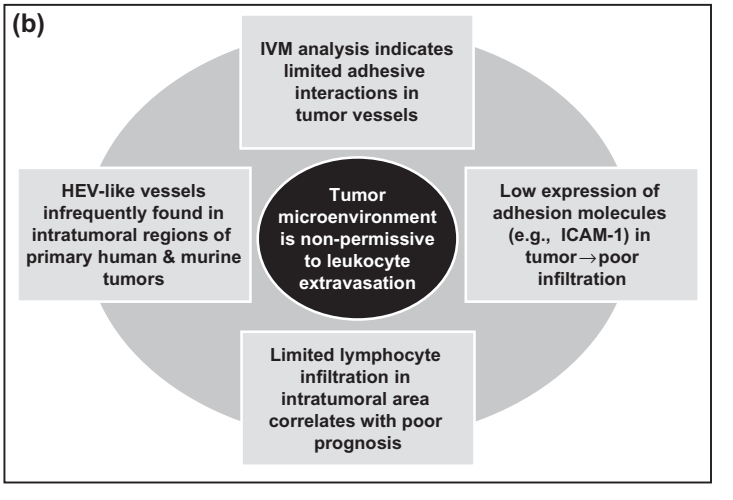


Figure 1. Comparative analysis of lymphoid organ HEV and tumour vessels. (a) Cryosections of peripheral lymph nodes (PLN) and transplanted colon 26 tumours from the same experimental mouse were stained with CD31-specific monoclonal antibody (mAb; 5 μg ml^{-1}) and FITC-labelled secondary antibody. ICAM-1 expression on the luminal surface of vessels was detected by intravenous injection of ICAM-1-specific mAb (500 μg per mouse) into colon 26 tumour-bearing mice. Tissues were harvested after 20 min and cryosetions were stained with TRITC-labelled secondary antibody. Immunofluorescent images were captured using the same exposure time in order to compare the relative expression levels of adhesion molecules by vessels. Arrows indicate cuboidal HEV in PLN or flat-walled squamous vessels in tumour tissues. ICAM-1 staining is low on tumour vessels compared to PLN HEV. Note that staining of ICAM-1 on tumour cells is also evident, which may result from access of ICAM-1 mAb to tissues outside the vascular compartment due to the inherent leakiness of tumour vessels [7]. (b) Schematic shows proposed inter-relationship between the parameters that limit lymphocyte-endothelial adhesion in the tumour micro-environment and clinical outcome.

HSP (HSP60, HSP65 or HSP70) of mammalian or bacterial origin act on HUVEC through an NF-κB signalling pathway to induce ICAM-1 expression and augment the ability to support lymphocyte adhesion [14–16]. One of the interesting findings to emerge is that increased shear forces also stimulate ICAM-1 expression by HUVEC *in vitro* [17].

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This may be relevant during hyperthermia responses in tissues when haemodynamic shear is influenced by changes in blood flow [18]. While the effects of heat shock or haemodynamic shear on endothelial adhesion have not been explored *in vivo*, it is tempting to speculate, based on *in vitro* endothelial systems, that high temperature thermal therapy could elicit changes in vascular adhesion and lymphocyte homing if delivered locally to tumour sites.

A recent series of *in vivo* studies have established that fever-range thermal therapy enhances lymphocyte-endothelial adhesion and site-specific trafficking [6, 19, 20]. An intriguing aspect is that fever-range thermal stress promotes homing by inducing independent changes in adhesion in both lymphocytes and target endothelium. In this regard, exposure of lymphocyte sub-sets (including naïve and central memory lymphocytes and $CD4^+$ and $CD8^+$ T cells) to febrile-range temperatures (38–40°C for 6 h) *in vitro* or *in vivo* stimulates the binding activity of lymphocyte homing receptors including L-selectin and $\alpha 4\beta 7$ integrin [19, 21–25]. This results in enhanced trafficking of lymphocytes across HEV in lymphoid organs [21, 26]. Thermal stimulation of lymphocyte adhesion was found to be dependent on the pro-inflammatory cytokine, interleukin-6 (IL-6), through its ability to initiate MEK-1/ERK-1-2 signalling downstream of the gp130 signal transduction molecule [25].

The adhesive properties of endothelial cells in HEV of lymphoid organs have further been shown to be amplified by fever-range whole body thermal therapy [26, 27]. Thus, elevation of mouse core temperatures to the febrile range (39.5–40°C for 6 h), using procedures originally developed by Dr. Repasky's laboratory [27], augments the capacity of HEV to support lymphocyte adhesion under shear and promotes lymphocyte trafficking to lymph nodes and Peyer's patches [19, 20, 26]. Enhanced lymphocyte trafficking to secondary lymphoid organs increases the probability that lymphocytes encounter cognate antigens within a micro-environment conducive to generating optimal adaptive immune responses. Related studies indicate that lymphocyte-endothelial adhesion is similarly enhanced in tumour vessels by fever-range thermal therapy (Q. Chen and S. S. Evans, unpublished observations). Notably, thermal stress does not promote changes in adhesion or trafficking across squamous, resting endothelium of non-malignant tissues *in vivo* [6, 26]. These observations suggest that control of vascular adhesion is influenced by exquisitely regulated molecular events within unique tissue micro-environments of lymphoid organs, tumour tissues and extra-lymphoid organs. Site-specific control of endothelial adhesion serves to focus the delivery of immune effectors cells to physiologically relevant tissues while preventing inappropriate encounters between cytolytic lymphocytes and normal tissues.

Conclusions and future directions

The emerging data support the concept that immune surveillance is promoted by physiological febrile responses that accompany infection and inflammation by virtue of the ability to improve lymphocyte trafficking across lymphoid organ HEV (Figure 2). Optimal immune protection is maintained under non-pathologic conditions since fever-range thermal stress does not indiscriminately amplify lymphocyte migration across squamous endothelium of all tertiary organs. Further study is required to determine if local or whole body thermal therapy can invoke similar adhesive mechanisms within heterogeneous tumour micro-environments, thereby augmenting delivery of tumour-specific $CD8^+$ cytotoxic T cells (Figure 2). Enhanced infiltration by $CD8^+$ T cells in primary and secondary lesions would be predicted to lead to improved tumour growth

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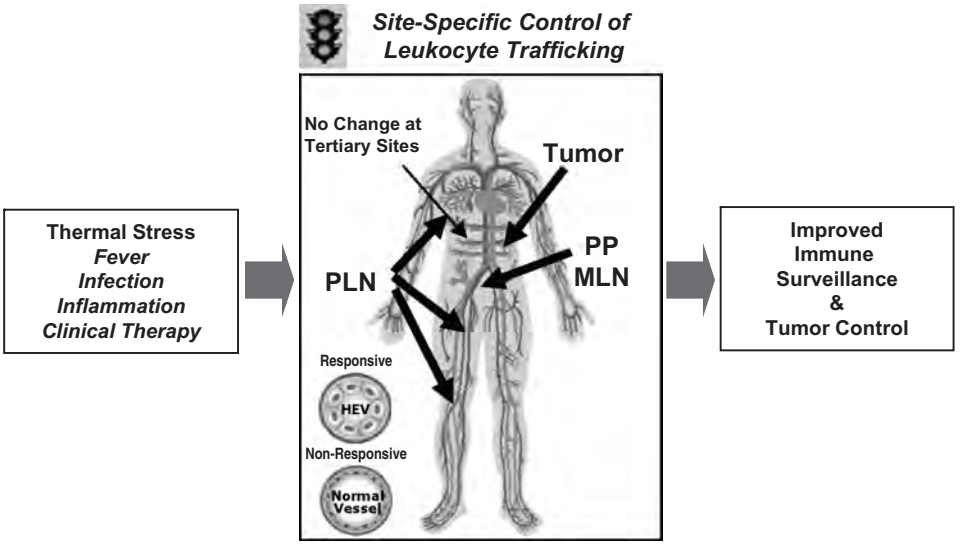


Figure 2. Model for thermal regulation of traffic signals that direct site-specific extravasation of lymphocytes across vascular beds. Thermal stress induced by inflammation or clinical therapy promotes lymphocyte trafficking across cuboidal HEV in lymphoid organs (PLN, mesenteric LN [MLN] and Peyer's Patches [PP]) and potentially in tumour vessels. In contrast, lymphocyte-endothelial adhesion and trafficking across non-activated squamous endothelium of extra-lymphoid organs is not augmented by fever-range thermal stress under non-pathological conditions. Thus, site-specific control of vascular adhesion by thermal stress has the potential to enhance immune surveillance and limit tumour progression.

control in immunologically responsive malignancies. The underlying mechanisms by which thermal stress stimulates endothelial adhesion within a select sub-set of blood vessels remain to be addressed. Likely candidate effector mechanisms are pro-inflammatory cytokines, since these molecules are known to be potent stimulators of endothelial adhesion. Additional investigation is required to determine the mode of thermal regulation of vascular endothelial adhesion. Resolution of this question depends on the identification of the specific traffic signals (i.e. adhesion molecules and chemokines) that are targeted by thermal stress in addition to mapping of the signal transduction pathways responsible for improving the delivery of lymphocytes across vascular roadways.

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SYMPOSIUM IN WRITING

Qing Chen · Daniel T. Fisher · Sylvia A. Kucinska
Wan-Chao Wang · Sharon S. Evans

Dynamic control of lymphocyte trafficking by fever-range thermal stress

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Abstract Migration of blood-borne lymphocytes into tissues involves a tightly orchestrated sequence of adhesion events. Adhesion molecules and chemokine receptors on the surface of circulating lymphocytes initiate contact with specialized endothelial cells under hemodynamic shear prior to extravasation across the vascular barrier into tissues. Lymphocyte–endothelial adhesion occurs preferentially in high endothelial venules (HEV) of peripheral lymphoid organs. The continuous recirculation of naïve and central memory lymphocytes across lymph node and Peyer's patch HEV underlies immune surveillance and immune homeostasis. Lymphocyte–endothelial interactions are markedly enhanced in HEV-like vessels of extralymphoid organs during physiological responses associated with acute and chronic inflammation. Similar adhesive mechanisms must be invoked for efficient trafficking of immune effector cells to tumor sites in order for the immune system to have an impact on tumor progression. Here we discuss recent evidence for the role of fever-range thermal stress in promoting lymphocyte–endothelial adhesion and trafficking across HEV in peripheral lymphoid organs. Findings are also presented that support the hypothesis that lymphocyte–endothelial interactions are limited within tumor microenvironments. Further understanding of the molecular mechanisms that dynamically promote lymphocyte trafficking in HEV may provide the basis for novel approaches to improve recruitment of immune effector cells to tumor sites.

Keywords Lymphocyte homing receptors · Adhesion molecules · High endothelial venules · Tumor microvessels · Fever

This article forms part of the Symposium in Writing "Thermal stress-related modulation of tumor cell physiology and immune responses", edited by Elfriede Noessner.

Q. Chen · D. T. Fisher · S. A. Kucinska · W.C. Wang
S. S. Evans (✉)
Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY, 14263-0001 USA
E-mail: sharon.evans@roswellpark.org
Tel.: +1-716-8453421

High endothelial venules: a locus of control for lymphocyte extravasation

High endothelial venules (HEV) are a major site of extravasation of blood-borne lymphocytes and thus provide a model for understanding the molecular mechanisms that control lymphocyte trafficking. HEV are restricted to peripheral lymphoid organs, i.e., lymph nodes (LN) and Peyer's patches (PP), and are morphologically and biochemically differentiated from the majority of vessels throughout the body [1–3]. The lumen of HEV is lined by cuboidal endothelial cells, in contrast to the squamous, elongated endothelial cells of vessels in extralymphoid organs. The irregular surface provided by HEV is thought to contribute to turbulent blood flow within vascular microdomains, thereby promoting margination of lymphocytes along vessel walls.

The molecular basis of lymphocyte extravasation across HEV has been extensively characterized by a combination of in vitro studies (including frozen tissue-section Stamper-Woodruff adherence assays and experiments employing purified surrogate substrates) and in vivo studies (i.e., short-term homing assays and intravital microscopy). These studies have revealed that an elegantly coordinated sequence of adhesion events initiates lymphocyte contact and ultimately, extravasation across HEV. These events include (1) initial tethering and rolling, (2) chemokine activation, (3) firm sticking, and (4) transendothelial migration [2, 4–6]. Each of these adherence steps is reversible. Thus, only a small percentage of cells that undergo tethering and rolling ultimately extravasate within a given vessel. The venular tree that extends through peripheral LN (PLN) organs is segregated into a hierarchy of functionally distinct levels based on the efficiency of the adhesive interactions that occur along the length of individual vessels. Order III–V

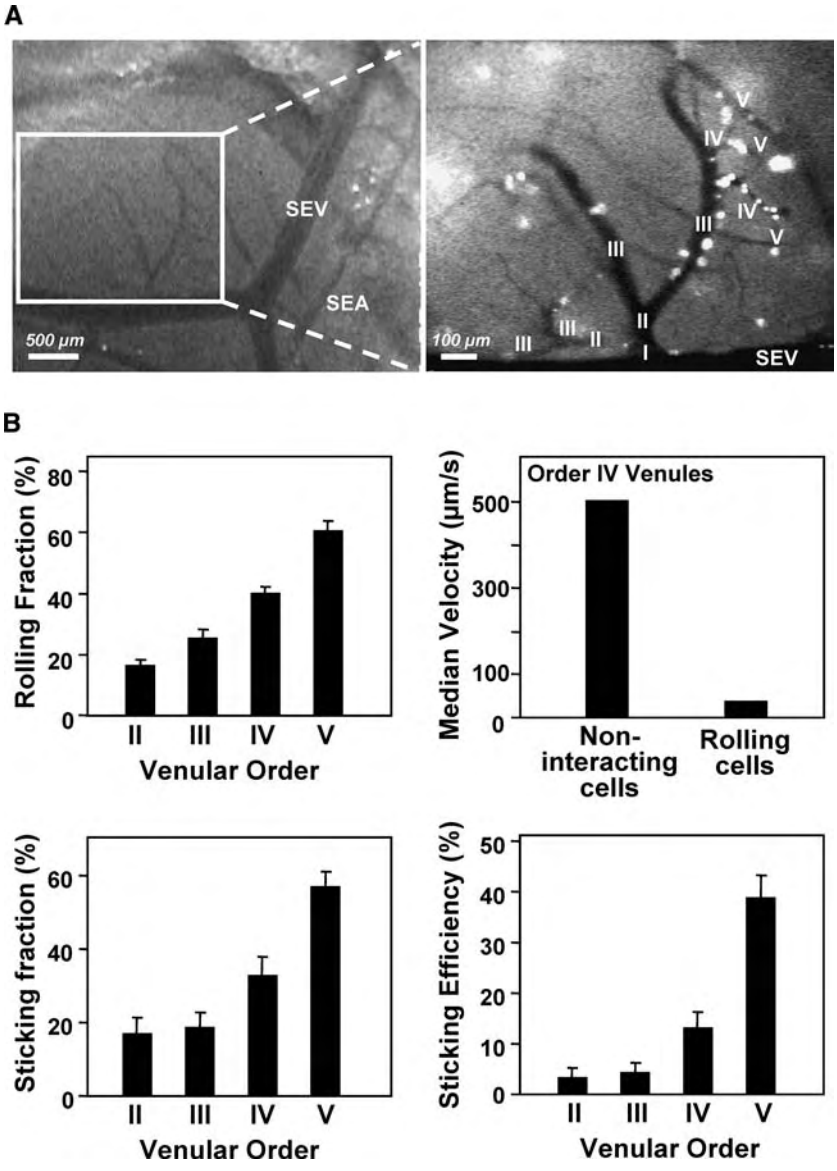


Fig. 1 Analysis of lymphocyte-endothelial interactions in nodal venules by intravital microscopy. **A** Superficial epigastric artery (SEA), superficial epigastric vein (SEV), and nodal venular structure were observed under low power (10X; left photomicrograph) in surface inguinal LN of C57BL/6 mice by epifluorescence intravital microscopy as described previously [11, 91]. Interactions between lymphocytes and nodal venules of different orders were visualized under high power (20×) in the same field following injection of fluorescent-labeled LN cells (~2.5×10^7 cells/mouse; labeled with calcein [1 μg/ml, Molecular Probes, Eugene, OR, USA]) via the femoral artery (right photomicrograph). The majority of fluorescent-labeled, firm sticking cells accumulate in order III–V vessels. **B** Rolling or sticking lymphocytes in different order venules were quantified in two mice. Rolling fraction was defined as the percentage of cells transiently interacting with HEV in the total number of cells passing through the vessel during the observation period, as described by von Andrian and M'Rini [91]. The median velocity of 30 non-interacting cells and 20 rolling cells in order IV venules is shown. Sticking fraction was the percentage of rolling cells that adhered in HEV for ≥ 30 s. Sticking efficiency was the percentage of total cells that arrest on vessel walls for ≥ 30 s [91]. See also Video 1, available at http://www.roswellpark.org/sse/cii2005.

vessels represent post-capillary HEV that are localized primarily in the T-cell-rich paracortical region while large, lower order I collecting venules are in the LN hilus [7, 8] (Fig. 1A). HEV are not detected in the B-cell-rich follicular region, supporting the long-standing notion that T and B cells jointly enter LN through HEV in the paracortex. Recent studies using multi-photon microscopy have shown that dendritic cells that enter draining LN via the afferent lymphatics congregate in the region proximal to HEV [6, 9, 10]. Thus, professional antigen (Ag)-presenting dendritic cells are spatially and temporally positioned to initiate contact with extravasating T cells, a scenario that is optimal for driving an efficient immune response by naïve or memory lymphocytes.

Intravital microscopy has shown that the majority of lymphocyte tethering/rolling and sticking interactions occur in order III–V vessels [7, 8, 11, 12] (Fig. 1B). Although it is difficult to quantify the velocity of lymphocytes in postcapillary order V HEV (because firmly

adherent cells obscure observations), the velocity of free-flowing lymphocytes in order IV venules has been quantified at ~500 μm/s [8]. A subfraction of lymphocytes undergo reversible tethering and rolling under hemodynamic flow in higher order venules such that lymphocyte rolling velocities are reduced to < 50 μm/s [8] (Fig. 1B). The transition from rolling cells to firmly sticking adherent cells (experimentally defined as cells that arrest on vessel walls for ≥30 s) occurs principally in higher order IV–V venules [7, 11, 12].

Transient tethering and rolling interactions in lymph node HEV are mediated by L-selectin molecules located on the microvillous projections of naïve and central memory lymphocytes [2, 4, 6, 13–15]. Positioning of L-selectin on microvilli facilitates contact with sialo-mucin-like adhesion molecules in HEV collectively termed peripheral lymph node addressins (PNAd). PNAd molecules are comprised of a group of proteins including CD34, GLYCAM-1, podocalyxin, endomucin and sgp200 [16]. Common post-translational modifications to these core proteins by core 2β-1,6-*N*-acetylglucosaminyltransferase-1 (C2GlcNAcT-I), high endothelial cell GlcNAc-6-sulfotransferase (HEC-GlcNAc6ST), and α1,3-fucosyltransferases (FucT-VII and FucT-IV) are required for optimal L-selectin-dependent trafficking to PLN [16]. The HEC-GlcNAc6ST-dependent sulfation determinant on PNAd molecules is recognized by MECA-79 mAb staining of high-walled cuboidal HEV [16–18] (Fig. 2). This epitope is highly expressed in order III–V venules and is required for lymphocyte-HEV adhesion and homing to PLN [6, 7]. L-selectin-dependent lymphocyte tethering and rolling in PP HEV is mediated by mucin domains within mucosal addressin cell adhesion molecule-1 (MAdCAM-1) [2, 4]. Interestingly, L-selectin/MAdCAM-1-dependent interactions do not appear to be as efficient as L-selectin/PNAd adhesion, and lymphocytes tend to roll at a higher velocity under strictly L-selectin-dependent mechanisms in PP HEV [2]. The $\alpha 4\beta 7$ integrin lymphocyte homing receptor binds to immunoglobulin-like domains at the N-terminus of MAdCAM-1 and collaborates with L-selectin to reduce the rolling velocity of lymphocytes as they move through PP HEV [2, 4, 19].

Tethering and rolling interactions increase the transit time of lymphocytes in HEV, allowing them to sample chemokine microenvironments on the luminal surface of these vessels. The CC chemokine ligand (CCL)21 (TCA-4/SLC/6C-kine/exodus 2) plays a primary role in triggering the transition of naïve and central memory lymphocytes from rolling cells to firmly adherent/sticking cells in LN and PP HEV [4, 6]. CCL21 secreted by high endothelial cells (HEC) becomes associated with the glycocalyx on the luminal surface of HEV [20, 21]. Ligation of CCL21 by CCR7 receptors on circulating lymphocytes leads to G-protein-dependent conformational changes in the $\beta 2$ integrin, leukocyte-function associated adhesion molecule-1 (LFA-1) [4, 6, 19]. This enables LFA-1 to engage its constitutively expressed endothelial counter-receptors, ICAM-1 and ICAM-2 (members of the immunoglobulin superfamily), on the surface of HEV.

The mechanisms supporting lymphocyte transendothelial migration have not been fully dissected at a molecular level in HEV. Analysis of these events is hampered by the fact that extravasation cannot be visualized by intravital microscopy for technical reasons [22]. Moreover, there are limited in vitro models available for the study of HEV-specific adhesion. It is speculated that LFA-1/ICAM-1-2 contribute to the extravasation process, along with selected chemokine/chemokine receptor interactions (e.g., CCL21/CCR7, and CXCL12/CXCR4) [2–4, 23]. High expression of junctional adhesion molecule-1 (JAM-1) on LN HEV [24] (Fig. 2) raises the possibility that this molecule also participates in transendothelial migration through its ability to function as an alternative ligand for LFA-1, as proposed for extralymphoid sites of inflammation [25].

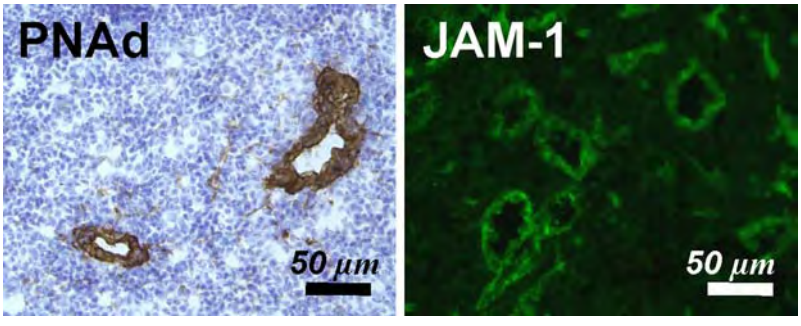


Fig. 2 Expression of PNAd and JAM-1 in PLN HEV. PNAd expression was analyzed on cuboidal HEV of peripheral LN cryosections (9 μm-thick) by immunohistochemical staining (*left panel*; note brown staining of individual HEC by rat anti-mouse PNAd primary mAb [BD Bioscience, San Diego, CA, USA] and biotin-conjugated goat anti-rat secondary Ab [BD Bioscience]). JAM-1 on HEV was detected by immunofluorescent staining (*right panel*; green fluorescent staining with goat anti-mouse JAM-1 primary antibody [R&D System, Minneapolis, MN, USA] and FITC-conjugated mouse anti-goat secondary Ab [Jackson ImmunoResearch, West Grove, PA, USA])

HEV-like vessels control trafficking in extralymphoid sites of inflammation

There are a number of parallels between the lymphocyte–endothelial interactions that continuously occur in

lymphoid organs and the inducible adhesive mechanisms in extralymphoid sites of inflammation. Under noninflammatory conditions, squamous endothelial cells in vessels of tertiary organs do not efficiently support lymphocyte adhesion under hemodynamic shear. However, cuboidal HEV-like vessels have been identified at multiple extralymphoid sites of acute and chronic inflammation [1, 16, 26, 27]. Moreover, these vessels are decorated with a vast array of adhesion molecules and chemokines that can mediate lymphocyte tethering/rolling, firm adherence, and extravasation. Proinflammatory cytokines produced locally in response to infection or inflammation such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), lymphotoxin, interferon-α (IFN-α), IFN-γ, or IL-6, regulate the morphology of these vessels as well as the synthesis or expression of numerous adhesion molecules (e.g., ICAM-1, E-selectin, VCAM-1, VAP-1) and chemokines (e.g., MIG [CXC9], IP-10 [CXC10], RANTES [CCL5]) that promote recruitment of effector/memory T cells to peripheral sites [16, 19, 28, 29].

Several endothelial adhesion molecules that were originally thought to be restricted to HEV of peripheral lymphoid organs have been found to be ectopically expressed on HEV-like vessels at extralymphoid sites of chronic inflammation [1, 16]. For example, HEV-like vessels that express PNAd or CCL21 have been identified in inflamed synovium of rheumatoid arthritis patients [30]. These vessels are associated with dense infiltrates of perivascular CD45RA$^+$ naïve T cells. Similar expression of PNAd, MAdCAM-1, or CCL21 has been documented at sites of chronic inflammation in patients with Crohn's disease, ulcerative colitis, diabetes, and thyroiditis, or in experimental animal models for these diseases [1, 16, 31, 32]. These results suggest that under the appropriate microenvironmental conditions, trafficking of L-selectin$^+$/CCR7$^+$ naïve or central memory T lymphocytes can be promoted to tertiary tissues through common mechanisms involved in the continuous recirculation of lymphocytes through lymphoid organs.

Fever-range thermal stress promotes lymphocyte trafficking across HEV

Febrile temperatures have been associated with improved survival in endothermic and ectothermic species, although the mechanisms underlying the physiologic benefit of fever are not well defined [33–35]. A recent series of studies, detailed below, have shown that fever-range hyperthermia actively promotes egress of blood-borne lymphocytes across HEV in LN and PP. The molecular mechanisms underlying thermal control of lymphocyte trafficking are complex and involve independent responses in both lymphocytes and HEC. These observations support the notion that febrile temperatures associated with infection, inflammation, or clinical thermal therapy act as a danger signal to heighten immune surveillance by regulating lymphocyte entry into secondary lymphoid organs.

Exposure of mice or cancer patients to fever-range whole body hyperthermia (WBH), using experimental methods developed by Repasky et al. to raise the core temperature to the range of physiologic fever [36], decreases the number of lymphocytes in the circulation [36–38]. In mice it was shown that these lymphocytes redistribute selectively to lymphoid organs that express HEV (i.e., to LN and PP, but not spleen) [38]. The mechanisms responsible for enhanced trafficking were first examined in lymphocytes. Culture of murine lymphoma cell lines (i.e., 300.19/L-selectin transfectant B cell line or $\alpha4\beta7^{hi}$/L-selectin^{lo} TK1 T cells) or primary lymphocyte populations (i.e., human peripheral blood lymphocytes [PBL] or mouse splenocytes) under conditions that simulate the temperature and duration of natural fever (i.e., 38–40°C for 2–6 h) causes a marked increase in the binding activity of L-selectin and $\alpha4\beta7$ integrin [14, 15, 38–42]. The binding function of these homing receptors was assessed in frozen-section in vitro adherence assays and in vivo homing studies, using blocking mAb directed against L-selectin/PNAd and $\alpha4\beta7$/MAdCAM-1 adhesion partners [14, 15, 38–40]. An example of this type of study is shown in Fig. 3. In these experiments, mouse lymphocytes from spleen or LN were cultured at febrile temperatures (40°C) for 6 h, and L-selectin binding function was evaluated by an in vitro adherence assay, as described in [14, 38, 41]. In both lymphocyte populations, heat-treatment markedly stimulated L-selectin-dependent adhesion under shear to HEV in mouse LN cryosections. These findings suggest that homing receptor function is regulated by thermal stress in lymphocytes of multiple organs during physiologic febrile responses. Importantly, thermal stimulation of homing receptor function is not restricted to in vitro studies. In this regard, splenocytes isolated from fever-range WBH-treated mice are characterized by enhanced L-selectin or $\alpha4\beta7$ integrin-dependent adhesion when compared with splenocytes from normothermal control animals [38, 41, 43].

Multiple lymphocyte subsets respond to thermal stimulation in vitro including CD45RA$^+$ naïve lymphocytes, CD45RO$^+$ memory lymphocytes, CD4$^+$ and CD8$^+$ T cells, CD19$^+$ B cells, and CD56^{bright} NK cells, while L-selectin-dependent adhesion is not increased by thermal stress in CD14$^+$ monocytes [41]. These observations are consistent with the notion that an important contribution of fever is to amplify the immune response by recruiting naïve and central memory lymphocytes to lymphoid organs. Moreover, thermal control of L-selectin-like adhesion is highly conserved in vertebrate species that diverged over 300 million years ago (i.e., mammals [humans and mice] and avian [chicken] species) [14, 35, 41], raising the possibility that this response confers a survival benefit that was retained during evolution.

Several lines of evidence are consistent with the hypothesis that fever-range thermal stress causes a

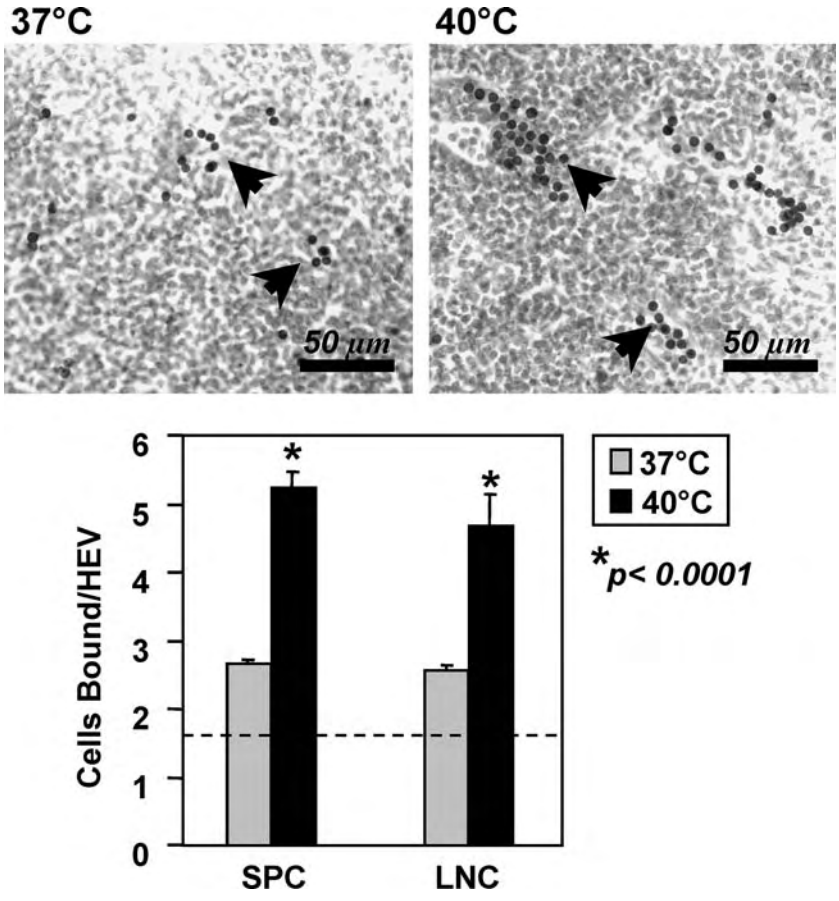


Fig. 3 Fever-range thermal stress activates lymphocyte adhesion to PLN HEV in vitro. Lymphocytes were isolated from spleen (SPC) or LN organs (LNC; pooled PLN and MLN) of BALB/c mice and then cultured in vitro at 37°C or 40°C for 6 h. Lymphocyte adherence to HEV in cryosections of BALB/c PLN was evaluated under mechanical shear as described in [14, 15, 41]. Photomicrographs show typical images of toluidine-stained lymph node cells (LNC) (*black arrows*) bound to HEV in PLN tissue cryosections. The number of adherent lymphocytes was quantified by light microscopy (Olympus, Spectra Services Inc., Ontario, NY, USA) in a total of 300–500 HEV in PLN cryosections. For consistency in double-blind evaluation, HEV were quantified only if they contained ≥ 1 adherent cell. The *dotted line* indicates the level of adhesion when lymphocytes were treated with functional blocking antibody to mouse L-selectin (Mel-14; American Type Culture Collection [ATCC, Rockville, MD, USA]). Data are the mean ± SE of triplicate samples in two experiments. Results are representative of ≥ 3 experiments. The differences between adhesion of untreated cells and hyperthermia-treated cells were significant, $*P < 0.0001$, using an unpaired two-tailed Student's t test

change in the avidity and/or affinity of lymphocyte homing receptors rather than affecting the synthesis or surface density of these molecules. In this regard, thermal stimulation of primary lymphocytes (human PBL, mouse splenocytes) or murine cell lines (TK1 cells, 300.19/L-selectin transfectant cells) does not affect the cell surface expression, mRNA levels, or total cellular content of L-selectin or $\alpha 4\beta 7$ integrin [14, 15, 39, 41]. Moreover, in the case of L-selectin, heat does not alter the lectin activity or positioning on microvillous projections [14]. Insight into the mechanisms controlling L-selectin adhesion is provided by findings that febrile temperatures cause L-selectin to become stably associated with the detergent-insoluble cytoskeletal matrix [15, 41, 42]. This is in contrast to observations under normothermal conditions where L-selectin is highly susceptible to extraction by mild detergents [15, 41, 44]. Thermal stimulation of L-selectin-cytoskeletal associations and L-selectin adhesion is dependent on an 11-amino acid region within the C-terminal cytoplasmic domain that contains a binding site for the cytoskeletal linker protein, α-actinin [15, 38, 45–47]. One interpretation of these findings is that thermal stress promotes L-selectin tensile strength and, thereby, the efficiency with which it withstands physiologic hemodynamic shear within HEV by stabilizing interactions between the cytoplasmic domain of L-selectin and the structural cytoskeleton.

Conditioned medium derived from heat-treated lymphocytes contains proadhesive factors that are responsible for activating L-selectin binding function [14, 40–42]. Thus, thermal effects on L-selectin adhesion cannot be attributed to direct effects of heat on plasma membrane dynamics or to the conformation of L-selectin or cytoskeletal proteins. These findings were initially surprising since soluble factors had not been previously shown to regulate L-selectin adhesion in lymphocytes. A role for both autocrine and paracrine-derived factors is

further implicated by findings that multiple cell types release proadhesive factors in response to heat including hematopoietic cells (B and T lymphocytes, monocytes) and stromal cells (endothelial cells, fibroblasts), while cell lines that represent parenchymal cells of various organs (skin, brain, liver, breast, lung) are non-responsive [40, 41].

IL-6 was identified as the central factor responsible for regulating L-selectin-cytoskeletal interactions and L-selectin adhesion in response to thermal stress in vitro and in vivo [41]. Other proinflammatory cytokines such as TNF-α, IL-1β, IL-11, oncostatin M, or leukocyte inhibitory factor (LIF) do not contribute to thermal stimulation of L-selectin adhesion under physiologic conditions. Notably, both IL-6 and a soluble form of the IL-6 receptor (sIL-6Rα) binding subunit are required to enhance L-selectin-dependent adhesion of lymphocytes to HEV in vitro and in vivo [41]. These observations support the concept that thermal control of lymphocyte adhesion depends on IL-6 trans-signaling whereby IL-6 and sIL-6Rα initiate lymphocyte responses through the transmembrane gp130 signal-transducing chain [48, 49]. Further studies positioned MAPK1/ERK1-2, but not other stress-related MAPK (p38 MAPK, JNK) in the trans-signaling pathway linking IL-6/sIL-6Rα-initiated extracellular responses to activation of L-selectin adhesion [41]. Unlike several acute or chronic inflammatory conditions (i.e., Crohn's disease, rheumatoid arthritis, bacterial infection, cancer), where elevated amounts of IL-6 or sIL-6Rα are detected [49, 50], thermal stress appears to enhance the bioactivity and/or bioavailability of IL-6/sIL-6R complexes without changing the molar concentrations of ligand or receptor [41].

Recent studies have revealed that fever-range thermal stress can also promote endothelial adhesion in HEV of LN and PP [38]. These studies demonstrate that elevation of mouse core body temperatures to a febrile range (39.5–40°C) by WBH treatment causes an increase in PNAd and MAdCAM-1-dependent adhesion in HEV of LN or PP that can be detected in frozen-section in vitro adherence assays. Thermal stimulation of HEV adhesion is not accompanied by any apparent change in the amount of PNAd or MAdCAM-1 displayed on HEV. Similar increases in HEV adhesion are observed during natural febrile responses to systemic (LPS) or local (turpentine) inflammatory stimuli [38]. Increases in HEV adhesion are also detected in response to hyperthermia treatment of LN and PP organ cultures in vitro [38]. These data suggest that HEV adhesion is regulated within the local lymphoid microenvironment and does not require involvement of other organ systems including the highly integrated hypothalamus-pituitary-adrenal axis which is known to contribute to the physiology of febrile responses [51].

Thermal stimulation of vascular adhesion is tightly controlled at multiple levels. Enhanced HEV adhesion requires sustained exposure to thermal stress [38]. Moderate effects are observed after fever-range WBH treatment for 2 h, whereas marked increases in HEV adhesion are detected after 6–8 h. Moreover, adhesion rapidly returns to basal levels following the removal of the heat stimulus, as would be predicted during natural febrile responses, where it is important to heighten lymphocyte trafficking and immune surveillance over a finite period of time.

Thermal effects on HEV adhesion are also tightly regulated with respect to the endothelial target. Robust increases in adhesion are observed following fever-range WBH treatment in cuboidal, differentiated HEV of LN and PP but not in squamous, less differentiated endothelium of non-lymphoid tissues (i.e., pancreas) [38]. Moreover, fever-range thermal stress does not alter the expression of adhesion molecules (ICAM-1, E-selectin, P-selectin, PECAM, VCAM-1, PNAd, or MAdCAM-1), chemokines (IL-8, RANTES, MCP-1, MIP-1β, MIG), or cytokines (IL-1β, IL-6, IL-11, IL-12, IL-13, TGF-β1) in non-activated (squamous) primary endothelial cells in vitro (i.e., macrovascular human umbilical vein endothelial cells [HUVEC] or microvascular human dermal microvascular endothelial cells [HMVEC]) [40]. These results are consistent with observations that fever-range WBH promotes lymphocyte trafficking to organs bearing HEV (i.e., LN or PP) but not to sites that lack HEV (i.e., spleen, pancreas) [38] (Fig. 4). Note that in these experiments, mice pretreated with WBH are allowed to revert to their normal basal temperature prior to adoptive transfer of fluorescent-labeled lymphocytes in order to assess vascular responses to elevated temperatures. Selective regulation of adhesion in differentiated HEV, but not squamous endothelium, would be expected to focus the immune response to lymphoid tissues and sites of infection while preventing the unproductive exodus of lymphocytes to other tissues during a physiologic febrile episode. On the basis of the estimate that the rate of extravasation across HEV under normothermal conditions in humans is $\sim 5\times10^6$ cells/s [1], it is predicted that the 2- to 5-fold increase in trafficking observed in response to fever-range WBH (Fig. 4) [38] reflects a physiologically significant enhancement in the number of lymphocytes that gain access to secondary lymphoid organs in the context of natural fever.

Collectively, findings that fever-range thermal stress dually regulates adhesion in lymphocytes and HEV provide evidence for a unifying mechanism whereby febrile temperatures dynamically modulate regional recruitment of circulating lymphocytes to peripheral lymphoid organs during infection and inflammation. An important question for future investigation relates to the molecular mechanisms underlying thermal control of HEV adhesion. It is tempting to speculate that local cytokine networks are involved since vascular adhesion is known to be regulated by proinflammatory cytokines [28, 29]. Moreover, thermal stress has been shown to control the synthesis or bioactivity of multiple cytokines (i.e., TNF-α, IL-1β, IL-6) [35, 41, 52–55], suggesting that one or more of these inflammatory agents contributes to thermal stimulation of HEV adhesion.

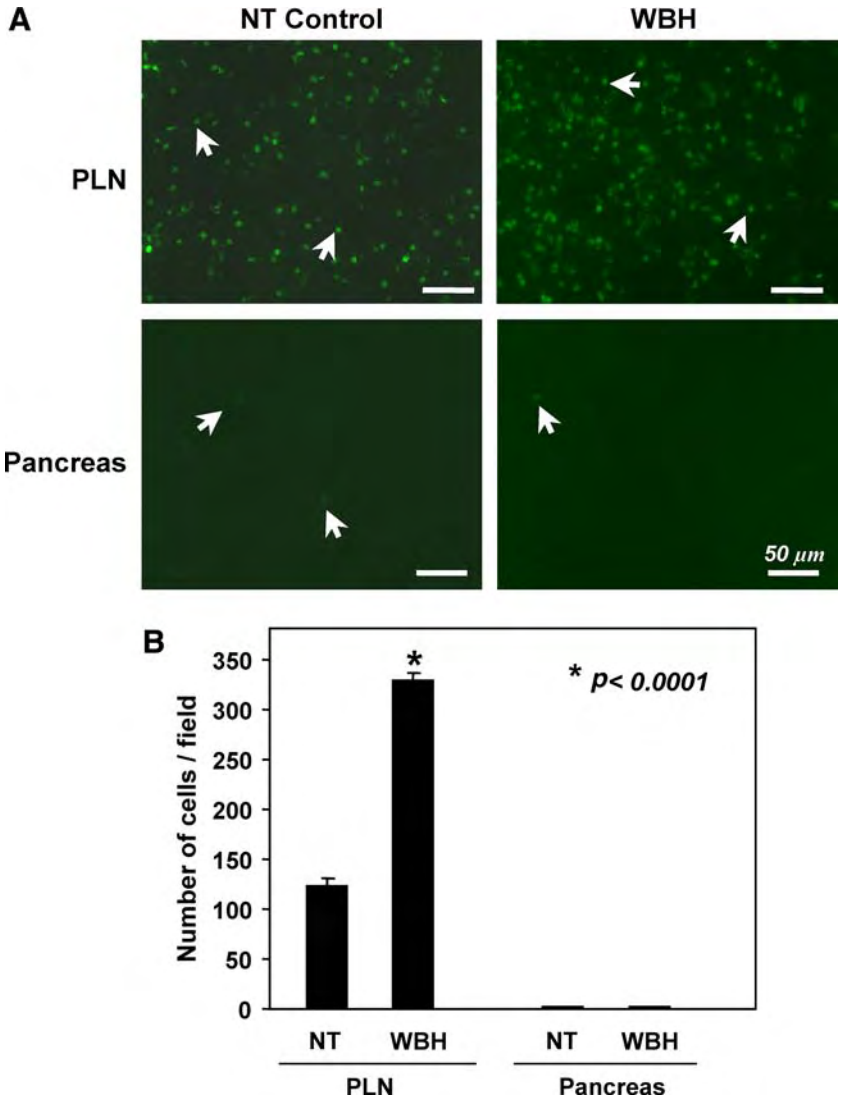


Fig. 4 Fever-range WBH stimulates lymphocyte homing to PLN in vivo. Calcein-labeled splenocytes were injected intravenously (5×10^7 cells/mouse) into normothermal (NT) control BALB/c mice (core temperature, 36.8 ± 0.2°C) or mice pretreated with fever-range WBH (core temperature, 39.5 ± 0.5°C, 6 h) and allowed to resume normothermal temperatures, as described in [38, 41]. After 1 h, PLN and pancreatic organs were removed and cryosections were prepared. Calcein-labeled green-fluorescent cells were observed and quantified by fluorescence microscopy. **A** Micrographs are images from different organs; the *arrows* indicate the typical morphology of calcein-labeled cells that were included in the quantification. **B** Numbers of fluorescent cells were counted in 10 fields (0.335 mm^2/field) of non-sequential tissue sections. Data are the mean ± SE ($n=2$ mice per group; data are representative of four independent experiments). The differences between splenocyte homing to PLN in NT control mice and WBH-treated mice were significant, $*p<0.0001$, using an unpaired two-tailed Student's *t* test

Perspectives on lymphocyte trafficking to tumor microenvironments

Control of tumor growth by the immune system involves a highly complex interplay between professional Ag-presenting cells, immune effector cells, tumor targets, and the tumor microenvironment [56–58]. Recognition of tumor Ag by T cells depends on efficient priming by Ag-presenting dendritic cells in regional lymph nodes. Moreover, productive interactions between cytotoxic T cells and tumor cells depend on sustained survival and retention of T cells within tumor sites. Emerging data suggest that regulatory T cells have a negative impact on anti-tumor immune responses in lymphoid organs and tumor tissues [59, 60]. One critical determinant of successful immune-based anti-tumor responses relates to the capacity of immune effector cells ($CD8^+$ cytolytic T cells, NK cells, neutrophils) to gain access to tumor tissues across the vascular endothelial barrier. The tumor microenvironment is often highly vascularized by convoluted, disorganized vessels, although intravital microscopy in experimental animal models reveals that these vessels are competent to support blood flow [61–63] (Fig. 5). Despite an extensive vasculature, leukocyte infiltration into the interior of tumor tissues is frequently limited. In this regard, dense leukocyte accumulations have been documented in the peritumoral region surrounding tumor nodules in cancer patients [64–68]. This regionalized localization correlates with the expression of adhesion molecules (E-selectin, ICAM-1, PNAd, P-selectin, and VCAM-1) and chemokines (MIG, IP-10) in peritumoral regions of human primary melanoma and colorectal cancer specimens or other tumor types [64, 65, 69–72]. In sharp contrast, these molecules are poorly expressed within intratumoral vessels, paralleling the overall exclusion of lymphocytes from this region. The failure of leukocytes to infiltrate tumor sites has been correlated with a poor prognosis in melanoma and lung cancer [66, 67, 73]. However, it is important to note that tumor growth potential cannot be predicted solely by the magnitude or nature of inflammatory infiltrates. Tumor

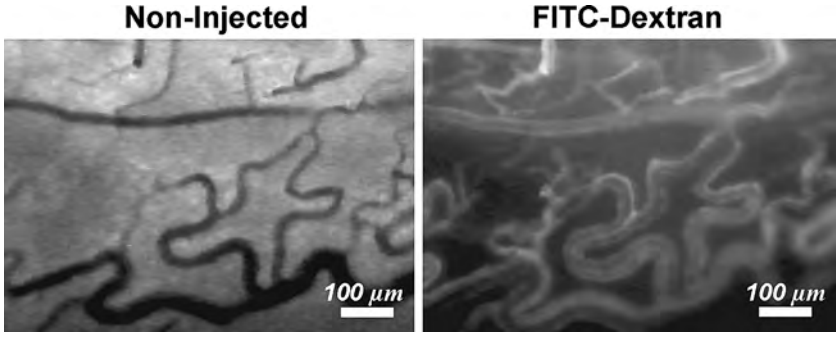


Fig. 5 Analysis of blood flow in *s.c.* murine colon 26 tumors by intravital microscopy. Dorsal skinfold window chambers were implanted in BALB/c mice as described in [92, 93]. In brief, a 12-mm-diameter hole was dissected through one layer of dorsal skinfold to expose the fascial plane in the other layer of skinfold. Colon 26 cells (2×10^4) were injected into the fascial plane at the time of surgery. In 9–14 days, tumors grew to 3–4 mm in diameter and were well vascularized inside the window chamber. The structure of tumor microvessels was observed under epifluorescence light microscopy (*left panel*). Blood flow in the same field was detected by injection of fluorescent-labeled FITC-dextran (10 mg/ml, 10 ml/kg body weight, Sigma-Aldrich, St. Louis, MO, USA) via the tail vein (*right panel*). See also Video 2, available at http://www.roswellpark.org/sse/cii2005.

tissues are highly heterogeneous with respect to lymphocyte infiltration among individuals and even within a single lesion [65]. Moreover, there are multiple reports indicating that local inflammation is positively correlated with tumor progression [74–76], which may reflect an imbalance in accumulation of proinflammatory macrophage or regulatory T cells compared with $CD8^+$ T cells and other immune effector cells.

Parallel findings of limited lymphocyte infiltration are observed in numerous murine experimental models. One example is RIP-Tag5 mice where expression of the SV40 large T antigen (Tag) transgene under control of the rat insulin promoter (RIP) drives the proliferation of pancreatic islet cells and the development of endocrine pancreatic tumors [77] (Fig. 6). The intratumoral region of pancreatic islet tumors is generally devoid of infiltrating leukocytes including $CD3^+$ T cells, whereas dense leukocyte accumulations can sometimes be detected in the peritumoral region [78–83] (Fig. 6). Although RIP-Tag5 tumors are highly vascularized (note that numerous vessels were stained for the pan-endothelial adhesion molecule, $CD31^+$, Fig. 6), these vessels are typically flat-walled structures compared to the cuboidal endothelium lining specialized HEV in PLN that support lymphocyte extravasation (Fig. 2). Moreover, there is a limited intratumoral expression of hallmark adhesion molecules (ICAM-1, VCAM-1) or chemokines (MIG, IP-10) which are known to recruit activated T cells [78–80] (Fig. 6). These findings are consistent with evidence that leukocytes do not interact efficiently with tumor vessels of RIP-Tag5 mice analyzed by intravital microscopy [79].

A major challenge is to identify mechanisms to promote trafficking to tumor sites while maintaining vascular selectivity. Systemic administration of potent inflammatory mediators such as cytosine-phosphate-guanine containing oligodeoxynucleotides (CpG) has been used based on the rationale that this agent can trigger an inflammatory milieu and promote lymphocyte infiltration by engagement of the innate immune system, particularly macrophages through toll-like receptor-9 [84]. Anti-tumor activity and enhanced trafficking induced by CpG could also be related to the generation of qualitatively improved T cell responses as a result of increased cytokine production, NK cell stimulation, and enhanced generation of Th1 T cell responses [80]. When systemic CpG was used in combination with the adoptive transfer of tumor specific (SV40 Tag) T cells in RIP-Tag mice, profound induction of ICAM-1 and VCAM-1 occurred in intratumoral lesions that was accompanied by significant intratumoral infiltration of tumor-specific $CD4^+$ and $CD8^+$ T cells [80]. Moreover, significantly improved survival was obtained when this treatment was applied in the early stages of carcinogenesis. Similar results were reported when RIP-Tag mice were treated with ionizing irradiation (at a dose that does not affect tumor growth) followed by adoptive transfer of Tag-specific T [79, 81–83]. An issue that remains to be addressed relates to the lack of selectivity of vascular responses to systemic inflammatory mediators. In this regard, CpG treatment has been shown to induce adhesion molecule expression on endothelial cells in the liver microenvironment and subsequent liver damage in a T-cell-mediated autoaggression mouse model [85]. If CpG and other systemic inflammatory mediators broadly promote lymphocyte adhesion in vascular beds of multiple organs, this could dilute the impact of the anti-tumor response while simultaneously promoting inappropriate contact between lymphocytes and normal bystander tissues.

An alternative strategy has been developed to use local inflammatory stimuli to promote leukocyte recruitment and impede tumor progression. In one example of this experimental approach, transfection of LIGHT (i.e., a TNF-α family member) into fibrosarcoma cells causes recruitment of $CD8^+$ T cells into transplanted tumors and overall improved survival [86]. Intratumoral infiltration by T cells was correlated with increased expression of chemokines and adhesion molecules at the protein and/or mRNA level (i.e., CCL21, MIG/IP-10, and MAdCAM-1). Intriguingly, T cell

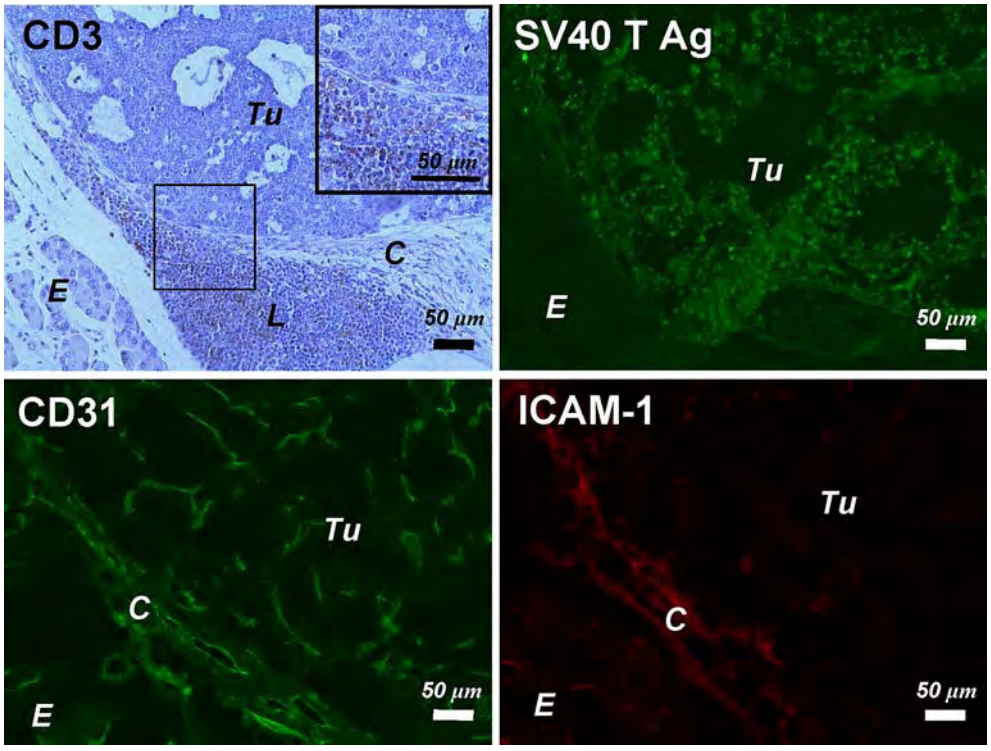


Fig. 6 CD3 lymphocyte infiltration and expression of adhesion molecules is restricted to the peritumoral region of RIP-Tag5 pancreatic tumors. Dense leukocyte (L) infiltrates containing $CD3^+$ T cells (indicated by brown staining obtained using rat anti-CD3 primary mAb [Serotec, Raleigh, NC, USA] and biotin-conjugated goat anti-rat secondary Ab [BD Bioscience, San Diego, CA, USA]) were detected in RIP-Tag5 mice (22–23 weeks) by immunohistochemical staining in the peritumoral region, outside of the edge of pancreatic islet tumors (Tu) demarked by the capsule (C). An enlargement of the designated region is shown in the inset in the *upper left panel*. $CD3^+$ T cells were rarely observed inside pancreatic islet tumors or in exocrine (E) pancreatic tissues. SV40 large T antigen expression was detected in pancreatic islet tumor cells, but not in exocrine pancreatic tissues by immunofluorescence staining (mouse anti-SV40 large T antigen primary mAb [BD Bioscience] and FITC-labeled goat anti-mouse secondary Ab [BD Bioscience]). Immunofluorescence microscopy revealed that vessels expressing the pan-endothelial adhesion molecule, CD31, (indicated by green fluorescence staining obtained using rat anti-mouse CD31 primary mAb [BD Bioscience] and FITC-labeled goat anti-rat secondary Ab [BD Bioscience]) are evident throughout the intratumoral region, in the exocrine pancreatic tissue and in the capsular region, while expression of ICAM-1 (indicated by red fluorescent staining obtained using hamster anti-mouse ICAM-1 mAb [BD Bioscience] and PE-conjugated mouse anti-hamster secondary Ab [BD Bioscience]) was primarily limited to the peritumoral region associated with the tumor capsule

proliferation is observed within LIGHT-transfected tumors, suggesting that activation and expansion of naïve or memory T cells can occur in situ. The introduction of inflammatory cytokines into a tumor microenvironment appears to be a powerful approach to induce regionalized lymphocyte recruitment. However, a limitation to the practical application of this approach is that it depends on the knowledge of the location of tumor nodules since potent inflammatory agents must be administered locally in the context of the tumor microenvironment to avoid indiscriminant activation of vascular adhesion and widespread inflammation in tertiary organs.

It will be of interest to determine if the mechanisms induced by thermal stress can bridge the gap between these two alternative strategies. Fever-range WBH has already been shown to improve leukocyte infiltration in murine tumor models [36, 43, 65, 87–89]. Moreover, fever-range WBH induces a moderate delay in tumor progression in a non-vaccine setting in rodent models where the frequency of tumor-reactive T cells is likely limited [36, 43, 87–90]. Improved clinical responses might be expected if thermal stress is used in combination with tumor vaccines where elevated numbers of tumor-reactive T cells would be available for recruitment.

A major question relates to whether thermally induced leukocyte trafficking to tumor tissues reflects acute changes in vascular adhesion within the tumor microenvironment. Further study is required to determine if tumor vessels behave like specialized HEV of lymphoid organs or like resting endothelium of extra-lymphoid sites that are refractory to fever-range thermal stress (Fig. 7). Preliminary data from our laboratory indicate that improved lymphocyte adhesion can be detected in tumor vessels following fever-range WBH (Q. Chen and S. S. Evans, unpublished observations), suggesting that the unique tumor microenvironment enables squamous endothelium to be dynamically regulated by thermal stress. Thus, fever-range thermal therapy has the potential to amplify adhesion and trafficking of immune cells to restricted vascular beds

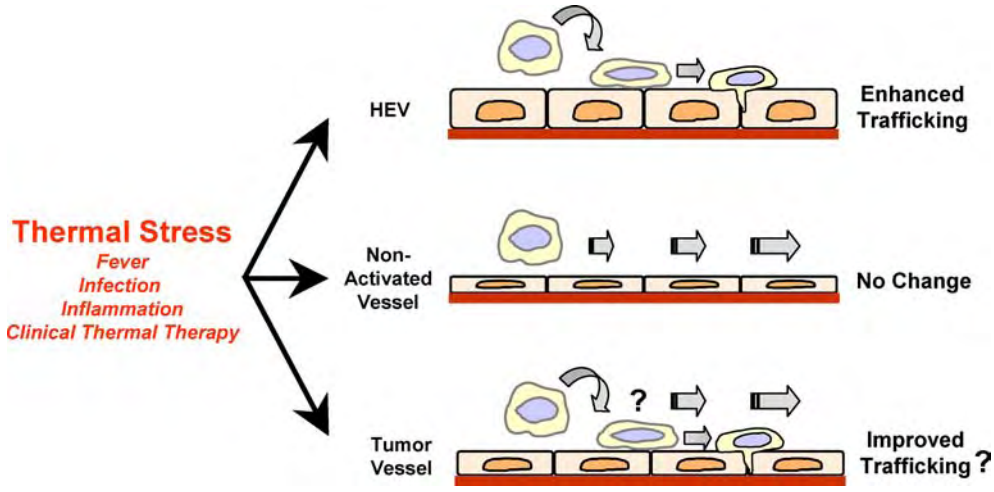


Fig. 7 Model for regulation of vascular adhesion and trafficking in response to fever-range thermal stress. Fever-range thermal stress acts independently on lymphocytes and cuboidal HEV to enhance trafficking in LN and PP HEV. No change in vascular adhesion or homing is observed in response to thermal stress across squamous, non-activated endothelium of extralymphoid organs. It remains to be determined if thermal enhancement of lymphocyte infiltration in tumor sites is mediated by changes in adhesion in tumor microvessels

including HEV of lymphoid organs and tumor microvessels while sparing non-activated endothelium in vessels of other organs (Fig. 7). Through these mechanisms, thermal stress in the context of clinical therapy, or during natural fevers associated with infection and inflammation, would be predicted to improve immune surveillance of peripheral tissues. Notably, the use of whole body thermal therapy obviates the need for information about the location of micrometastases and is theoretically not limited by the total tumor volume or tissue depth. Moreover, there is the potential to enlist multiple mechanisms by stimulating adhesion in tumor microvessels as well as the homing potential of tumor-specific lymphocyte subsets at distal sites of T cell priming by tumor antigens (i.e., draining LN, spleen). An important consideration will be to determine if fever-range thermal stress affects adhesion or survival programs in tumor cells, per se, which could clearly have an impact on tumor progression and metastasis. In this regard, published studies, discussed above, have shown that fever-range temperatures promote L-selectin and $\alpha 4\beta 7$ integrin-dependent trafficking of murine lymphoma cells to lymphoid organs (i.e., related to blood-borne metastatic mechanisms) [15, 38, 39]; however, the response of malignant cells comprising solid tumors remains to be investigated. Future understanding of how fever-range thermal stress contributes to lymphocyte trafficking is expected to have considerable clinical relevance for the development of novel strategies to either promote immune surveillance of peripheral tissues (i.e., during treatment of acute infections or cancer) or interfere with lymphocyte trafficking during pathologic conditions associated with chronic inflammation (e.g., autoimmune disorders).

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Hurdles to Lymphocyte Trafficking in the Tumor Microenvironment: Implications for Effective Immunotherapy

Daniel T. Fisher,[1] Qing Chen,[1] Michelle M. Appenheimer,[1] Joseph Skitzki,[2] Wan-Chao Wang,[1] Kunle Odunsi,[1,3] and Sharon S. Evans[1]

[1]Department of Immunology, Roswell Park Cancer Institute, Buffalo, New York
[2]Department of Surgery, Case Western Reserve University, Cleveland, Ohio
[3]Department of Gynecologic Oncology, Roswell Park Cancer Institute, Buffalo, New York

An important consideration in the development of T cell-based cancer immunotherapy is that effector T cells must efficiently traffic to the tumor microenvironment in order to control malignant progression. T cell trafficking to target tissues is orchestrated by dynamic interactions between circulating lymphocytes and endothelial cells lining blood vessels. It is informative, in this regard, to compare and contrast the molecular mechanisms governing lymphocyte extravasation at distinct vascular sites: (1) high endothelial venules (HEV) of secondary lymphoid organs, which are portals for efficient trafficking of naive and central memory T lymphocytes; (2) non-activated endothelium of normal tissues that mediate relatively low basal levels of trafficking but are rapidly transformed into HEV-like vessels in response to local inflammatory stimuli; and (3) vessels within the intratumoral region and the surrounding peritumoral areas. These vessels can be distinguished by differential expression of hallmark trafficking molecules that function as molecular beacons directing lymphocyte migration across vascular barriers. This article reviews evidence that recruitment of effector T cells to the intratumoral microenvironment is impeded by sub-threshold expression of trafficking

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Address correspondence to Sharon S. Evans, Roswell Park Cancer Institute, Buffalo, New York, 14263-0001, USA; E-mail: sharon.evans@roswellpark.org

molecules on tumor microvessels. Emerging data support the thesis that when considered from the perspective of extravasation, vessels embedded within the intratumoral microenvironment of established tumors do not exhibit stereotypical characteristics of a chronic inflammatory state. A major challenge will be to develop therapeutic approaches to improve trafficking of effector T lymphocytes to tumor sites without skewing the balance in favor of a chronic inflammatory milieu that facilitates tumor maintenance and progression.

Keywords Fever-range thermal stress, immunotherapy, lymphocyte trafficking, tumor microenvironment, vascular adhesion.

INTRODUCTION

Inflammation has emerged as a double-edged sword in cancer biology. On the one hand, inflammatory stimuli are considered a driving force underlying early progression and long-term maintenance of cancer. This topic has been comprehensively reviewed in several recent articles (Balkwill et al., 2005; de Visser et al., 2006; Joyce, 2005; Philip et al., 2004; Vakkila and Lotze, 2004). The prevailing concept is that cellular components of the innate immune response (i.e., mast cells, neutrophils, and tumor-associated macrophages [TAM]), contribute to the malignant process by releasing inflammatory mediators (e.g., cytokines, chemokines) within the tumor microenvironment that support the proliferative and survival programs of tumor cells as well as host cells such as vascular endothelial cells. A strong correlation exists between inflammation and certain malignancies, further supporting the view that proinflammatory factors are causally related to cancer development. Patients with chronic inflammatory disorders of the gastrointestinal tract such as Crohn's and colitis have a high incidence of colon cancer; infections by human papilloma virus and hepatitis C predispose patients to cervical cancer and hepatocellular cancer, respectively; and infection by the gram-negative bacterium, *Heliobacter pylori*, has been associated with gastric cancers (Balkwill et al., 2005; Coussens and Werb, 2002; Joyce, 2005). These observations provide a rationale for the investigation of anti-inflammatory therapeutic strategies to disrupt cancer progression.

An alternative, but equally compelling thesis is that inflammatory infiltrates consisting of immune effector cells such as cytotoxic T lymphocytes (CTL), natural killer cells (NK cells), and cytolytic macrophages, can profoundly limit tumor progression by directly targeting tumor cells or destroying supportive elements such as vascular structures within tumor tissues. Paradoxically, entry of immune effector cells into nonlymphoid tissues depends on a local inflammatory response, the very process that is believed to drive malignant progression. The sequence of events required for effector cell recruitment to tumor tissues parallels natural tissue responses to infectious agents (viruses, bacteria) where an influx of innate inflammatory cells is required to initiate cues on blood vessels that, in turn, recruit additional waves of

immune effector cells. While the concept of immune surveillance for the elimination of cancer has gone through multiple phases of acceptance and discredit since it was first proposed by Burnet (1970), there is now strong evidence to indicate that local effector T cells can influence the clinical course in cancer patients, as discussed in several extensive reviews (Boon et al., 2006; Chen et al., 2003; Dunn et al., 2004; Marincola et al., 2003; Pardoll, 2002). Data that support this hypothesis include: (1) the identification of tumor antigens that are recognition determinants for $CD4^+$ T helper cells and $CD8^+$ cytolytic T lymphocytes; (2) findings that adoptive transfer of tumor-reactive T cells confers antitumor responses in preclinical studies and patients, and (3) evidence in experimental animal models and patients that extensive T cell infiltration of tumor tissues correlates with a favorable prognosis.

Important factors that likely contribute to positive or negative clinical outcomes include the relative balance of the types of innate and effector immune cells that infiltrate tumor sites and the precise microanatomical location of infiltrates within neoplastic lesions (i.e., intratumoral versus stroma rich peritumoral regions). The process that controls leukocyte entry into compartmentalized regions of tumor tissues remains an underexplored area of investigation. This review will compare and contrast T cell trafficking in tumor sites with the well established mechanisms that direct T cell migration in lymphoid organs and extralymphoid organs under normal and inflammatory conditions. A central theme will be that, in contrast to high-walled HEV-like vessels in lymphoid organs and sites of acute or chronic inflammation, flat-walled vessels at intratumoral sites do not express high levels of hallmark trafficking molecules such as intercellular adhesion molecule-1 (ICAM-1), thereby severely limiting T cell access to tumor sites (Figure 1). These observations support the notion that tumor microvessels do not have the full complement of inflammatory characteristics necessary to mobilize blood-borne immune effector cells to the tumor microenvironment. Thus, vascular barriers to efficient entry of effector lymphocytes into intratumoral regions represent a potential mechanism of immune escape. Additional discussion will be on therapeutic approaches that are under investigation in preclinical models to promote T cell homing to tumor tissues. The unique inflammatory environment within tumor tissues may predispose vessels to respond to proadhesive signals, thereby dynamically altering their ability to capture immune effector cells within the circulating pool. A major challenge for future studies will be to develop novel strategies to direct T cell recruitment to tumor sites without tipping the balance in favor of a proinflammatory environment that supports tumor progression.

Limitations to T Cell-Based Immunotherapy

Cell-mediated antitumor immunity depends on the activation of cytolytic immune effector cells including $CD8^+$ T cells and NK cells that recognize cancer

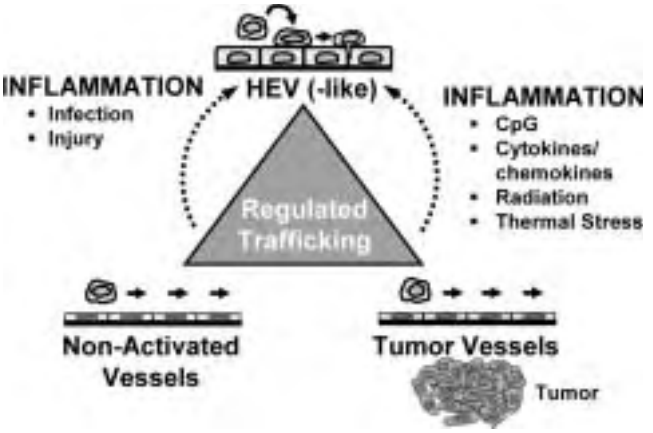


Figure 1: Schematic of lymphocyte-endothelial interactions at distinct vascular sites. In HEV of lymphoid organs, naïve and central memory T cells initiate the full sequence of adhesion events leading to extravasation. This is in contradistinction to non-activated vessels of extra-lymphoid organs or intratumoral vessels that do no support efficient T cell interactions. Inflammatory signals convert non-interacting squamous vessels into HEV-like vessels that can mediate lymphocyte entry into tissues.

cells. $CD8^+$ T cells are effective mediators of tumor immunity because their function is dependent on recognition of antigen-specific targets. This specificity arms the immune system with the ability to destroy target cells, while leaving normal tissues intact. Significant progress has been made toward developing vaccines to activate populations of tumor-reactive $CD8^+$ T cells. These vaccines have proven to be effective at generating tumor-specific CTL that are fully competent to kill tumor cells in in vitro assays (Boon et al., 2006; Dudley et al., 2002; Marincola et al., 2003; Rosenberg et al., 2004). While vaccine treatments can strongly activate patient immune systems, they have not translated into effective clinical outcomes and disappointingly few patients show tumor regression or marked improvement (Armstrong and Jaffee, 2002; Boon et al., 2006; Davis et al., 2003; Laheru and Jaffee, 2005; Marincola et al., 2003; Mocellin, 2005).

A frequently overlooked issue is that tumor-reactive CTL must extravasate through the vascular endothelium and infiltrate the tumor parenchyma in order to initiate contact-dependent lysis of target cancer cells. Dense accumulations of lymphocytes are often found in the peritumoral areas surrounding highly vascularized lesions, demonstrating an ongoing yet ineffective immune response (Carlos, 2001; Chen et al., 2006c; Chen et al., 2003; Clark et al., 1989; Johnson et al., 2000). An example of restricted localization of leukocytes in distinct microanatomical regions is shown in pancreatic islet tumors of transgenic RIP-Tag 5 mice (Figure 2A). In this experimental model, leukocytes including $CD3^+$ T cells rarely infiltrate intratumoral regions although they can be found at high density in the peritumoral region of some islet tumors (Chen et al., 2006c; Ganss et al., 2002; Garbi et al., 2004; Onrust

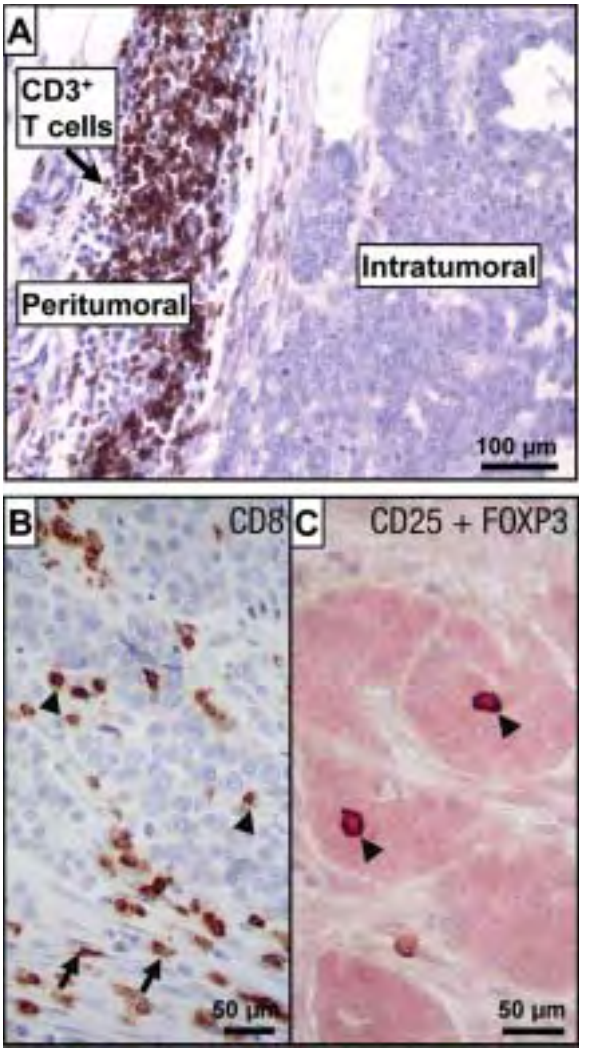


Figure 2: T cell infiltration into the tumor microenvironment. (A) Paraffin sections of pancreatic islet tumors of RIP-Tag 5 transgenic mice (23 weeks of age) were stained with CD3 specific mAb and biotin-conjugated secondary Ab (brown). Dense $CD3^+$ T cell infiltrates were detected in the peritumoral region that surrounds the tumor mass whereas rare $CD3^+$ cells were observed in the intratumoral region. (B) $CD8^+$ tumor infiltrating lymphocytes (TIL) were observed both in tumor epithelium (i.e., intratumoral) (arrow head) and within tumor stroma (arrow) in human epithelial ovarian cancer. High frequency of intraepithelial $CD8^+$ TILs was associated with improved survival in patients. (C) $CD25^+FOXP3^+$ regulatory T cells (T_{reg}) (arrow head) were detected by double immunohistochemical staining of CD25 (brown) and FOXP3 (red) in ovarian cancer. T_{reg} cells were not prognostic for survival, but a high intraepithelial $CD8^+/T_{reg}$ ratio was found to be associated with improved survival.

et al., 1996). Similar findings in multiple murine tumors and human cancer suggest that unique features of the intratumoral microenvironment contribute to this region being non-permissive to lymphocyte extravasation (Carlos, 2001; Chen et al., 2006c; Chen et al., 2003; Clark et al., 1989; Johnson et al., 2000; Mortarini et al., 2003).

Multiple independent variables appear to have an impact on the complex relationship between leukocyte infiltration of tumor tissues and patient outcome. Important factors include the subtype of immune cell infiltrates and the site of infiltration within microanatomically distinct regions of tumor tissues. In a study of 41 lung cancer tumor specimens, intratumoral macrophage density positively correlated with microvessel counts and negatively correlated with patient relapse-free survival ($p < 0.05$) (Chen et al., 2005). In breast and cervical cancer, there is also strong evidence to indicate that intratumoral localization of dense infiltrates of tumor-associated macrophages (TAMs) is associated with a poor prognosis (Ben-Baruch, 2006). In contrast, another report on 175 patients with non-small cell lung cancer (NSCLC) indicated 5-year survival of 52.9% and 7.7% respectively in patients with high versus low islet macrophage density ($p < 0.0001$), suggesting that tumor islet $CD68^+$ macrophage density is a powerful independent predictor of survival from surgically resected NSCLC (Welsh et al., 2005). Further, T cell infiltration of intratumoral regions, indicative of spontaneous T cell responses, has been associated with a favorable prognosis in human melanoma, non-small cell lung carcinoma, colon cancer, breast cancer and ovarian cancer (Chen et al., 2006c; Chen et al., 2003; Chiba et al., 2004; Clark et al., 1989; Johnson et al., 2000; Kuroda et al., 2005; Marrogi et al., 1997; Menon et al., 2004; Naito et al., 1998; Sato et al., 2005; Zhang et al., 2003). Notably, there is no correlation between the extent of T cell infiltration of peritumoral regions and survival in these tumor types. Studies in melanoma and colon cancer patients have shown that $CD8^+$ T cells within intratumoral sites contain cytolytic granules, suggesting that these cells are engaged in cytotoxic effector functions against tumor targets (Mortarini et al., 2000; Naito et al., 1998).

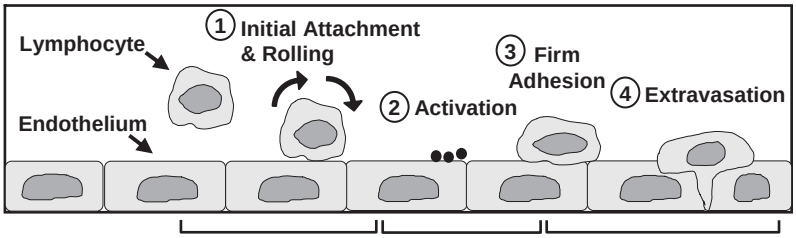
The relationship between the location of tumor-infiltrating T cell subsets and patient outcome has been explored in a recent study of epithelial ovarian cancer. Analysis of 117 ovarian cancer patients revealed that the presence of $CD8^+$ T cells in the intraepithelial regions of tumors (Figure 2B) was a strong prognostic indicator for improved survival, whereas the extent of $CD8^+$ T cell infiltration in the surrounding stroma was not a predictive indicator (Sato et al., 2005). Although the results indicate that intraepithelial infiltration by regulatory T cells (T_{reg}) (Figure 2C) alone was not associated with worse survival as reported previously (Curiel et al., 2004; Zou, 2005, 2006), a high intraepithelial $CD8^+/T_{reg}$ ratio was associated with improved survival. Taken together with the findings of Curiel et al. (2004), these results indicate that the ratio between $CD8^+$ T cells and T_{reg} cells is a crucial determinant of outcome in ovarian cancer patients.

Limited access to tumor tissues may also to be a determining factor for the efficacy of active T-cell based immunotherapy. Examination of non-responding patients after vaccination or adoptive transfer has shown that effector cells failed to infiltrate the tumor even though populations of tumor-reactive T cells could be detected in the blood (Boon et al., 2006; Dudley et al., 2002; Lurquin et al., 2005; Thurner et al., 1999; Yu et al., 2001). The extent of T cell infiltration of tumor tissues relies on the dynamic interplay between multiple factors including (a) the rate of entry of blood-borne lymphocytes across tumor microvessels, (b) lymphocyte retention in tumor beds, (c) proliferation of lymphocytes in situ, and (d) survival. The majority of studies performed to date have assessed the overall accumulation of T cells in tumor tissues which provides a snapshot of the steady-state equilibrium in a heterogeneous tumor microenvironment. Relatively few studies have investigated the mechanisms that regulate trafficking of blood-borne lymphocyte to tumor sites or how the tumor microvasculature can be modified to improve the efficiency of immune effector cell migration at these sites.

Molecular Mechanisms Controlling T Lymphocyte Extravasation Across Endothelial Barriers

Lymphocyte extravasation at all sites of the body is mediated by a common sequence of stepwise adhesion events that includes: (1) primary tethering and rolling of lymphocytes along the luminal surface of vascular endothelium, (2) lymphocyte activation by chemokines displayed on endothelial cells, (3) transition to secondary, firm adhesion, and ultimately (4) extravasation of lymphocytes through intercellular junctions between adjacent endothelial cells (Figure 3) (Butcher and Picker, 1996; Chen et al., 2006c; Miyasaka and Tanaka, 2004; Springer, 1994; von Andrian and Mempel, 2003). Complementary arrays of both common and unique homing molecules participate in primary and secondary adhesion events at different tissue destinations which provide the basis for site-specific trafficking of lymphocyte subsets to distinct tissue locales. The molecular mechanisms that control extravasation have been most clearly delineated for the adhesion pathways that support trafficking of T cells across HEV in peripheral lymphoid organs including peripheral lymph nodes (PLN) and Peyer's patches (PP). These organs are strategically positioned to protect the body from challenge by environmental pathogens. HEV support efficient recirculation of naïve and central memory T cells as a component of immune surveillance, thereby serving as a reference model for understanding extravasation mechanisms across vascular endothelial barriers.

Trafficking Across HEV under Homeostatic and Inflamed Conditions. Naïve and central memory T cells continuously gain access to secondary lymphoid organs by engagement of trafficking molecules constitutively expressed on cuboidal postcapillary HEV (Figure 3) (Butcher et al., 1999; Girard and



Lymphoid Tissues	PLN	PNAd	CCL21	ICAM-1, ICAM-2 CD31
	PP	MAdCAM-1	CCL21	ICAM-1 MAdCAM-1, CD31
Extra-lymphoid Tissues	Skin	E-selectin	CCL17/27 CXCL9/10	ICAM-1, VCAM-1 CD31
	Gut	MAdCAM-1	CCL25 CXCL9/10	ICAM-1, MAdCAM-1, CD31
	Tumor Sites	E-selectin P-selectin VCAM-1	CXCL9/10 CCL5, CCL22 CXCL12	ICAM-1, VCAM-1 CD31

Figure 3: Paradigm of the four-step adhesion cascade that mediates extravasation of circulating leukocytes through endothelial barriers. Common and unique sets of trafficking molecules dictate the nature of adhesion events that occur in vessels at distinct tissue sites.

Springer, 1995; Miyasaka and Tanaka, 2004; Rosen, 2004; Springer, 1994; von Andrian and Mempel, 2003). In PLN, homeostatic recirculation is initiated by the lymphocyte homing receptor, L-selectin, which forms catch-bonds with endothelial counter-receptors termed peripheral lymph node addressins (PNAd) that decorate cuboidal high endothelial cells. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) supports lymphocyte tethering and rolling in HEV of PP and mesenteric LN (MLN) through dual engagement with L-selectin as well as $\alpha_4\beta_7$ integrin on circulating lymphocytes. These primary adhesive interactions cause lymphocytes to slowly roll along the endothelium enabling them to sample the chemokine environment of endothelial cells.

Engagement of the G-protein coupled chemokine receptor, CCR7, on lymphocytes by the CCL21 chemokine on the surface of HEV precipitates the switch from primary to secondary adhesion by inducing conformational changes in integrin molecules that increase their affinity and avidity for endothelial ligands (Butcher et al., 1999; Carman and Springer, 2003; Johnston and Butcher, 2002; Mackay, 2001; Miyasaka and Tanaka, 2004; von Andrian and Mempel, 2003). The binding of the β_2 integrin leukocyte-function associated antigen-1 (LFA-1) to its endothelial ligands ICAM-1/2 has emerged as a central secondary adhesion event that regulates firm adhesion and transendothelial

migration at all tissue sites including secondary lymphoid organs (Figure 3) (Butcher and Picker, 1996; Springer, 1994). In addition to providing a binding interface for LFA-1, ICAM-1 actively modulates the affinity of LFA-1 molecules, strengthening the molecular interactions between lymphocytes and the endothelium (Carman and Springer, 2003; Luster et al., 2005; Sarantos et al., 2005). The precise mechanisms controlling transendothelial migration in HEV are not fully understood. ICAM-1 has been shown in vitro to be a major component of cup-like structures that surround transmigrating lymphocytes at intercellular junctions (Carman and Springer, 2004; Shaw et al., 2004; Yang et al., 2005). These ICAM-1-rich structures are thought to provide traction and guidance to lymphocytes migrating through endothelial barriers. Junctional proteins such as JAM-1, JAM-2 and CD31 are also likely to play a role in the transendothelial migration process (Aurrand-Lions et al., 2001; Miyasaka and Tanaka, 2004; Muller, 2001; Ostermann et al., 2002).

The dynamics of leukocyte trafficking to LN are dramatically altered as a consequence of local tissue inflammation. Improved recirculation of T cells through inflamed LN increases the probability that rare antigen-restricted T cells will encounter cognate antigens presented by dendritic cells (DC). These interactions lead to productive activation and expansion of T cell clones. Multiple mechanisms contribute to the transient increase in T cell accumulation observed in draining LN (dLN) following local antigen challenge or infection by bacterial or viral pathogens. Rapid release of TNF-α by resident mast cells at sites of bacterial infection causes an increase in T cell accumulation in dLN which is dependent on expression of vascular cell adhesion molecule-1 (VCAM-1) in nodal tissues (Mackay et al., 1992). Mast cells at sites of antigen challenge also produce the monocyte-inflammatory chemokine CCL4 that is delivered via the afferent lymphatics to dLN where it mediates improved T cell trafficking (Tedla et al., 1998). Viral infection has recently been shown to enhance the recirculation of $CD4^+$ and $CD8^+$ T cells by remodeling the feeding arterioles that supply postcapillary HEV in dLN. The resultant increase in arteriole diameter (from ~ 100 to 150 μm), together with an increase in the total number of $PNAd^+$ HEV in enlarged dLN, triggers 'inflammation-induced recirculation' which augments the pool of naïve T lymphocytes available for screening of foreign antigens trapped in LN compartments (Soderberg et al., 2005).

In addition to promoting entry of naïve or central memory T cells, local tissue inflammation opens the gateway for cell types normally excluded from LN. In this regard, under normal conditions monocytes, NK cells, and T cell subsets that lack CCR7 are unable to initiate firm adhesion and transendothelial migration across HEV of non-inflamed LN (Sallusto and Mackay, 2004; von Andrian and Mempel, 2003). However, introduction of antigen-pulsed mature DC into the skin leads to an increase in the accumulation of NK cells and T cells in dLN through an L-selectin-dependent, CCR7-independent mechanism

(Martin-Fontecha et al., 2003, 2004; Sallusto and Mackay, 2004). These NK cells are recruited to inflamed LN in a CXCR3-dependent manner where they provide IFN-γ to polarize Th1 responses. Local tissue inflammation induced by complete Freund's adjuvant stimulates TNF-α-dependent synthesis of the CXCR3 ligand, CXCL9 in dLN (Janatpour et al., 2001; McEvoy et al., 1997; Sallusto and Mackay, 2004; von Andrian and Mempel, 2003). Although it is not known which nodal cells produce CXCL9, presentation of this prototypical inflammatory chemokine on HEV augments monocyte adhesion under shear. Monocyte trafficking across HEV is also enhanced following antigen challenge through a CCL2/CCR2-dependent mechanism (Palframan et al., 2001; von Andrian and Mempel, 2003). Intravascular display of CCL2 on HEV occurs by remote control whereby chemokine produced in inflamed tissues is delivered via the afferent lymphatics to dLN and ultimately reaches the luminal surface of HEV by transcytosis. Taken together, these studies indicate that proinflammatory cytokines and chemokines within inflamed tissues and dLN have a profound influence on the composition of immune cells present in LN tissue microenvironments.

A unifying feature of the inflammatory mechanisms described above is that the response is restricted to HEV in LN that drain infected tissues or sites of antigen challenge. While dLN are the first line of defense against invading pathogens, there may also be an advantage to heightening immune surveillance of distal secondary lymphoid organs to prevent dissemination of rapidly multiplying infectious agents or to arm the host when it is vulnerable to subsequent microbial infections. Recent studies have suggested that fever, one of the cardinal but least understood features of inflammation, can elicit systemic effects on the recruitment properties of the HEV-axis throughout the body (Appenheimer et al., 2005; Chen and Evans, 2005; Chen et al., 2006c). These studies demonstrate that temperatures in the range of physiologic fever act as an alert system to enhance the level of homeostatic recirculation across HEV in LN and PP.

Fever-range thermal stress exerts direct effects on naïve or central memory T cells to increase the binding function of trafficking molecules, i.e., L-selectin and $\alpha_4\beta_7$ integrin, that support transient tethering and rolling interactions in HEV (Appenheimer et al., 2005; Chen et al., 2004; Evans et al., 1999, 2000, 2001; Wang et al., 1998). This increase in lymphocyte homing potential is not accompanied by a change in the expression level of trafficking molecules (L-selectin, $\alpha_4\beta_7$ integrin, or LFA-1) or their position on distinct regions of lymphocyte surface membranes (i.e., microvillus processes versus planar membranes) (Chen et al., 2004; Evans et al., 1999, 2000; Wang et al., 1998). In the case of L-selectin, thermal stress anchors this trafficking molecule to the detergent-insoluble cytoskeletal matrix by a mechanism that depends on the C-terminal 11-amino acid domain (Chen et al., 2004; Evans et al., 1999). This domain contains a binding site for α-actinin, a cytoskeletal linker protein

(Dwir et al., 2001; Kansas and Pavalko, 1996; Pavalko et al., 1995). These observations support the speculation that thermal induction of stable interactions between the L-selectin cytoplasmic domain and the actin-based cytoskeleton enhance the tensile strength of this molecule, and thereby its ability to withstand hemodynamic shear in HEV. These studies further revealed an unexpected role for the proinflammatory cytokine, IL-6, in amplifying homeostatic trafficking of naïve and central memory T cells. In this respect, thermal stimulation of L-selectin adhesion depends on an IL-6 trans-signaling mechanism in which engagement of the gp130 signal transduction receptor chain by IL-6 and a soluble form of the IL-6 receptor binding subunit results in activation of downstream MEK1/ERK1–2 pathways (Chen et al., 2004, 2006c).

The effects of fever-range thermal stress on lymphocyte trafficking are mirrored in the HEV. This issue was addressed using whole body hyperthermia (WBH) protocols to raise the core temperature of mice or advanced cancer patients to the range of natural fever (39–40°C for 6 h) (Kraybill et al., 2002; Pritchard et al., 2004). These mild hyperthermic conditions, in the absence of pathogenic challenge, result in a transient decrease in the number of lymphocytes in the circulating pool (Evans et al., 2001; Kraybill et al., 2002; Ostberg and Repasky, 2000). A concomitant increase in the adhesive capacity of HEV was demonstrated by frozen tissue-section in vitro adherence assays and short-term in vivo homing studies (Chen et al., 2006a; Chen et al., 2006c; Evans et al., 2001). In the study shown in Figure 4, lymphocytes labeled with a fluorescent tracking dye were adoptively transferred into the venous circulation of mice that were pretreated with WBH. These mice were allowed to revert to their normal core temperature prior to adoptive transfer in order to evaluate stable changes in HEV adhesion without contributions from other parameters that also influence trafficking such as direct heat effects on lymphocyte adhesion or hemodynamic parameters (vasodilation, blood flow). A substantial increase was detected in the number of fluorescent-tagged lymphocytes that accumulate in $PNAd^+$ PLN HEV of heat-treated mice 5–15 min. after adoptive transfer (Figure 4) (Chen et al., 2006b). Thermal stress also caused ~ a 2-fold increase in the number of cells that subsequently extravasated into the underlying tissue parenchyma (Chen et al., 2006b; Chen et al., 2006c; Evans et al., 2001). This increase represents a profound affect on an already efficient homeostatic trafficking mechanism in which ~ 1 in every 4 lymphocytes that enter HEV undergo the full sequence of adhesion events culminating in extravasation (Miyasaka and Tanaka, 2004; von Andrian and Mempel, 2003).

The precise mechanisms underlying thermal regulation of HEV adhesion have not been fully resolved. Elevated temperatures do no alter the level of expression of molecules such as PNAd or MAdCAM-1 that support primary adhesion in PLN or PP HEV, respectively (Figure 4) (Evans et al., 2001). Initial findings indicate that thermal stress increases the intravascular density of ICAM-1 on HEV, thereby promoting integrin-dependent trafficking of naïve

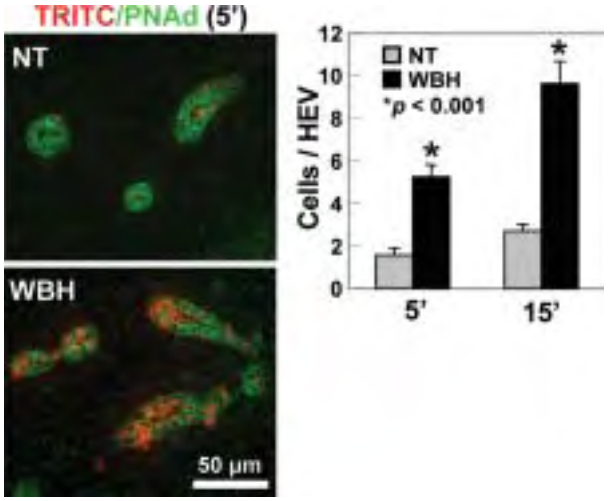


Figure 4: Enhanced interactions of blood-borne lymphocytes within PLN HEV in response to the thermal component of fever. Splenocytes isolated from BALB/c mice (8 weeks) were labeled ex vivo with fluorescent tracking dye (TRITC, red) and injected intravenously through the tail-vein into normothermic (NT) control mice or mice pretreated for 6 hours with fever-range whole body hyperthermia (WBH; core temperature of 39.5–40.0°C). PLN organs were isolated 5 and 15 minutes after adoptive cell transfer. Frozen tissue sections were counterstained with PNAd-specific mAb (MECA-79) and FITC-labeled secondary Ab (green). The numbers of $TRITC^+$ lymphocytes bound to $PNAd^+$ cuboidal HEV were quantified under fluorescence microscopy. A minimum 40 HEV were observed under each condition. WBH treatment significantly enhanced binding of $TRITC^+$ cells to HEV at both 5 and 15 minute time points (unpaired two-tailed Student's *t*-test).

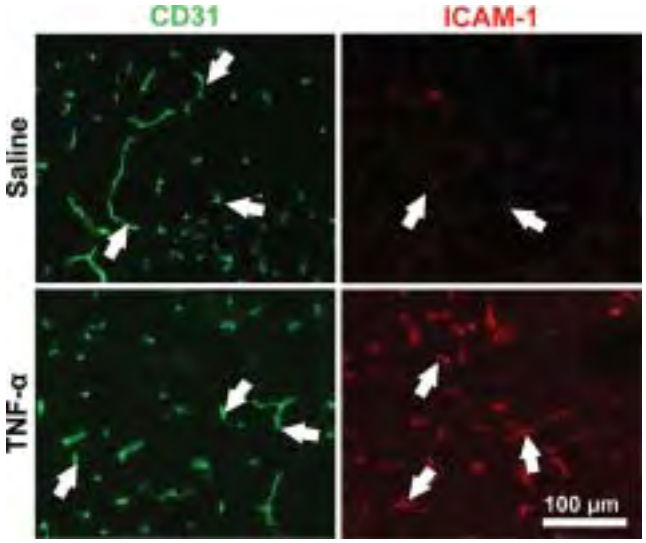


Figure 5: Induction of ICAM-1 expression on vessels in extralymphoid organs by treatment with the prototypical inflammatory cytokine TNF-α. BALB/c mice (8 weeks) were treated with TNF-α (administered intraperitoneally, 10 μg/kg) for 6 hours. Cryosections of pancreas were stained for the pan-endothelial CD31 adhesion molecule (green) or ICAM-1 (red). TNF-α treatment substantially upregulated ICAM-1 expression on pancreatic vessels without altering CD31 density.

and central memory lymphocytes (Chen et al., 2006a). These changes in ICAM-1-dependent adhesion in HEV in response to a mild inflammatory stimulus are remarkable in light of evidence that ICAM-1 induction is considered a hallmark of profound inflammation triggered by cytokines such as TNF-α, IL-1β, IFN-γ, and IL-6 (Pober and Cotran, 1990; Roebuck and Finnegan, 1999; Springer, 1995). An important feature of the thermal response is that stimulation of trafficking in this non-pathogenic experimental model is restricted to specialized vascular beds, i.e., HEV, while squamous endothelium lining non-inflamed vessels are refractory (Chen et al., 2006b, 2006c; Evans et al., 2001; Shah et al., 2002). Mechanisms that maintain highly focused lymphocyte trafficking to secondary lymphoid organs during febrile inflammatory responses would be expected to generate robust immune protection by optimizing the chance that antigen-specific T cells come into contact with cognate antigens.

Inflammation-Induced Trafficking to Extralymphoid Organs. The majority of blood vessels in the body are lined by squamous endothelial cells that express insufficient levels of the adhesion molecules and chemokines to support recruitment of circulating lymphocytes. However, during inflammatory responses, effector lymphocytes traffic to extralymphoid sites at high frequency due to dynamic changes in the vascular presentation of homing molecules (Luster et al., 2005; Springer, 1995; von Andrian and Mempel, 2003). These vessels are transformed into HEV-like structures by proinflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-6) released locally by resident stromal or immune cells in response to infection or vascular damage (Figure 1) (Luster et al., 2005; Pober, 2002; Pober and Cotran, 1990; Roebuck and Finnegan, 1999). Prototypical inflammatory cytokines such as TNF-α induce the vascular expression of molecules that support primary (e.g., P/E-selectin, VCAM-1) and secondary adhesion events including hallmark molecules such as ICAM-1 (Figure 5) and other molecules (e.g., CXCL9, CXCL10, CCL25, VCAM-1) (Mackay, 2001; Pober, 2002; Pober and Cotran, 1990; Springer, 1995). IL-6 has emerged as a particularly interesting cytokine, in this regard, due to its requisite and non-redundant role in triggering T cell recruitment during acute and chronic inflammatory responses (Appenheimer et al., 2005; Chen et al., 2004; Hurst et al., 2001; Jones, 2005; Jones et al., 2005; McLoughlin et al., 2005; Modur et al., 1997; Romano et al., 1997).

Differential pairings of lymphocyte and endothelial adhesion molecules and chemokines/chemokine receptors dictate which leukocytes gain access to extralymphoid tissue destinations. Site-specific control of trafficking serves to avoid unproductive cell recruitment or collateral tissue damage. Following activation by antigen in secondary lymphoid organs, T cells are directed away from peripheral lymphoid organs due to the rapid down-regulation of L-selectin and CCR7 (Butcher and Picker, 1996; von Andrian and Mempel, 2003; Weninger et al., 2001). Subsequent upregulation of other trafficking molecules reroute activated $CD8^+$ T cells to extralymphoid sites of infection or injury.

Recent studies suggest that the site where naïve lymphocytes encounter cognate antigen determines the homing destination of activated lymphocytes, creating tropisms towards selected peripheral sites (Calzascia et al., 2005; Campbell et al., 2003; Luster et al., 2005; Mora et al., 2003). For example, a lymphocyte activated in a PLN that drains the skin will express skin-tropic homing receptors (e.g., PSGL-1, which binds E-selectin, and the chemokine receptors CXCR3 and CCR4 which bind CXCL9/10 and CCL17, respectively), enabling these cells to traffic efficiently to inflamed skin (Figure 3) (Mora et al., 2005). Skin-tropic lymphocytes have a limited capacity to traffic to gut-associated tissues, however, since they lack gut-tropic homing molecules such as $\alpha 4\beta 7$ integrin and CCR9 (which bind MAdCAM-1 and CCL25, respectively) (Figure 3) (Campbell et al., 2003; Mora et al., 2005). The regional priming of effector T cells serves to compartmentalize the active immune response, making the dynamics of T cell imprinting an important consideration in determining the optimal route of vaccination in the development of effective T cell-based tumor immunotherapy.

Lymphocyte Trafficking Across Tumor Microvessels. The mechanisms limiting the infiltration of effector T cells into intratumoral sites are poorly understood. Tumor vessels are typified by an irregular tortuous structure (Brizel et al., 1993; Chambers et al., 2002; Jain et al., 2002). These vessels are lined by squamous, flat $CD31^+$ endothelial cells that express low levels of adhesion molecules (such as ICAM-1) or chemokines (CXCL9/10) (Figure 6A) (Chen and Evans, 2005; Chen et al., 2003, 2006c; Ganss et al., 2002; Garbi et al., 2004; Gollnick et al., 2003; Lugade et al., 2005; Onrust et al., 1996). Thus, tumor vessels do not exhibit many of the hallmark characteristics of an acute or chronic inflammatory state. Visual analysis of vessels in intratumoral regions of murine tumors indicates that they are virtually indistinguishable from normal vasculature in terms of their low constitutive level of expression of ICAM-1 or their inability to interact efficiently with circulating leukocytes (Figure 6A, B) (Chen and Evans, 2005; Chen et al., 2006c; Ryschich et al., 2002; Wu et al., 1992). This is in contrast to observations in $ICAM\text{-}1^+$ LN HEV (Figure 6A) or vessels of inflammatory sites where substantial numbers of T cells rapidly interact with vessel walls and extravasate into the underlying parenchyma (Figure 4) (Butcher and Picker, 1996; Chen et al., 2006c; Luster et al., 2005; von Andrian and Mempel, 2003). In studies that tracked the fate of activated T cells during the first hour after intravenous adoptive transfer, it was determined that an extremely low frequency of these cells gain immediate entry into tumor nodules (Skitzki et al., 2004) (Q. Chen, D. Fisher, and S. Evans, unpublished). The low frequency of lymphocyte extravasation in the tumor microenvironment has hampered the dissection of trafficking mechanisms at this site. Compounding the difficulties encountered in actively studying extravasation in the tumor microenvironment is the fact that both human and mouse tumors can be highly heterogeneous with respect to the extent of $CD8^+$ T cell

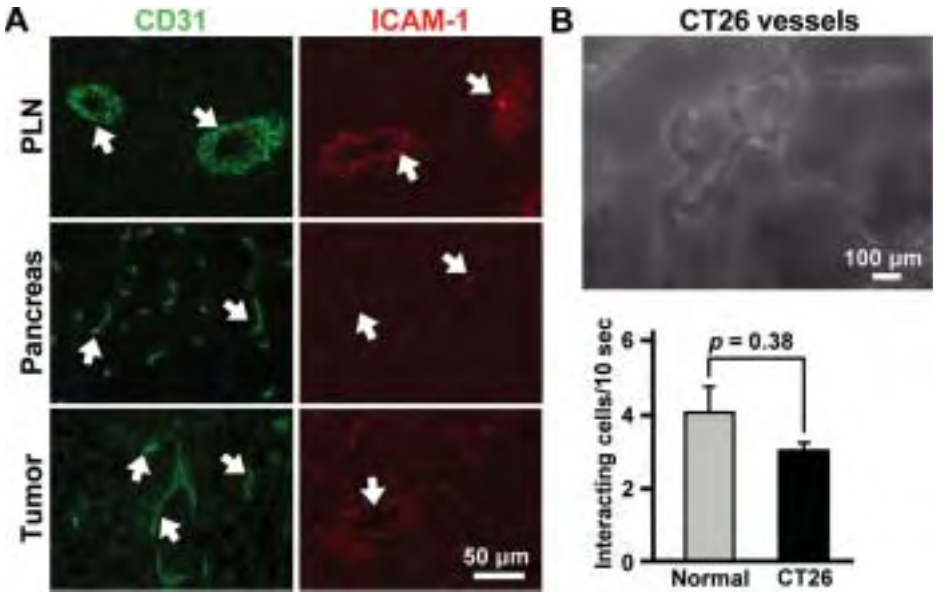


Figure 6: Comparative analysis of ICAM-1 expression and leukocyte-endothelial interactions at distinct vascular sites. (A) Intravascular expression of ICAM-1 was examined by injecting ICAM-1-specific mAb intravenously into Colon 26 (CT26) tumor-bearing BALB/c mice. Tissues were harvested after 30 minutes and cryosections were counterstained using rhodamine (red)-conjugated secondary Ab. Vascular structures were also stained with anti-CD31 mAb (green). Photographic images were taken using identical exposure settings so that the relative expression levels of CD31 and ICAM-1 could be compared between different tissues sites. While tissues at all three sites were highly vascularized (indicated by numerous $CD31^+$ vessels), flat-walled vessels in CT26 tumors and extralymphoid organs (e.g., pancreas) expressed relatively low levels of ICAM-1 compared with cuboidal HEV of PLN. (B) Dorsal skin-flap window chambers were surgically implanted in BALB/c mice as described previously (Chen et al., 2006c; Moeller et al., 2004; Wu et al., 1992). CT26 cells were injected into the fascial plane at the time of surgery and vascularized tumors were visible after 9–14 days. Normal vessels were also observed in the fascial plane of skin of non-tumor-bearing mice 9 days after window chamber implantation. Circulating leukocytes were labeled in situ by intravenous injection of the fluorescent dye rhodamine-6-G. Leukocyte-endothelial interactions were examined by intravital microscopy. (Supplementary material is available for this article. Go to the publisher's online edition of *Immunological Investigations* for a free video clip). The number of rolling leukocytes was quantified over 10 seconds at a set reference point in vessels of equivalent diameter (n = 3 vessels per condition). Circulating leukocytes failed to interact with normal subcutaneous vessels and tumor vessels and no statistical difference was observed between these vessel types (unpaired two-tailed Student's *t*-test).

infiltration in tumors of the same histological grade and even within different regions of a given tumor (Chen et al., 2003; Johnson et al., 2000; Menon et al., 2004; Sato et al., 2005; Yu et al., 2005).

Tumor tissues can be segregated into distinct microanatomical regions based on fundamental differences in vascular parameters. In sharp contrast to the intratumoral region, the microvessels in peritumoral regions frequently have an HEV-like morphology and express high levels of primary and secondary

adhesion molecules and chemokines (e.g., E-selectin, ICAM-1, CXCL9) (Figure 3) (Carlos, 2001; Chen et al., 2003; Chen et al., 2006c; Kunz et al., 1999; Nelson et al., 1994). These peritumoral vessels that surround tumor nodules are often associated with dense leukocyte infiltrates. While the mechanisms limiting adhesion in intratumoral microvessels are not fully understood, several in vitro or in vivo studies have suggested that angiogenic factors (i.e., bFGF, HGF, VEGF-C and VEGF-D) or anti-inflammatory cytokines (IL-10, TGF-β) present in the tumor microenvironment prevent the expression of endothelial adhesion molecules such as ICAM-1 or VCAM-1 (Bouma-ter Steege et al., 2004; Griffioen et al., 1996; Melder et al., 1996; Min et al., 2005; Piali et al., 1995; Roebuck and Finnegan, 1999). It is not clear if intratumoral vessels are being actively repressed or merely remain refractory to the prevailing inflammatory environment present in tumor sites. Recent findings that the presence of tumor tissues decreases CCL21 expression and naïve T cell homing in HEV of tumor-draining LN suggest that tumor-derived soluble factors have a negative impact on trafficking mechanisms (Carriere et al., 2005).

Insight into the relationship between T cell trafficking and clinical outcome is provided by a recent study in which g-protein mediated chemokine signaling was globally inhibited by administration of pertussis toxin at a definitive time point relative to adoptive transfer of tumor-specific T cells (Skitzki et al., 2004). Pretreatment of lymphocytes with pertussis toxin, an irreversible inhibitor of g-protein signaling, prior to adoptive transfer inhibited early entry of $CD8^+$ T cell in pulmonary micrometastases and markedly reduced the antitumor response. In contrast, intravenous pertussis toxin given 24 hours after adoptive transfer completely blocked the ongoing recruitment of tumor reactive $CD8^+$ T cells from the periphery to the site of pulmonary micrometastases, but only had a minimal effect on the clinical efficacy of the adoptive transfer. Apparently, early entry of tumor-specific T cells into sites of metastases had a disproportionate effect on the degree of tumor suppression as subsequent recruitment contributed minimally. This study further supports the concept that tumor-specific T cells utilize established chemokine-dependent lymphocyte trafficking mechanisms to gain access into tumor sites. These findings suggest that a therapeutic benefit could be achieved in T cell-based immunotherapy by promoting early entry mechanisms that hinge on the lymphocyte-endothelial cell interface.

Several experimental approaches have been taken to exploit the mechanism of action of acute inflammatory stimuli in an attempt to transform tumor microvessels into HEV-like vessels that support recruitment of immune effector cells (Figure 1). One approach has been to use potent inflammatory mediators that normally accompany bacterial infection such as oligodeoxynucleotides that contain cytosine-phosphate-guanine (CpG-ODN) motifs. These DNA motifs are detected by innate immune cells, particularly macrophages,

through toll-like receptor-9 which then activates a profound innate immune response accompanied by massive release of proinflammatory cytokines (Hemmi et al., 2000). Systemic administration of CpG-ODN in combination with adoptive transfer of tumor-specific T cells (i.e., specific for SV40 large T antigen [Tag]) has recently been used to treat pancreatic islet tumors in Rip-Tag 5 transgenic mice (Garbi et al., 2004). Trafficking molecules such as ICAM-1, VCAM-1, CXCL9 and CXCL10 are poorly expressed by intratumoral vessels in untreated Rip-Tag 5 mice, correlating with limited infiltration by T cells in this tumor model (Chen et al., 2006c; Ganss et al., 2002; Garbi et al., 2004; Onrust et al., 1996). CpG-ODN administration was found to induce ICAM-1 and VCAM-1 on intratumoral vessels in Rip-Tag 5 mice (Garbi et al., 2004). When combined with the adoptive transfer, this inflammatory response was accompanied by strong intratumoral infiltration of tumor-specific $CD4^+$ and $CD8^+$ T cells. Increased survival was noted when treatment was applied in the early stages of carcinogenesis, suggesting that entry of tumor-reactive immune effector cells into intratumoral sites can support an effective anti-tumor immune response.

Direct damage to tissues in the tumor microenvironment is also a strong stimulus leading to an acute inflammatory response. An effective method to induce localized tissue damage in the tumor microenvironment is ionizing radiation (IR), a common treatment for solid mass tumors. Several preclinical studies have examined the interaction between IR and immunotherapy. Mice treated with IR followed by the adoptive transfer of tumor-specific T cells showed improved survival when the two treatments were combined (Cao et al., 2002; Ganss et al., 2002; Lugade et al., 2005). Examination of the tumor sites following treatment revealed profound intratumoral infiltration by $CD8^+$ T cells which was accompanied by the upregulation of chemokines (CXCL9 and CXCL10) as well as adhesion molecules such as VCAM-1 or ICAM-1 on tumor microvessels.

An alternative strategy has been to genetically modify tumor cells to express inflammatory agents so that active inflammation is restricted to the tumor microenvironment. For example, genetic modification of fibrosarcoma cells to express LIGHT (a TNF-α family member) enhances recruitment of $CD8^+$ T cells into tumor sites and improves overall survival (Yu et al., 2004). Intratumoral infiltration by T cells directly correlates with increased chemokine and adhesion molecule expression at the protein and/or mRNA level (i.e., CCL21, CXCL9/10, and MAdCAM-1) in tumor tissues at subcutaneous sites. Intriguingly, T cell proliferation is observed within LIGHT-expressing tumors, suggesting that activation and expansion of naïve or memory T cells can occur in situ.

A novel two-pronged strategy has recently been developed in order to stimulate T cell recruitment to tumor tissues in preclinical models. For these studies, mice implanted with renal carcinoma cells engineered to express

CXCL9 showed delayed tumor growth and moderate lymphocyte infiltration (Pan et al., 2006). When these mice were also treated with IL-2 to expand the circulating pool of T cells that express CXCR3 (the counter-receptor for CXCL9), strong intratumoral infiltration by $CD4^+$ T cells, $CD8^+$ T cells and NK cells was observed which correlated with tumor regression.

A common drawback of many of the experimental strategies taken, thus far, to regulate vascular adhesion and T cell recruitment is the inability to direct the response with pinpoint accuracy to vascular beds within tumor lesions. Systemic treatment with potent proinflammatory agents such as CpG-ODN results in indiscriminant activation of vascular adhesion and widespread inflammation in tertiary organs that can lead to bystander effects in normal tissues (Garbi et al., 2004; Sacher et al., 2002). The introduction of inflammatory cytokines or chemokines into a tumor microenvironment is a powerful approach to induce regionalized lymphocyte recruitment. However, a limitation to the practical application of this tactic is that it depends on knowledge of the location of primary or metastatic tumor nodules. Thus, a major challenge is to develop systemic treatments that induce site-specific changes in vascular receptivity that promote improved trafficking to widely dispersed primary and metastatic nodules without activating normal endothelium.

Hints for new directions for overcoming this daunting obstacle to tumor immunity are provided by evidence that the unique features of the tumor microenvironment predispose intratumoral vessels to respond to proadhesive inflammatory cues such as fever-range thermal stress. Tumor-bearing mice treated with fever-range thermal therapy show an increase in leukocyte infiltration (NK cells, neutrophils, lymphocytes) of intratumoral regions which is accompanied by delayed tumor growth (Burd et al., 1998; Chen et al., 2003, 2006b; Ostberg et al., 2005). This is in sharp distinction to normal vessels that are refractory to the proadhesive effects of thermal stress and thus, do not support improved lymphocyte trafficking (Chen and Evans, 2005, 2006c; Evans et al., 2001). While the mechanisms that govern the accumulation of lymphocytes in tumor tissues during thermal therapy remain under investigation, there is preliminary evidence that ICAM-1 is substantially upregulated on intratumoral vessels, correlating with improved trafficking of $CD8^+$ T lymphocytes to the tumor microenvironment (Q. Chen, D. Fisher, and S. Evans, unpublished). These findings raise the possibility that mild thermal therapy, which has limited toxicity in patients (Kraybill et al., 2002), is an attractive partner for T cell-based immunotherapy regimens.

PERSPECTIVES AND FUTURE DIRECTIONS

Most studies in T cell-based cancer immunotherapy have focused on developing new strategies to expand the frequency and repertoire of tumor-specific T cells. Major unresolved questions relate to how obstacles can be overcome in

mobilizing entry of these battle-ready T cells to the tumor microenvironment. There is mounting evidence that chronic inflammation is a common feature of tumor sites; indeed, inflammation is believed to play an important role in tumorigenesis and growth. Nevertheless, the intratumoral microenvironment does not display all the major characteristics of inflamed tissues that are required to support T cell recruitment. This is especially true of the tumor vasculature, which more closely resembles non-activated vessels found in normal extralymphoid tissues. Emerging evidence suggests that tumor microvessels can be distinguished from normal vessels of nonlymphoid organs by their responsiveness to certain inflammatory stimuli such as fever-range thermal stress. A promising avenue of investigation will be to identify the molecular basis for differences between intratumoral vessels and other vascular sites. It is likely that unique attributes of the tumor microenvironment (e.g., chemokines, cytokines, hypoxia, interstitial pressure) predispose tumor vascular elements to respond to inflammatory cues to change their adhesiveness, and thereby their ability to capture circulating effector T cells. Thus, it may be feasible to exploit these differences to develop therapeutic approaches to target the immune system to primary and metastatic lesions.

Another major hurdle for the development of effective T cell based immunotherapy is that the mechanisms that govern trafficking of specific leukocyte subsets to the tumor microenvironment remain unclear. The recent explosion in the understanding of the function of trafficking molecules, particularly chemokines, will likely provide the underpinning for exciting new research on the dynamics of T cell homing to complex tumor tissues. A potential challenge may be that strategies that improve effector T cell trafficking across the vascular gateway in tumor tissues will also mobilize immunosuppressive T_{reg} cell subsets or inflammatory leukocytes that support malignant progression. It remains to be determined if distinct homing mechanisms can be enlisted to enhance the recruitment of preferred leukocyte subsets in tumor beds. Ultimately, the success or failure of T cell-based cancer immunotherapy will likely depend on identifying inflammatory strategies that walk the tightrope between promoting immune effector cell recruitment and driving tumor progression within the tumor microenvironment.

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Fever-range thermal stress promotes lymphocyte trafficking across high endothelial venules via an interleukin 6 *trans*-signaling mechanism

Qing Chen[1], Daniel T Fisher[1], Kristen A Clancy[1], Jean-Marc M Gauguet[2], Wan-Chao Wang[1], Emily Unger[1], Stefan Rose-John[3], Ulrich H von Andrian[2], Heinz Baumann[4] & Sharon S Evans[1]

Fever is an evolutionarily conserved response during acute inflammation, although its physiological benefit is poorly understood. Here we show thermal stress in the range of fever temperatures increased the intravascular display of two 'gatekeeper' homing molecules, intercellular adhesion molecule 1 (ICAM-1) and CCL21 chemokine, exclusively in high endothelial venules (HEVs) that are chief portals for the entry of blood-borne lymphocytes into lymphoid organs. Enhanced endothelial expression of ICAM-1 and CCL21 was linked to increased lymphocyte trafficking across HEVs. A bifurcation in the mechanisms controlling HEV adhesion was demonstrated by evidence that the thermal induction of ICAM-1 but not of CCL21 involved an interleukin 6 *trans*-signaling pathway. Our findings identify the 'HEV axis' as a thermally sensitive alert system that heightens immune surveillance during inflammation by amplifying lymphocyte trafficking to lymphoid organs.

The continuous recirculation of lymphocytes across specialized high endothelial venules (HEVs) in lymphoid organs is critical for the maintenance of immune homeostasis and immune surveillance. HEVs lined by cuboidal endothelial cells are distinguished from flat-walled vascular beds throughout the body by their ability to support efficient extravasation of lymphocytes into underlying tissues[1]. Lymphocyte entry across HEVs involves a highly ordered sequence of adhesion events that includes tethering and rolling along vessel walls; chemokine-dependent activation; firm arrest; and transendothelial migration[2–4].

Primary tethering and rolling of naive and central memory lymphocytes in HEVs of peripheral lymph nodes (PLNs) and mesenteric lymph nodes (MLNs) is initiated via the engagement of sialomucin-like endothelial molecules, collectively called 'peripheral lymph node addressin' (PNAd), by L-selectin on lymphocytes[2,3,5]. Mucosal addressin cell adhesion molecule 1 (MAdCAM-1) on HEVs of MLNs and Peyer's patches also supports primary adhesion through interactions with L-selectin or $\alpha_4\beta_7$ integrin on circulating lymphocytes[2]. Secondary firm arrest is triggered mainly by interactions between CCL21 chemokine displayed on the lumenal surfaces of HEVs with $G_{\alpha i1}$ protein–coupled CCR7 chemokine receptors on lymphocytes[2–4]. Chemokine activation increases the affinity of leukocyte function–associated antigen 1 (LFA-1) for its endothelial ligands, intercellular adhesion molecule 1 (ICAM-1) and ICAM-2, which have redundant functions during steady-state trafficking across HEVs[2,3]. The final process of transendothelial migration in HEVs is not fully understood, but is suggested by *in vitro* models to involve molecules located in interendothelial junctions, including ICAM-1, ICAM-2, CD31 and junctional adhesion molecules 1 and 2 (refs. 3,6).

Local and systemic increases in temperature are cardinal features of inflammation. The evolutionarily conserved febrile response has been linked to improved survival during infection in endothermic and ectothermic species[7–10]. A key unresolved issue relates to the physiological benefit of fever. Studies have suggested that febrile temperatures provide a 'danger signal' that mobilizes the entry of blood-borne lymphocytes into secondary lymphoid organs, where the probability of encountering cognate antigens or pathogens is enhanced. Temperatures that mimic febrile episodes (38–40 °C) act directly on T lymphocytes and B lymphocytes to enhance L-selectin-dependent and $\alpha_4\beta_7$ integrin–dependent homing across lymph node and Peyer's patch HEVs[11–13]. Thermal modulation of L-selectin adhesion involves a tightly orchestrated *trans*-signaling mechanism initiated by engagement of the glycoprotein gp130 signal-transducing subunit by interleukin 6 (IL-6) and a soluble form of the IL-6 receptor-α binding subunit (sIL-6Rα)[13]. Those findings support the present view that IL-6 *trans* signaling provides a molecular 'switch' that governs lymphocyte trafficking during acute inflammation or chronic inflammatory disorders[14,15].

Although febrile temperatures can improve the vascular delivery of inflammatory cells to tissues by regulating hemodynamic parameters

[1]Departments of Immunology, Roswell Park Cancer Institute, Buffalo, New York 14263, USA. [2]The CBR Institute for Biomedical Research and Department of Pathology, Harvard Medical School, Boston, Massachusetts, 02115, USA. [3]Department of Biochemistry, Christian Albrechts University Kiel, D-24098 Kiel, Germany. [4]Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263, USA. Correspondence should be addressed to S.S.E. (sharon.evans@roswellpark.org).

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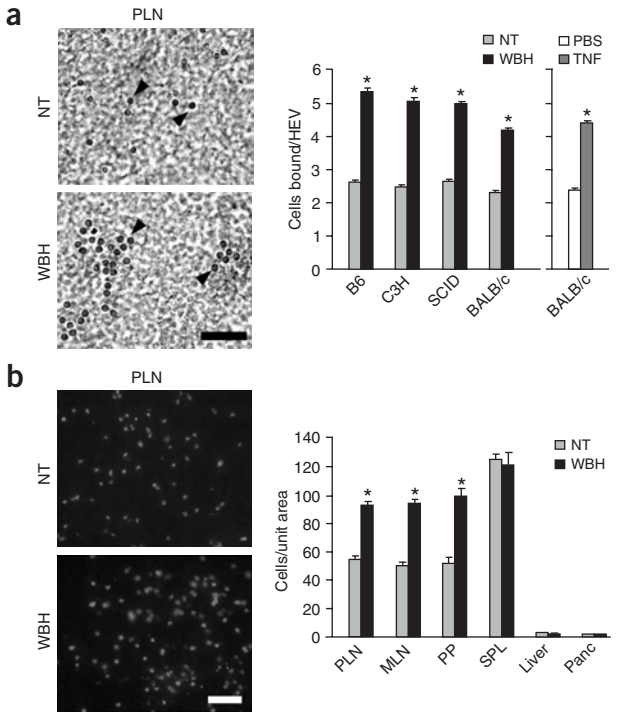


Figure 1 Fever-range thermal stress enhances HEV adhesion and homing of lymphocytes to lymphoid organs with HEV structures. (**a**) *In vitro* frozen-section adherence assay of the binding of splenocytes to HEVs in pooled PLNs from individual normothermic control mice (NT), WBH-treated mice or TNF-treated mice. Arrowheads in representative photomicrographs (left) indicate toluidine-stained splenocytes bound to individual HEVs in PLNs from BALB/c mice. Right, quantification (mean ± s.e.m.) of cells bound to HEVs ($n = 3 \times 10^2$ HEVs per mouse). B6, C57BL/6; SCID, severe combined immunodeficiency. (**b**) *In vivo* short-term (1-hour) homing of fluorescence-labeled splenocytes to secondary lymphoid organs (pooled PLNs, MLNs, Peyer's patches (PP) and spleen (SPL)) and nonlymphoid organs (liver and pancreas (Panc)) in individual normothermic control BALB/c mice or WBH-treated BALB/c mice. Left, representative photomicrographs of PLNs. Right, quantification (mean ± s.e.m.) of calcein-labeled cells in organ cryosections, by fluorescence microscopy ($n = 10$ fields per mouse). *, $P < 0.0001$, NT versus WBH. Scale bars (**a**,**b**), 50 μm. Data are representative of three or more (**a**) or ten (**b**) independent experiments.

such as vasodilation and blood flow, the contribution of thermal stress to endothelial adhesion remains mostly unexplored. The binding function of 'gatekeeper' cuboidal HEVs is augmented by thermal stress in the range of fever temperatures *in vivo*[16,17], whereas febrile temperatures do not influence the ability of squamous endothelium of nonlymphoid organs to support leukocyte adhesion *in vivo* or *in vitro*[16,18,19]. Here we report that the thermal element of fever controls the molecular mechanisms that underlie firm adhesion and transendothelial migration of blood-borne lymphocytes across HEVs. We identified an integrated function for IL-6 *trans* signaling as a regulator of trafficking by demonstrating that IL-6–sIL-6Rα mediated thermal induction of ICAM-1-dependent extravasation of lymphocytes selectively in HEVs of lymphoid organs. Our results support the idea that HEVs act as sentinels during febrile inflammatory responses by heightening trafficking of naive and central memory lymphocytes to secondary lymphoid organs.

RESULTS

Thermal stress stimulates lymphocyte-HEV interactions

We used several experimental approaches to assess the effects of thermal stress on HEV adhesion independently of other factors that may also influence trafficking, such as hemodynamic parameters. In the first series of studies, we treated mice for 6 h with whole-body hyperthermia (WBH) to raise the core temperature to the range of fever temperatures ('fever-range'; 39.5 ± 0.5 °C). We quantified the binding activity of individual HEVs by an *in vitro* assay in which we allowed splenocytes (from untreated mice) to adhere to HEVs in PLN

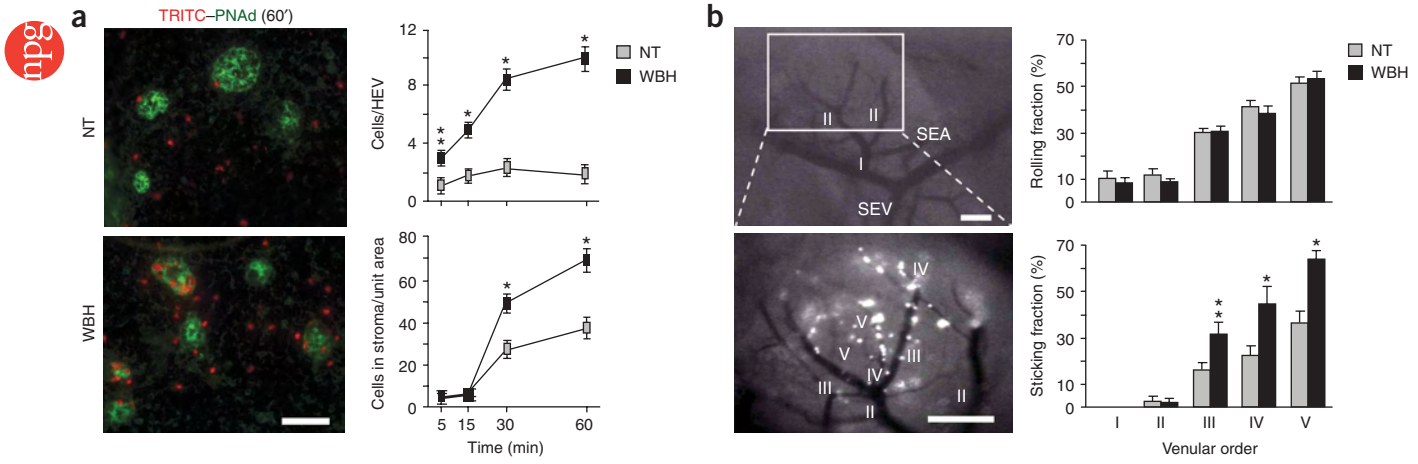


Figure 2 Fever-range thermal stress enhances lymphocyte-HEV interactions. (**a**) Kinetic analysis of lymphocyte homing. PLNs were isolated 5–60 min after adoptive transfer of TRITC-labeled splenocytes (red) into normothermic control BALB/c mice or WBH-treated BALB/c mice. Tissue cryosections (left) are stained with mAb specific for PNAd to visualize HEVs (green) and are representative photomicrographs of TRITC-labeled cells associated with HEVs or infiltrated into the tissue parenchyma at 60 min (60'); right, quantification of those cells by fluorescence microscopy. Data (mean ± s.e.m.) are of ten fields analyzed of pooled PLNs from individual mice and are representative of three or more independent experiments. (**b**) Intravital microscopy (left) of the interactions of calcein-labeled splenocytes with the lymph node venular tree of a WBH-treated mouse, showing the vascular structure, including the superficial epigastric artery (SEA), superficial epigastric vein (SEV) and venular branches (I–V) in an inguinal lymph node (**Supplementary Video 1** online). Right, rolling fractions and sticking fractions in normothermic control and WBH-treated C57BL/6 mice. Data (mean ± s.e.m.) are from three mice per treatment group in three independent experiments. Scale bars, 50 μm (**a**) or 200 μm (**b**). *, $P < 0.0001$, and **, $P < 0.01$, normothermic versus WBH.

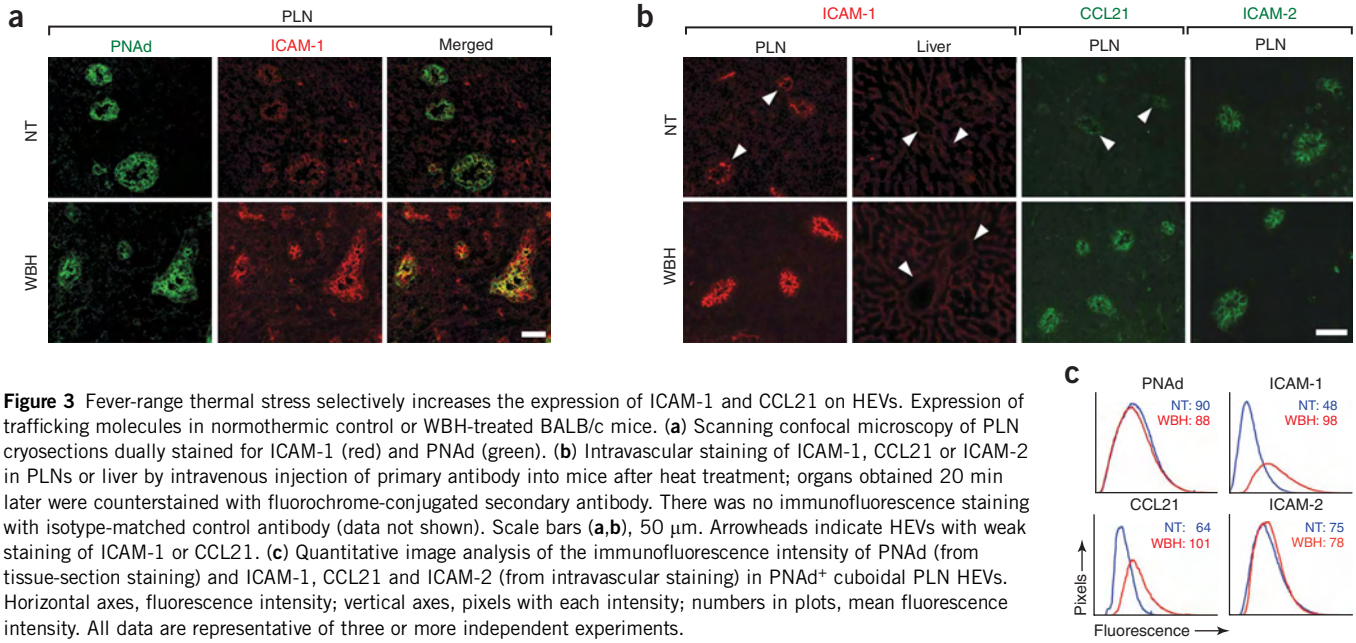


Figure 3 Fever-range thermal stress selectively increases the expression of ICAM-1 and CCL21 on HEVs. Expression of trafficking molecules in normothermic control or WBH-treated BALB/c mice. (**a**) Scanning confocal microscopy of PLN cryosections dually stained for ICAM-1 (red) and PNAd (green). (**b**) Intravascular staining of ICAM-1, CCL21 or ICAM-2 in PLNs or liver by intravenous injection of primary antibody into mice after heat treatment; organs obtained 20 min later were counterstained with fluorochrome-conjugated secondary antibody. There was no immunofluorescence staining with isotype-matched control antibody (data not shown). Scale bars (**a**,**b**), 50 μm. Arrowheads indicate HEVs with weak staining of ICAM-1 or CCL21. (**c**) Quantitative image analysis of the immunofluorescence intensity of PNAd (from tissue-section staining) and ICAM-1, CCL21 and ICAM-2 (from intravascular staining) in PNAd$^+$ cuboidal PLN HEVs. Horizontal axes, fluorescence intensity; vertical axes, pixels with each intensity; numbers in plots, mean fluorescence intensity. All data are representative of three or more independent experiments.

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organ cryosections from control mice at normal temperature ('normothermic' mice) or heat-treated mice. Fever-range WBH increased the ability of PLN HEVs to support lymphocyte adhesion in several immunocompetent mouse strains (C57BL/6, C3H and BALB/c) as well as in mice with severe combined immunodeficiency that lacked B lymphocytes and T lymphocytes (**Fig. 1a**). We detected similar increases in HEV adhesion in response to thermal stress or to the administration of recombinant tumor necrosis factor (TNF; **Fig. 1a**), a potent stimulator of vascular adhesion *in vitro* and *in vivo*[20].

We next examined the thermal regulation of lymphocyte trafficking in short-term (1-hour) *in vivo* homing assays in which we labeled splenocytes *ex vivo* with fluorescent tracking dyes (TRITC or calcein) and then injected the cells intravenously into normothermic control or WBH-treated BALB/c mice. We allowed recipient mice to equilibrate to normal body temperature before adoptively transferring labeled cells to reverse the thermal effects on blood flow and to avoid heat activation of L-selectin or $\alpha_4\beta_7$ integrin adhesion in labeled indicator cells[11–13]. Thermal stress caused an increase of approximately twofold in the trafficking of labeled cells selectively in secondary lymphoid organs with HEVs (PLNs, MLNs and Peyer's patches) but not in spleen or extralymphoid organs (such as liver or pancreas), which lack HEV structures (**Fig. 1b** and **Supplementary Fig. 1** online). WBH treatment did not alter the composition of cells that trafficked to PLNs, suggesting that homeostatic trafficking mechanisms were maintained. Thus, thermal stress did not result in enrichment for specific leukocyte populations that gained entry to PLNs, including T cells (CD4$^+$ and CD8$^+$) that traffic more efficiently than B cells (B220$^+$)[21,22], or monocytes (CD11b^{hi}Gr1^{lo}) and neutrophils (Gr1^{hi}), which were essentially excluded[2,3] (**Supplementary Fig. 1**). Naive cells (L-selectin-high CD44^{lo}) or central memory cells (L-selectin-high CD44^{hi}) constituted the main T cell subsets that homed to PLNs in control and hyperthermic conditions, whereas effector-memory T cells (L-selectin-low CD44^{hi}; **Supplementary Fig. 1**) and activated T cell populations (L-selectin-low CXCR3^{hi}CCR7^{lo}; data not shown) were excluded from this site.

We assessed the influence of thermal stress on the kinetics of lymphocyte interactions with gatekeeper HEVs after intravenous injection of fluorescence-labeled splenocytes into normothermic or WBH-treated mice. We counterstained tissue sections with monoclonal antibodies (mAbs) specific for molecules on PLN HEVs (PNAd) or Peyer's patch HEVs (MAdCAM-1) to determine whether labeled cells were located in HEVs or were extravasated into the tissue parenchyma. We found that WBH treatment increased the number of lymphocytes associated with PLN HEVs (**Fig. 2a**) and Peyer's patch HEVs (**Supplementary Fig. 2** online) within 5 min of transfer and that the increase in lymphocyte-HEV interactions was sustained over 1 h. Notably, more lymphocytes completed the multistep process culminating in extravasation into the stroma of PLNs or Peyer's patches by 30 min in WBH-treated mice than in normothermic control mice at 1 h ($P < 0.002$; **Fig. 2a** and **Supplementary Fig. 2**).

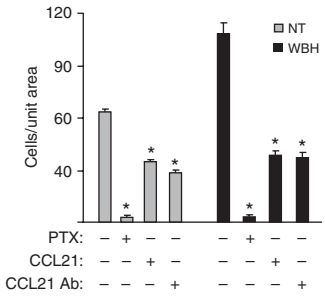


Figure 4 CCL21 is required for the thermal stimulation of lymphocyte trafficking across HEVs. Short-term (1-hour) homing of TRITC-labeled splenocytes. Cells were pretreated with (+) or without (–) pertussis toxin (PTX) or were pretreated with (+) or without (–) a desensitizing concentration of CCL21 before adoptive transfer into normothermic control or WBH-treated BALB/c mice. Alternatively, mice were treated for 20 min with (+) or without (–) CCL21-neutralizing antibody (CCL21 Ab) before cell transfer. Isotype-matched control antibody did not affect lymphocyte homing (data not shown). Cryosections of pooled PLNs from individual mice were counterstained for PNAd, and TRITC-labeled cells infiltrating the parenchyma were quantified by fluorescence microscopy ($n = 10$ fields per mouse). *, $P < 0.0001$, function-blocking reagent versus untreated control. Data (mean ± s.e.m.) are representative of three or more independent experiments.

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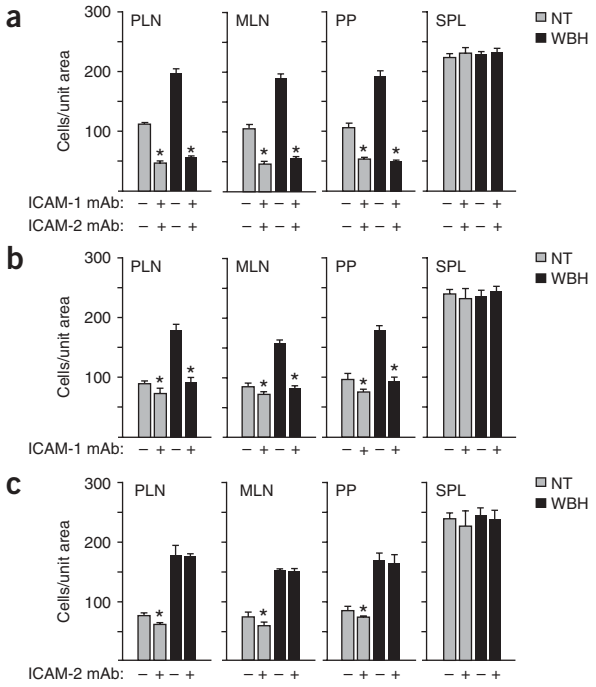


Figure 5 ICAM-1 is required for the thermal enhancement of trafficking across HEVs. Short-term (1-hour) homing of TRITC-labeled splenocytes in normothermic control or WBH-treated BALB/c mice. At 20 min before adoptive transfer, recipient mice were treated with function-blocking mAbs (+) specific for ICAM-1 and ICAM-2 (**a**) or for ICAM-1 only (**b**) or ICAM-2 only (**c**) or with isotype-matched control antibody (–). TRITC-labeled cells in tissue cryosections from individual mice were quantified by fluorescence microscopy ($n = 10$ fields per mouse). *, $P < 0.0001$, function-blocking mAb versus control antibody. Data (mean ± s.e.m.) are representative of three or more independent experiments.

We used intravital microscopy of inguinal lymph nodes to pinpoint the nature of the adhesive interactions regulated by thermal stress (**Fig. 2b**). High-order (III–V) postcapillary HEVs detected in this organ preparation reside in the paracortical region, whereas low-order (I and II) flat-walled venules drain into the superficial epigastric vein in the medulla[23]. WBH pretreatment did not affect the frequency of rolling interactions throughout the venular tree (**Fig. 2b**). However, heat treatment substantially increased the fraction of cells that transitioned from primary rolling to secondary firm sticking exclusively in order III–V venules (HEVs). The velocity of noninteracting lymphocytes in order III venules was equivalent in normothermic and WBH-treated mice (1,195 ± 340 μm/s and 1,130 ± 549 μm/s, respectively (mean ± s.e.m.); $n > 60$ cells analyzed in three experiments), indicating that the increase in sticking interactions could not be attributed to sustained effects of heat pretreatment on blood flow. Thus, thermal stimulation of lymphocyte homing in lymphoid organs is correlated with enhanced secondary firm adhesive interactions selectively in HEVs.

Thermal stress enhances ICAM-1 and CCL21 display in HEVs

To investigate the molecular basis of the thermal stimulation of lymphocyte-HEV interactions, we examined the expression of trafficking molecules known to participate in the multistep adhesion sequence in HEVs. Two-color confocal immunofluorescence microscopy of tissue cryosections showed that fever-range thermal stress did not alter the relative expression of PNAd or MAdCAM-1, which mediate primary tethering and rolling in PLN or Peyer's patch HEVs, respectively (**Fig. 3a** and **Supplementary Fig. 2**). In contrast, the expression of ICAM-1, which participates in secondary adhesion and transendothelial migration, was enhanced considerably by heat in PNAd$^+$ and MAdCAM-1$^+$ HEVs (**Fig. 3a** and **Supplementary Fig. 2**). We detected similar ICAM-1 staining in HEVs after thermal stress or systemic administration of recombinant TNF (**Supplementary Table 1** online). Although TNF treatment upregulated ICAM-1 indiscriminately on the vessels of all organs examined, the response to thermal stress was site specific, such that only HEVs of lymphoid

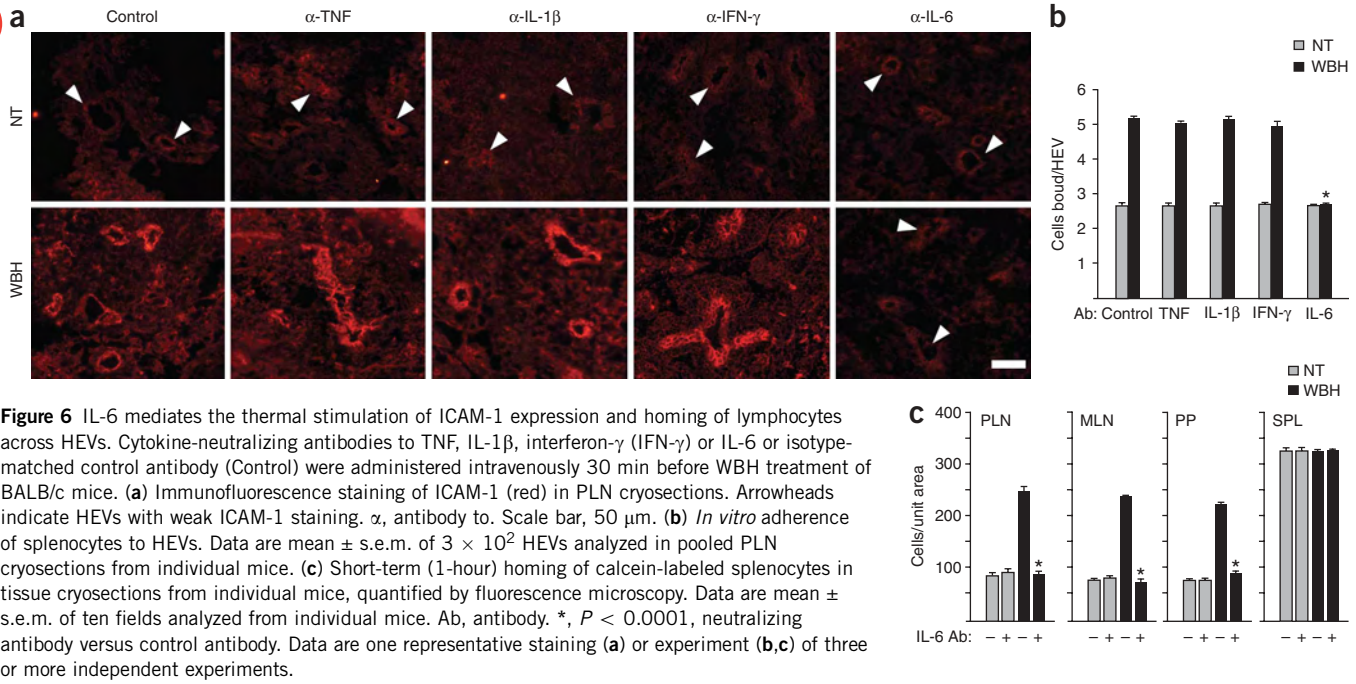


Figure 6 IL-6 mediates the thermal stimulation of ICAM-1 expression and homing of lymphocytes across HEVs. Cytokine-neutralizing antibodies to TNF, IL-1β, interferon-γ (IFN-γ) or IL-6 or isotype-matched control antibody (Control) were administered intravenously 30 min before WBH treatment of BALB/c mice. (**a**) Immunofluorescence staining of ICAM-1 (red) in PLN cryosections. Arrowheads indicate HEVs with weak ICAM-1 staining. α, antibody to. Scale bar, 50 μm. (**b**) *In vitro* adherence of splenocytes to HEVs. Data are mean ± s.e.m. of 3×10^2 HEVs analyzed in pooled PLN cryosections from individual mice. (**c**) Short-term (1-hour) homing of calcein-labeled splenocytes in tissue cryosections from individual mice, quantified by fluorescence microscopy. Data are mean ± s.e.m. of ten fields analyzed from individual mice. Ab, antibody. *, $P < 0.0001$, neutralizing antibody versus control antibody. Data are one representative staining (**a**) or experiment (**b**,**c**) of three or more independent experiments.

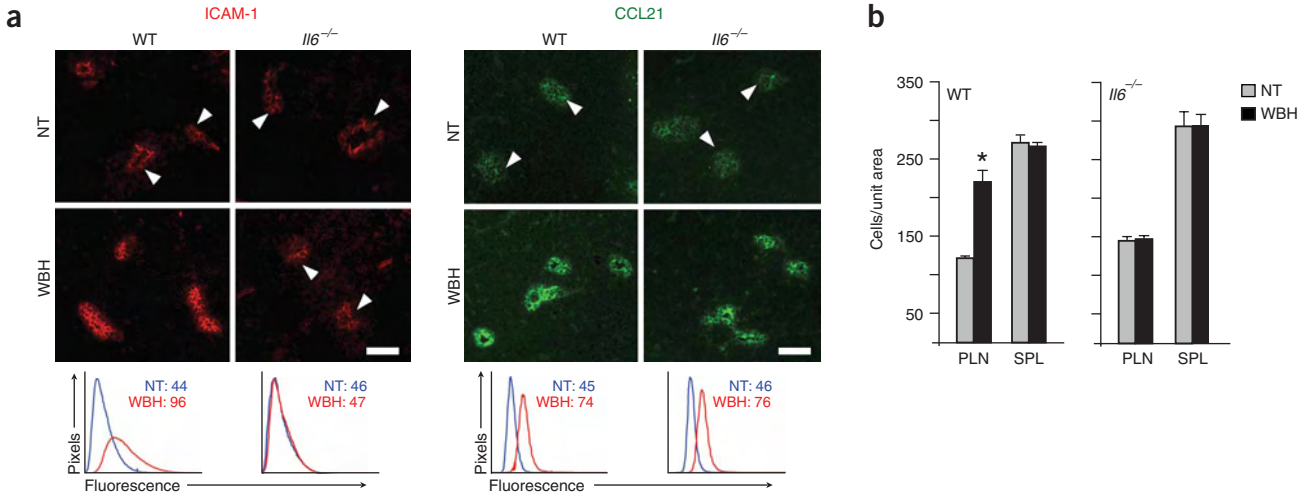


Figure 7 Thermal induction of ICAM-1 and lymphocyte homing does not occur in IL-6-deficient mice. (**a**) Intravascular staining of ICAM-1 (red) and CCL21 (green) in PLNs from normothermic control or WBH-treated *Il6*$^{-/-}$ and wild-type (WT) C57BL/6 mice. Arrowheads indicate HEVs with weak staining of ICAM-1 or CCL21. Scale bars, 50 μm. Bottom, quantitative image analysis of ICAM-1 and CCL21 staining: horizontal axes, fluorescence intensity; vertical axes, pixels with each intensity; numbers in plots, mean fluorescence intensity. (**b**) Short-term (1-hour) homing of TRITC-labeled splenocytes in tissue cryosections from individual *Il6*$^{-/-}$ and wild-type mice with (WBH) or without (NT) WBH treatment, quantified by fluorescence microscopy. Data are mean ± s.e.m. of ten fields analyzed from individual mice. *, $P < 0.0001$, NT versus WBH. All data are representative of three or more independent experiments.

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organs had increased ICAM-1 expression (**Supplementary Table 1**). WBH treatment did not alter the vascular expression of other adhesion molecules that mediate transendothelial migration, including CD31 (**Supplementary Table 1**) and junctional adhesion molecules 1 and 2 (data not shown). Thermal stress also did not modify the HEV expression of molecules associated with inflammatory responses, such as vascular cell adhesion molecule 1 (**Supplementary Table 1**), E-selectin and Duffy antigen–related receptor for chemokines (data not shown), which has been linked to regulation of the activity and/or availability of inflammatory chemokines (CXCL1, CXCL5, CCL2, CCL5 and CCL7) but not of homeostatic chemokines (CCL21, CCL19, CXCL12 and CXCL13)[3,4,24].

We did intravascular staining *in situ* by injecting ICAM-1-specific mAb into the vascular compartments of recipient mice, then counterstaining tissue sections with fluorescence-labeled detection antibody. Hyperthermia augmented ICAM-1 expression on the lumenal and junctional surfaces of high endothelial cells (HECs; **Fig. 3b**), which are the contact sites for blood-borne lymphocytes undergoing firm adhesion or transendothelial migration. Thermal stress also substantially increased intravascular presentation of the homeostatic chemokine CCL21 (**Fig. 3b**), the principal chemokine required for the transition of lymphocytes from primary rolling interactions to secondary arrest in HEVs, without affecting the weak-to-nondetectable staining for other homeostatic chemokines (CCL19, CXCL12 and CXCL13) on HEVs (data not shown). Quantitative image analysis showed that thermal stress increased the mean fluorescence staining intensity for ICAM-1 and CCL21 on PNAd^{+} cuboidal HEVs by about 2-fold and 1.5-fold, respectively, whereas PNAd and ICAM-2 staining was unchanged (**Fig. 3c**). The finding that the intravascular density of ICAM-2 was not altered established that WBH pretreatment did not generally increase the access of mAbs to epitopes in the HEV microcompartment (**Fig. 3b,c**). There was no change in the lumenal presentation of ICAM-1 (**Fig. 3b**) or CCL21 (data not shown) on the vessels of extralymphoid organs such as liver. Consistent with the observation that thermal stress altered ICAM-1 and CCL21 expression only in HECs that constitute a minor population in nodal tissues (less than 4% of stromal cell–rich fractions[25]), we did not detect a change in the total amounts of those proteins in whole-PLN lysates (**Supplementary Fig. 3** online). These data suggested that increased intravascular display of CCL21 and ICAM-1 contributes to enhanced lymphocyte trafficking across HEVs in response to thermal stress.

Enhanced homing depends on CCL21 and ICAM-1

We used a 'multipronged' approach to determine the relative contributions of CCL21 and its ligand, CCR7, to improved trafficking by thermal stress. We initially used pertussis toxin, an irreversible inhibitor of $G_{\alpha i1}$ protein signaling, to globally inhibit chemokine-dependent entry of lymphocytes into lymphoid organs. Pretreatment of fluorescence-labeled splenocytes with pertussis toxin fully blocked their ability to extravasate in PLNs in both normothermic and hyperthermic conditions (**Fig. 4**). To directly target the CCR7 chemokine receptor for CCL21, we pre-exposed fluorescence-labeled splenocytes to a high concentration of recombinant CCL21 (10 μg/ml) *ex vivo* before transfer into recipient mice (**Fig. 4**). That treatment did not alter the surface expression of L-selectin or LFA-1 on lymphocytes (data not shown). As reported before[26], CCR7 desensitization by that strategy reduced the homing of lymphocytes to PLNs (**Fig. 4**) and Peyer's patches (data not shown) in normothermic conditions, albeit only partially, which probably reflected incomplete desensitization or resensitization *in vivo*. The extent of reduction in lymphocyte trafficking was similar in normothermic and WBH-pretreated mice, indicating a common CCR7-dependent mechanism during trafficking across HEVs in both temperature conditions. Lymphocyte extravasation in control and heat-treated mice was also reduced substantially by a complementary strategy targeting CCL21 *in vivo* with a function-blocking antibody (**Fig. 4**). We conclude that CCR7-CCL21 is the main chemokine receptor–chemokine pair responsible for improved lymphocyte entry across HEVs in response to thermal stress.

Those observations raised the issue of whether CCL21 induction by thermal stress was the sole determinant of physiological importance for homing in HEVs or if heat-inducible ICAM-1 contributed to this

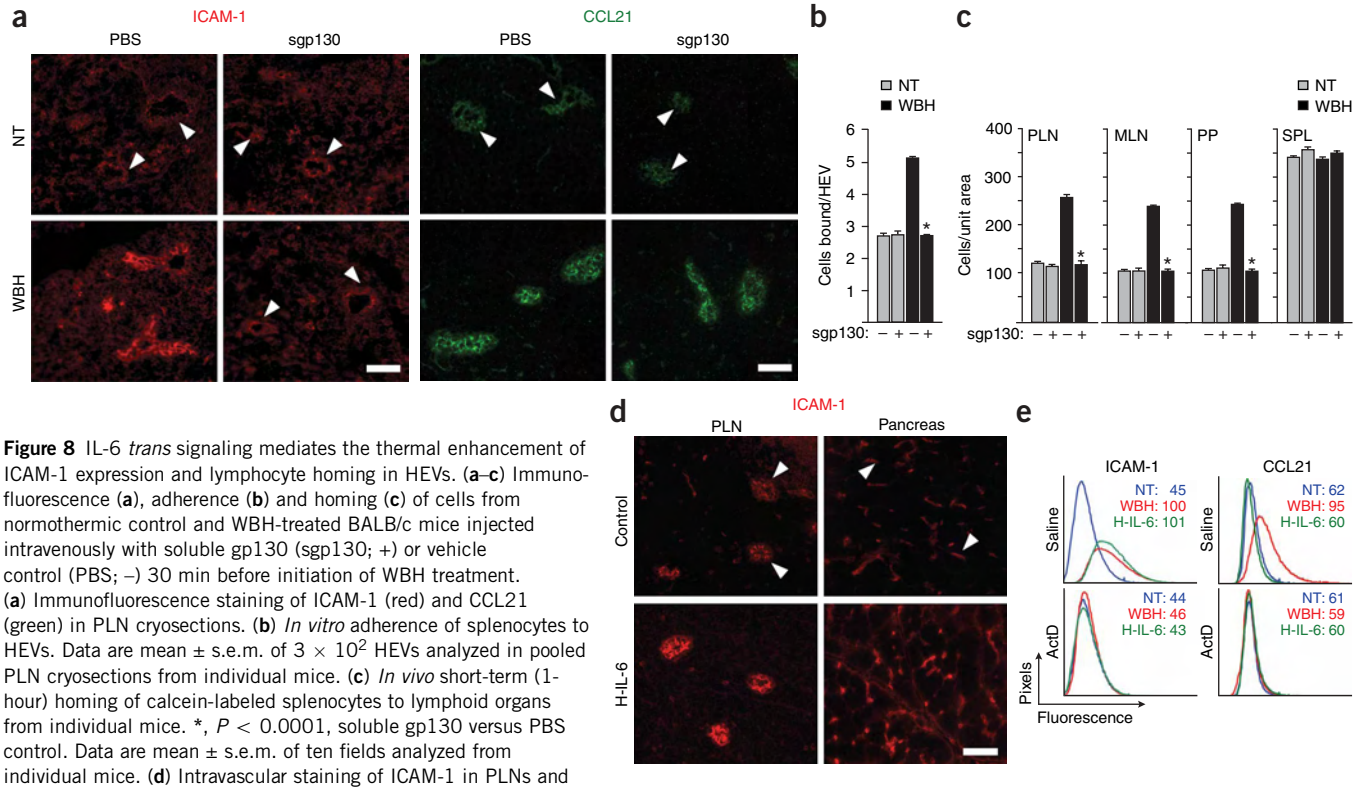


Figure 8 IL-6 *trans* signaling mediates the thermal enhancement of ICAM-1 expression and lymphocyte homing in HEVs. (**a**–**c**) Immunofluorescence (**a**), adherence (**b**) and homing (**c**) of cells from normothermic control and WBH-treated BALB/c mice injected intravenously with soluble gp130 (sgp130; +) or vehicle control (PBS; –) 30 min before initiation of WBH treatment. (**a**) Immunofluorescence staining of ICAM-1 (red) and CCL21 (green) in PLN cryosections. (**b**) *In vitro* adherence of splenocytes to HEVs. Data are mean ± s.e.m. of 3×10^2 HEVs analyzed in pooled PLN cryosections from individual mice. (**c**) *In vivo* short-term (1-hour) homing of calcein-labeled splenocytes to lymphoid organs from individual mice. *, $P < 0.0001$, soluble gp130 versus PBS control. Data are mean ± s.e.m. of ten fields analyzed from individual mice. (**d**) Intravascular staining of ICAM-1 in PLNs and pancreata from BALB/c mice treated with hyper-IL-6 (H-IL-6) and from control BALB/c mice. (**e**) Image analysis quantification of the intravascular staining intensity of ICAM-1 and CCL21 in HEVs of pooled PLNs from individual BALB/c mice given actinomycin D (ActD) or vehicle control (Saline) intraperitoneally 30 min before treatment for 6 h with WBH or hyper-IL-6. Horizontal axes, fluorescence intensity; vertical axes, pixels with each intensity; numbers in plots, mean fluorescence intensity. Arrowheads (**a**,**d**) indicate HEVs with weak staining of ICAM-1 or CCL21; scale bars (**a**,**d**), 50 μm. Data are one representative staining (**a**,**d**) or experiment (**b**,**c**,**e**) of three or more independent experiments.

process. We addressed that issue by assessing the relative requirements for LFA-1 and its cognate receptors, ICAM-1 and ICAM-2, in short-term homing studies. Combined mAb blockade of ICAM-1 and ICAM-2 reduced lymphocyte trafficking across HEVs considerably in both normothermic and hyperthermic conditions (**Fig. 5a**), paralleling results obtained with an LFA-1-blocking mAb (data not shown). Independent mAb blockade of ICAM-1 or ICAM-2 had only modest effects on trafficking to lymphoid organs in normothermic conditions (**Fig. 5b,c**), supporting reports that those molecules can substitute for each other as HEV ligands for LFA-1 during steady-state homing in HEVs[27,28]. Interference with ICAM-1 activity, either by neutralizing mAb (**Fig. 5b**) or by genetic targeting in ICAM-1-deficient mice (**Supplementary Fig. 4** online), completely prevented the thermal stimulation of trafficking to PLNs, MLNs and Peyer's patches. In contrast, functional inhibition of ICAM-2 failed to reduce trafficking across HEVs in WBH-treated mice (**Fig. 5c**). We confirmed the requirement for ICAM-1 in heat-induced HEV adhesion by a frozen-section *in vitro* adherence assay (**Supplementary Fig. 4**). Although this assay mainly measures PNAd-dependent adhesion in PLN HEVs in normal conditions[5], LFA-1–ICAM–dependent binding interactions have been characterized in HEVs of inflamed lymph nodes with this method[29]. Evidence that HEVs of control and hyperthermia-treated mice supported equivalent PNAd-dependent adhesion after concomitant blockade of ICAM-1 and ICAM-2 in frozen-section assays (**Supplementary Fig. 4**) was consistent with intravital studies (**Fig. 2b**) demonstrating that thermal stress did not augment primary adhesive interactions in HEVs. These results collectively demonstrated that ICAM-1 is the 'preferential' binding partner for LFA-1 in HEVs in thermal conditions and that high-density presentation of CCL21 is not sufficient, in the absence of ICAM-1, to support improved trafficking to lymphoid organs.

IL-6 *trans* signaling mediates enhanced trafficking

Inflammatory cytokines, including TNF, IL-1β, interferon-γ and IL-6, are potent stimulators of endothelial expression of ICAM-1 and inflammatory chemokines *in vitro* or *in vivo*[30] (**Supplementary Table 1**). To determine if those candidate cytokines regulate HEV adhesion during thermal stress, we treated mice with cytokine-neutralizing mAbs before initiating WBH and then examined expression of ICAM-1 or CCL21 by HEVs. There was thermal induction of ICAM-1 in PLN HEVs (**Fig. 6a**) and Peyer's patch HEVs (data not shown), despite the presence of mAbs specific for TNF, IL-1β or interferon-γ, whereas there was no induction in mice treated with an IL-6 function–blocking mAb. Similarly, only IL-6-neutralizing mAb blocked the thermal enhancement of HEV adhesion detected by frozen-section *in vitro* adherence assays (**Fig. 6b**). The IL-6-neutralizing mAb also prevented the thermal stimulation of lymphocyte homing across HEVs in PLNs, MLNs and Peyer's patches (**Fig. 6c**). Improved trafficking was not accompanied by a measurable increase in total nodal content of IL-6 during heat treatment (IL-6 in PLNs of normothermic control mice and in mice after WBH treatment at 2, 4 and 6 h: 1.1 ± 0.2 pg/mg total protein, 0.9 ± 0.2 pg/mg total protein, 0.6 ± 0.1 pg/mg total protein and 1.0 ± 0.2 pg/mg total protein, respectively (mean ± s.e.m.); $n = 3$ mice). We confirmed the requirement for IL-6 in IL-6-deficient mice, as thermal stress failed to induce ICAM-1 expression or lymphocyte trafficking in HEVs in

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these mice (**Fig. 7a**,**b**). In contrast, IL-6-deficient mice (**Fig. 7a**) and wild-type mice treated with IL-6-neutralizing mAb (data not shown) had normal CCL21 induction in response to thermal stress.

IL-6 regulation of ICAM-1 occurs in primary venular endothelial cells *in vitro* that lack the membrane form of the IL-6Rα binding subunit by means of a *trans*-signaling mechanism that depends on the availability of both IL-6 and a soluble form of IL-6Rα[14,31,32]. To test whether IL-6 *trans* signaling contributes to ICAM-1-mediated homing during thermal stress *in vivo*, we administered recombinant soluble gp130 before WBH treatment. Soluble gp130 competitively inhibits *trans* signaling by IL-6–sIL-6Rα without interfering with the classical signaling pathway mediated by membrane-anchored IL-6Rα[14,15]. Soluble gp130 fully blocked the thermal induction of ICAM-1 expression on HEVs but did not prevent CCL21 upregulation (**Fig. 8a**). Moreover, disruption of IL-6 *trans* signaling by soluble gp130 prevented the thermal enhancement of HEV adhesion detected in frozen-section *in vitro* adherence assays as well as trafficking across HEVs *in vivo* without affecting the baseline activity of HEVs in normothermic conditions (**Fig. 8b**,**c**).

To determine whether thermal regulation of trafficking molecules in HEVs involves transcriptional control, we treated mice with actinomycin D, a pharmacological inhibitor of transcription *in vivo*[33]. We 'mapped' the requirement for transcriptional events in the IL-6 *trans*-signaling pathway leading to ICAM-1 induction in parallel studies that examined the effect of actinomycin D on IL-6 *trans*-signaling responses triggered directly by 'hyper-IL-6', a recombinant fusion protein in which IL-6 is covalently linked to sIL-6Rα[34]. We detected similar ICAM-1 induction on HEVs in response to hyper-IL-6 or thermal stress (**Fig. 8d**,**e**). However, in contrast to the site-specific response of thermal stress, ICAM-1 was also widely induced by hyper-IL-6 on vessels of extralymphoid organs (such as pancreas, liver, kidney and heart; **Fig. 8d** and data not shown). Actinomycin D abrogated ICAM-1 upregulation at all vascular sites in response to thermal stress or hyper-IL-6 (**Fig. 8e** and data not shown) without disrupting cellular processes that do not depend on new transcription, such as activation of 'downstream' signaling molecules (such as STAT3) by hyper-IL-6 (data not shown). The thermal enhancement of intravascular CCL21 in HEVs was also sensitive to actinomycin D treatment (**Fig. 8e**). Consistent with the observation that the thermal upregulation of CCL21 did not involve endogenous IL-6 *trans*-signaling mechanisms, administration of hyper-IL-6 failed to increase the presentation of CCL21 on HEVs (**Fig. 8e**). Overall, these data support a model whereby the vascular-specific effects of fever-range thermal stress on ICAM-1-mediated lymphocyte trafficking across HEVs are orchestrated by IL-6 *trans* signaling, whereas IL-6 is dispensable for enhanced intravascular display of CCL21 (**Supplementary Fig. 5** online).

DISCUSSION

Secondary lymphoid organs are strategically positioned to provide the first line of defense against invading pathogens. Microbes entering the skin are a source of antigens that are transported by Langerhans cells to draining lymph nodes through the afferent lymphatics. These antigen-presenting cells localize proximal to HEVs[3], the main gateways for recirculating B lymphocytes and T lymphocytes. Enteric antigens have immediate access to gut-associated Peyer's patches and MLNs. The continual flux of pathogen-derived antigens and immune cells through lymphoid organs increases the probability that cognate antigens will be encountered by rare antigen-specific lymphocytes present at a frequency of only 1 in 1×10^5 to 1×10^6 cells.

Our study has provided insight into the beneficial mechanism of action of the thermal component of fever that has generally been relegated to a bystander function during infection and inflammation. A central finding was that fever-range thermal stress augmented the capture efficiency of gatekeeper HEVs for naive and central memory lymphocytes by increasing the intravascular density of CCL21 and ICAM-1. Notably, the approximately twofold increase in recruitment induced by thermal stress represented a profound enhancement in an already efficient process in which about one of every four lymphocytes that enter HEVs completes the multistep cascade leading to extravasation[1,3]. Thus, the acute effects of fever on lymphocyte trafficking would be predicted to enhance substantially the repertoire of antigen-specific B lymphocytes and T lymphocytes that screen lymphoid organs for the presence of foreign antigens. That mechanism complements the stimulatory effects of thermal stress on the migration of skin-derived Langerhans cells to lymph nodes[10,35].

Our findings expand on the long-recognized function of lymph nodes in mobilizing the immune system during inflammation[3,36]. Local release of TNF or CCL4 by mast cells at sites of bacterial challenge or antigen stimulation enhances T cell entry into draining lymph nodes[37,38]. Viral infection has also been reported to amplify $CD4^+$ and $CD8^+$ T cell recirculation by increasing the diameter of the feeding arterioles that supply postcapillary HEVs in draining lymph nodes (from about 100 μm to 150 μm)[39]. A fundamental difference between our findings and published results obtained in inflamed lymph nodes relates to the scope of the vascular response. Local inflammation at sites of infection or antigen challenge influences trafficking only in draining lymph nodes[3,36]. In contrast, fever-range WBH, which mirrors the thermal component of systemic fever, augments the recruitment properties of the 'HEV axis' throughout the body. Heightened immune surveillance of distal secondary lymphoid organs during fever would provide a substantial advantage by protecting the host against widespread dissemination of rapidly multiplying infectious agents. A notable distinction is that systemic thermal stress maintains homeostatic trafficking mechanisms, whereas local tissue inflammation 'opens the gateway' for $CCR7^-$ cell types normally excluded from trafficking across HEVs in draining lymph nodes[3,36]. Thus, thermal stress mainly improves the recruitment of naive and central memory $CD4^+$ and $CD8^+$ T cells and B cells across lymph node HEVs without disproportionately enhancing the homing of monocytes, neutrophils, effector-memory T cell subsets or activated T cells.

The maintenance of homeostatic lymphocyte recirculation by thermal stress is explained by the nature of the trafficking molecules upregulated on HEVs. Fever-range WBH 'preferentially' enhanced the intravascular density of two molecules involved in homeostatic trafficking (CCL21 and ICAM-1). Those data are consistent with *in vitro* studies showing that CCL21 and ICAM-1 act cooperatively to optimize LFA-1 binding activity[40–43]. CCL21 functions in a dose-dependent way *in vitro* to activate 'inside-out' signaling, leading to ligand-independent conformational changes that convert LFA-1 from a low-affinity state to an intermediate-affinity state[40,42]. Transition of LFA-1 to a high-affinity state depends on 'outside-in' signaling initiated by engagement of LFA-1 by ICAM-1 through a mechanism that is highly dependent on ICAM-1 density[40,41].

A central finding of our study was that CCL21 was necessary but not sufficient to support improved trafficking across HEVs in response to thermal stress. Interference with ICAM-1-dependent adhesion abrogated the thermal effects on trafficking despite high intravascular density of CCL21 and the availability of ICAM-2. The critical requirement for ICAM-1 during lymphocyte egress across HEVs demonstrated by thermal stress was unexpected, given that ICAM-1 and ICAM-2 can substitute for each other during steady-state trafficking in normothermic conditions[27,28]. Fundamental differences

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between ICAM-1 and ICAM-2 may explain why ICAM-1 is the 'preferential' binding partner for LFA-1 during thermal responses. The high intravascular density of ICAM-1 resulting from thermal stimulation *in vivo* could theoretically favor the formation of stable dimers and higher-order multimers, as demonstrated biochemically in endothelial cells *in vitro* after ICAM-1 upregulation by TNF or by genetic overexpression[43,44]. Notably, ICAM-2 cannot form dimers, which may relate to intrinsic differences in the hydrophobicity of the N-terminal regions of ICAM-2 and ICAM-1 (refs. 43,45). ICAM-1 dimer formation reinforces molecular interactions with high-affinity LFA-1 conformers; thus, the bond lifetimes of monomeric and dimeric ICAM-1 for LFA-1 are about 25 s and 330 s, respectively[46]. The regulation of ICAM-1 density by thermal stress may also affect its ability to redistribute to cup-like structures that are proposed to provide traction for cells undergoing transmigration[47,48]. In contrast to ICAM-1, ICAM-2 is present in only moderate amounts in endothelial microdomains juxtaposed to LFA-1 on migrating lymphocytes[47].

Our studies here have demonstrated a nonredundant function for IL-6 *trans* signaling in controlling lymphocyte access to lymphoid organs during thermal stress. Disruption of IL-6 *trans*-signaling mechanisms prevented the thermal upregulation of ICAM-1 on HEVs and enhancement of lymphocyte homing. It remains to be determined if increased ICAM-1 expression results from the direct engagement of gp130 molecules on HECs by IL-6–sIL-6Rα and subsequent STAT3-mediated activation of the ICAM-1 promoter, as reported for primary endothelial cells (human umbilical vein endothelial cells) *in vitro*[30–32], or if other IL-6-responsive intermediary cells participate in that response. The finding that actinomycin D inhibited ICAM-1 upregulation on HEVs by both thermal stress and hyper-IL-6 indicated a transcriptional component of the pathway downstream of gp130 ligation by IL-6 and sIL-6Rα that controls new ICAM-1 synthesis. Our results excluding the possibility of involvement of IL-6–sIL-6Rα during thermal induction of the homeostatic chemokine CCL21 are not entirely unexpected, as IL-6 *trans* signaling has been linked mainly to regulation of the production of inflammatory chemokines (CCL2, CCL8, CXCL5 and CXCL6) *in vitro* or *in vivo*[14,15]. The mechanisms controlling CCL21 expression on the lumenal surface of HEVs are unknown[3,4]. Data indicating that the thermal control of intravascular presentation of CCL21 depended on newly initiated transcriptional events without altering the total nodal content of CCL21 are consistent with a scenario in which chemokine synthesis is induced only in limited cell populations (such as HECs or perivascular cells) or in which heat controls the transcription of as-yet-unidentified molecules involved in the transport, presentation or turnover of that homeostatic chemokine[3,4].

Many lines of evidence indicate that the IL-6 *trans*-signaling response initiated by thermal stress involves local microenvironmental control of adhesion in discrete vascular beds. Notably, the thermal effects on firm sticking of lymphocytes were restricted to high-order (III–V) venules (HEVs) in PLNs. That result was in contrast to studies demonstrating downstream low-order (I–II) segments in the same venular tree that were refractory to thermal stress. Immunolocalization studies established that upregulation of ICAM-1 occurred solely in the numerically minor population of HECs without affecting the total ICAM-1 content in nodal tissues. Moreover, ICAM-1 was not induced indiscriminately by hyperthermia in vessels of extralymphoid organs. A possible key to the tight control of vascular-specific responses lies in the dual requirement for IL-6 and sIL-6Rα in the initiation of thermal responses. Notably, the squamous endothelium of extralymphoid organs is able to upregulate ICAM-1 in response to IL-6 *trans* signaling initiated by hyper-IL-6. One possible explanation for the finding that thermal upregulation of ICAM-1 occurs exclusively in HEVs without affecting the overall IL-6 concentration in lymphoid organs is that site-specific vascular responses to hyperthermia are dictated by the local bioavailability of IL-6 and/or sIL-6R in microanatomically restricted sites. Many cell types are a potential source of IL-6 in lymphoid organs, including monocytes, lymphocytes, dendritic cells, endothelial cells, fibroblasts or pericytes located in the fibroreticular network surrounding HEVs, whereas sIL-6Rα derives mainly from leukocytes[13,49].

In conclusion, we have provided evidence that temperatures in the range of physiological fever act as an alert system to enhance the frequency of the homeostatic recirculation of lymphocytes across HEVs. The febrile response involves an integrated function for IL-6 *trans* signaling in controlling not only HEV adhesion, as reported here, but also the binding function of the L-selectin homing receptor in lymphocytes[9,13,17]. Our findings enlarge on the function of IL-6 *trans* signaling in controlling lymphocyte trafficking during acute and chronic inflammation[14,15]. IL-6–sIL-6Rα activity has been linked to T cell recruitment at sites of acute inflammation as well as to the pathogenesis of chronic inflammatory disorders, including inflammatory bowel disease, rheumatoid arthritis, peritonitis and diabetes[14,15]. Thus, our finding that IL-6 regulates ICAM-1-dependent lymphocyte trafficking in the context of acute febrile responses may have broad relevance to the mechanisms underlying chronic inflammation.

METHODS

Mice. The following age-matched (8–12 weeks of age) female mice were purchased from Jackson Laboratory or Taconic: BALB/c, C3H, C57BL/6, severe combined immunodeficient (BALB/c background), IL-6-deficient (B6.129S2-*Il6*^{tm1Kopf}/J on a C57BL/6 background) and ICAM-1-deficient (Icam1^{tmJegr}/J on a C57BL/6 background). Mice were maintained in pathogen-free barrier conditions. All animal protocols were approved by the Roswell Park Cancer Institutional Animal Care and Use Committee.

Treatment with fever-range WBH, cytokine-neutralizing reagents, recombinant cytokines or transcription inhibitor. Mice were treated with fever-range WBH (core temperature of 39.5 ± 0.5 °C for 6 h) by being placed in an environmental chamber at 38.8 °C (model BE5000; Memmert) as described[16,35]. Normothermal control mice (core temperature, 36.8 ± 0.2 °C) were maintained at 22 °C in a darkened cabinet for the experimental period. Mice were injected intraperitoneally with 1 ml sterile saline to avoid dehydration, and the core temperatures of 'sentinel' mice in all experimental groups were monitored with a subcutaneously implanted microchip thermotransponder (14 mm × 2.2 mm; implanted 1 week or more before WBH treatment) and a programmable data-acquisition system (Bio Medic Data Systems). Neutralizing mAbs specific for IL-6, TNF, IL-1β, interferon-γ (100 μg/mouse in 200 μl PBS; R&D Systems) or recombinant soluble gp130 (2.5 μg/mouse in 250 μl PBS; R&D Systems) were injected intravenously into mice 30 min before WBH treatment. Mice were injected intraperitoneally with 1 ml of recombinant TNF (10 μg/kg body weight in sterile saline; R&D Systems) or hyper-IL-6 (80 μg/kg body weight) 6 h before organs were collected. Details for the production of hyper-IL-6 are in the **Supplementary Methods** online. Actinomycin D (Sigma) was injected intraperitoneally (2 mg/kg body weight in 1 ml saline, a dose reported to arrest transcription *in vivo*[33]) 30 min before the initiation of WBH or hyper-IL-6 treatment.

Frozen-section *in vitro* adhesion assay. Lymphocyte adhesion to HEVs was evaluated by a frozen-section *in vitro* adhesion assay as described[11,13,16]. A total of 5 × 10^6 mouse splenocytes were overlaid onto 12-μm-thick cryosections of PLNs (pooled inguinal, brachial, axillary, sciatic, superficial and deep cervical nodes) from normothermic control mice or WBH-treated mice. Selected lymphoid tissue specimens were blocked with mAb specific for PNAd (MECA79; 50 μg/ml), ICAM-1 (3E2; 5 μg/ml), ICAM-2 (3C4; 5 μg/ml) or isotype control antibody from BD Biosciences. The assay was done at 4 °C for 30 min with

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mechanical rotation (112 r.p.m.; DS-500 Orbital Shaker; VWR). After removal of nonadherent cells, sections were fixed in 3% glutaraldehyde and were stained with 0.5% toluidine. Lymphocyte adhesion was quantified by light microscopy (Olympus, Spectra Services) for analysis of a total of 3×10^2 HEVs per lymphoid tissue sample. For consistency in 'double-blind' evaluations, HEVs were quantified only if they contained one adherent cell or more.

***In vivo* homing assay.** Homing of lymphocytes to lymphoid and nonlymphoid organs was assessed by a short-term homing assay[16,17]. Mouse splenocytes at a density of 5×10^7 cells per ml were labeled for 20 min at 37 °C with 180 μg/ml of TRITC (tetramethylrhodamine-6-isothiocyanate; Molecular Probes) or 1 μg/ml of calcein (Molecular Probes) in RPMI 1640 medium (Invitrogen), and labeling was stopped by centrifugation through a 'cushion' of FCS (Invitrogen). Equivalent numbers of labeled cells (1×10^7 to 2×10^7 cells in 300 μl PBS) were injected intravenously into WBH-treated mice and normothermic control mice, and organs were collected 1 h after cell transfer unless otherwise indicated. PLNs consisted of pooled inguinal, brachial, axillary, sciatic, superficial and deep cervical nodes. The number of fluorescence-labeled cells was quantified (by researchers 'blinded' to sample identity) with a BH2/RFL fluorescence microscope (Olympus Optical) in ten fields or more (unit area of each field, 0.34 mm^2) of nonsequential cryosections 9 μm in thickness. The percentage of fluorescence-labeled cells in single-cell suspensions of recipient lymphoid organs was also analyzed by flow cytometry with a FACScan (BD Biosciences). Equivalent results were obtained by microscopic quantification and flow cytometry; all results were confirmed by both methods. The constant ratio (about 1:1) of transferred cells in spleens of control mice and WBH-treated mice provided an internal reference standard for the number of input cells in homing studies. Details of treatment with adhesion-blocking antibodies, chemokines and pertussis toxin are provided in the **Supplementary Methods**. For studies involving the homing of an enriched population of $CD4^+$ or $CD8^+$ T cells that was L-selectin-low, $CXCR3^{hi}$, mouse splenocytes were activated for 2 d *in vitro* with plate-bound mAb to mouse CD3 (145-2C11; BD Biosciences), followed by treatment for 3 d with recombinant IL-2 (12.5 ng/ml; R&D Systems)[50].

Flow cytometry. Multiparameter flow cytometry was used for 'phenotyping' of TRITC-labeled cells before or after transfer in single-cell suspensions of PLNs and spleen from recipient mice. Details are provided in the **Supplementary Methods**.

Intravital microscopy. Intravital microscopy of inguinal PLNs was done as described[17,23]. C57BL/6 mice were anesthetized by intraperitoneal injection of 1 mg/ml of xylazine and 10 mg/ml of ketamine (10 ml per kg body weight). The left inguinal lymph node was exposed in an abdominal skin flap and the surrounding fatty tissue was removed to expose the lymph node microvasculature. Approximately 2.5×10^7 calcein-labeled splenocytes were injected through a catheter inserted into the right femoral artery and were visualized with a customized intravital microscopy system (Spectra Services). Brightfield microscopy was used to identify the vascular structure and blood flow status in venular branches; fluorescent microscopy was used to visualize calcein-labeled cells. At the end of the observation period, 150-kilodalton fluorescein isothiocyanate–conjugated dextran (10 mg/ml; Molecular Probes) was injected to define the venular structure. All images were captured with an EB charge-coupled device camera (Hamamatsu Photonics) and were recorded with a digital videocassette recorder (DSR-11, Sony) for analysis of cell activity. The rolling fraction was defined as the percentage of total cells passing through the vessel that transiently interacted with HEVs during the observation period[17,23]. The sticking fraction was defined as the percentage of rolling cells that adhered to HEVs for 30 s or more. For rolling and sticking fractions, data were generated from three normothermic control mice (venules analyzed: order I, 3; order II, 8; order III, 15; order IV, 16; order V, 12) and three WBH-treated mice (venules analyzed: order I, 4; order II, 7; order III, 11; order IV, 13; order V, 11). The velocity of more than 20 noninteracting (fast-moving) cells in order III venules was measured in each mouse.

Immunofluorescence analysis. Organs were embedded in optimum cutting temperature compound (Sakura Finetek). Tissue cryosections 9 μm in thickness were fixed for 10 min at –20 °C in methanol/acetone (3:1), and the expression of homing molecules was detected by immunofluorescence staining. Details of the procedures for staining of organ cryosections or intravascular staining are in the **Supplementary Methods**. Digital images were captured with an Olympus BX50 upright fluorescence microscope equipped with a SPOT RT camera (Spectra Services). Confocal images were obtained with the Leica TCS SP2 spectrophotometer confocal microscope. All images were captured with identical exposure times and image settings in each experiment. Images were analyzed with ImageJ software[51] (http://rsb.info.nih.gov/ij) for determination of the relative fluorescence staining intensity for trafficking molecules on all HEVs in a single 9-μm-thick cross-section of 14 pooled PLNs (paired inguinal, brachial, axillary, sciatic, superficial and deep cervical nodes). For normalization of the varying sizes of individual HEVs in cryosections, fluorescence intensity is expressed in terms of pixels (reflecting a fixed unit area). For this, $PNAd^+$ cuboidal structures were circled (with Intuos3 USB Pen Tablet; Wacom) and each pixel in those defined regions was assigned a fluorescence intensity value (based on a scale from 0 to 255). Histograms represent the data from all pixels analyzed (range, 1×10^6 to 1.7×10^6) for the total HEVs per cryosection (range, 1×10^2 to 1.75×10^2); similar numbers of HEVs and pixels were analyzed for each treatment condition in individual experiments.

Immunoblots and enzyme-linked immunosorbent assay. PLNs were sonicated to obtain total tissue lysates. For immunoblot analysis of CCL21, equal amounts of protein (20 μg) were fractionated by reducing SDS-PAGE (12% acrylamide), and membrane blots were probed with antibodies specific for CCL21 (R&D Systems) or β-actin (Sigma). Recombinant CCL21 (R&D Systems) was used as a positive control. ICAM-1 protein in PLN tissue lysates was measured by enzyme-linked immunosorbent assay (R&D Systems). IL-6 concentrations in PLN tissue lysates were measured by multiplex immunoassay (Luminex 100) using mouse Fluorokine MAP assays (R&D Systems). Phosphorylated STAT3 was detected by immunoblot analysis[13] in liver and spleen extracts of mice pretreated with actinomycin D for various times (0, 0.5, 1, 2 and 4 h), followed by 30 min of treatment with hyper-IL-6.

Statistical analysis. Statistical analysis used the unpaired, two-tailed Student's *t*-test.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

Q.C. and S.S.E. conceptualized and designed the research; S.S.E. supervised the research; Q.C. did all experiments unless stated otherwise; D.T.F. contributed to the experimental design for quantitative image analysis and did the phenotypic analysis in short-term homing assays and enzyme-linked immunosorbent assay for ICAM-1; K.A.C assisted in immunofluorescence staining and kinetic analysis in short-term homing assays; E.U. contributed to the analysis of ICAM-1 staining; W.-C.W. did frozen-section adherence assays and provided technical assistance for organ retrieval; U.H.v.A. and J.-M.G. helped with intravital microscopy studies; S.R.J. provided the hyper-IL-6 expression construct; H.B. provided recombinant hyper-IL-6 and contributed to discussions regarding IL-6 regulation of lymphocyte trafficking; and all authors contributed to discussions and to the preparation of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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High-Endothelial Venules

Master Regulators of Lymphocyte Trafficking and Targets of Fever-Range Thermal Stress

Qing Chen, Kristen Clancy, Wan-Chao Wang, and Sharon S. Evans

Department of Immunology, Roswell Park Cancer Institute, Buffalo, New York

A longstanding question in immunology revolves around the physiological benefit of the ancient fever response. Despite the fact that fever occurs at great metabolic cost, it is associated with improved survival during infection in endothermic and ectothermic vertebrate species (1). The prevailing paradigm with regard to leukocyte trafficking has been that febrile temperatures influence leukocyte delivery to tissues principally through bystander effects on hemodynamic parameters (i.e., vasodilation and increased blood flow). This chapter focuses on emerging evidence for a proactive role of temperatures in the range of natural fever (i.e., 38–40°C) in regulating the molecular events that support lymphocyte adhesion in high-endothelial venules (HEVs). HEVs are major sites of lymphocyte extravasation, serving as a locus for recirculation of blood-borne lymphocytes through peripheral lymphoid organs. Continuous entry of naïve and central memory lymphocytes across HEVs is crucial for immune homeostasis and immune surveillance. Notably, the mechanisms by which fever-range thermal stress promote lymphocyte–endothelial interactions are tightly regulated with respect to the type of vessels involved. Sustained exposure to fever-range thermal stress selectively targets adhesion in HEVs, whereas squamous endothelial cells (ECs) of noninflamed extralymphoid organs are not responsive. The physiological fever-range thermal stimulus described here is distinct from heat shock conditions (e.g., ≥43°C) that globally promote adhesion in nonactivated EC. These observations support the concept that HEVs act as sentinels during febrile inflammatory responses by heightening the delivery of naïve and central memory lymphocytes to secondary lymphoid organs.

OVERVIEW OF THE MOLECULAR MECHANISMS ORCHESTRATING LYMPHOCYTE TRAFFICKING ACROSS HIGH-ENDOTHELIAL VENULES

HEVs function as gatekeepers controlling the egress of lymphocytes out of the peripheral blood compartment and into secondary lymphoid organs where pathogens and cognate antigens are encountered (2–4). These specialized postcapillary venules are localized in T cell–enriched zones of all secondary lymphoid organs except the spleen. The ECs lining HEVs are morphologically and biochemically distinct from the flat, squamous ECs of the majority of the vessels throughout the body. These so-called high ECs (HECs) exhibit a cuboidal morphology, allowing them to extrude into the lumen of vessels and provide an irregular surface topography. These biophysical parameters theoretically contribute to turbulent blood flow, facilitating margination of leukocytes along the vessel wall. The vasculature in vertebrate species provides an extensive surface area for potential sites of extravasation. However, under noninflammatory steady-state conditions, the majority of constitutive lymphocyte extravasation occurs preferentially across HEVs that compose a relatively small percentage of the total vasculature. The mechanisms controlling lymphocyte extravasation in HEVs of lymphoid organs is briefly discussed in the next section. Additional details regarding the molecular basis of trafficking across lymphoid organ HEVs or HEV-like vessels of inflamed tissues are described in Chapters 155 and 170.

Lymphocyte migration across HEVs is coordinated by adhesion molecules and chemokine/chemokine receptor partners. These molecules participate in a stepwise sequence of reversible adhesion events that include (a) initial tethering and rolling, (b) chemokine-mediated activation and firm adhesion, and (c) transendothelial migration (2,5,6). Extravasation of naïve and central memory lymphocytes in peripheral lymph nodes (PLN) is initiated by the L-selectin leukocyte homing receptor. Positioning of L-selectin on microvillous projections enables this molecule to initiate contact between free-flowing lymphocytes and the walls of HEVs. L-selectin binds transiently to counter-receptors on HEVs collectively termed PLN addressins (PNAd) that include CD34, glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), podocalyxin, endomucin, and sgp200 (3). The N-terminal

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calcium-binding lectin domain of L-selectin interacts directly with negatively charged mucin domains of PNAd molecules generated by post-translational enzymatic modifications (i.e., glycosylation, fucosylation, sulfation, sialylation) (3). The sulfation determinant on PNAd recognized by MECA-79 monoclonal antibody (mAb) is absolutely required to support lymphocyte–HEV interactions in PLN (2,3,5). MECA-79-reactive PNAd molecules are not expressed on the lumenal surface of Peyer's patches (PP) HEVs. Instead, L-selectin binds to the mucin stalk of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in PP HEVs (5). The efficiency of lymphocyte tethering and rolling in PP HEVs is enhanced by binding of a second leukocyte homing receptor, $\alpha 4\beta 7$ integrin, to a negatively charged aspartic acid residue in the N-terminal immunoglobulin domain of MAdCAM-1 (5). Lymphocyte homing in mesenteric lymph nodes (MLN) is mediated by combined interactions of L-selectin with PNAd and MAdCAM-1 as well as $\alpha 4\beta 7$ integrin binding to MAdCAM-1 (5).

Intravital microscopy studies have elegantly demonstrated that the process of tethering and rolling dramatically reduces the velocity of lymphocytes in HEVs (5,6) (see also Chapter 170). This is most evident in higher-order venules that are located downstream from the capillary bed. Here, free-flowing lymphocytes, moving at a rate of >400 to 500 μm/sec, transition to slow rolling cells with a speed of $\leq$50 μm/sec (7,8). The increased transit time allows lymphocytes to sample the chemokine microenvironment on the surface of HEVs. A single chemokine synthesized by HECs and stromal cells, the CC chemokine ligand (CCL)21 (TCA-4/SLC/6C-kine/exodus 2) plays a predominant role in supporting the progression from slow-rolling to firm-sticking cells in LN and PP HEVs (4,6).

Engagement of CCL21 by CCR7, a 7-transmembrane-spanning chemokine receptor on naïve and central memory lymphocytes, triggers G-protein-dependent conformational changes in the $\beta 2$-integrin, leukocyte function adhesion molecule-1 (LFA-1). This enables LFA-1 to bind with high affinity to its constitutively expressed counter-receptors, intercellular adhesion molecule (ICAM)-1 and ICAM-2, on the walls of HEVs (2,4,6). ICAM-1 and ICAM-2 appear to have redundant functions in HEVs based on evidence that either molecule can substitute during homeostatic trafficking under conditions in which the other molecule is not operative (i.e., during mAb blockade or in genetically deficient mice) (9). In PLN HEVs, firm adhesion of lymphocytes is primarily mediated by LFA-1/ICAM-1–2 interactions, whereas in MLN and PP, both LFA-1/ICAM-1–2 and $\alpha 4\beta 7$ integrin/MAdCAM-1 contribute to firm adherence (5). LFA-1/ICAM-1–2 interactions have also been implicated in supporting transendothelial migration although the molecular mechanisms controlling this process in HEVs are not well understood (4–6). At extralymphoid sites of injury or inflammation, ICAM-1 is highly induced by inflammatory cytokines on cuboidal, HEVs-like vessels, where it predominates over ICAM-2 in recruitment of neutrophils, macrophages, and lymphocyte subsets (9).

FEVER-RANGE THERMAL STRESS AMPLIFIES LYMPHOCYTE–HEVs ADHESION AND LYMPHOCYTE HOMING

Local increases in temperature at sites of inflammation and, in some cases, systemic fever are cardinal features of a host response to infection or inflammation. A recent series of studies has shown that fever-range temperatures alter the tissue distribution of lymphocytes in vivo. In this regard, significant decreases in the number of lymphocytes are observed in the circulating pool following elevation of the core temperature of mice (10–12). This was accomplished experimentally using whole body hyperthermia (WBH) protocols developed by Pritchard et al. to simulate the temperature and duration of physiologic fever (13). Transient decreases in the number of peripheral blood lymphocytes have also been reported in patients with advanced cancer undergoing clinical fever-range WBH therapy (12,13). In mice, it was shown that these cells redistribute to HEV-bearing organs (LN and PP) but not to organs that lack HEVs, such as spleen or pancreas (8,10). Investigation of the underlying mechanisms has revealed that fever-range thermal stress influences trafficking by independently stimulating adhesion in two distinct cellular targets – lymphocytes (i.e., B and T cells) and HEC (Figure 52.1). Collectively, these findings support the notion that fever-range thermal stress provides a danger signal during inflammation to proactively regulate lymphocyte egress across HEVs in secondary lymphoid organs (1,8).

Figure 52.1

THERMAL STRESS STIMULATES LYMPHOCYTE HOMING RECEPTOR FUNCTION

To investigate the effect of fever-range thermal stress on lymphocyte adhesion, primary lymphocyte populations or lymphocyte cell lines expressing a defined profile of adhesion molecules (Table 52-1) were cultured in vitro under fever-like temperature conditions (i.e., 40°C; 104°F) for 6 hours (1,8,10,14–18). This reductionist approach of solely modifying temperature conditions allowed for the identification of a role for the *thermal component* of fever in regulating lymphocyte trafficking. Changes in adhesion were evaluated under shear in frozen-section in vitro adherence assays where lymphoid tissue HEVs serve as substrates. Alternatively, lymphocytes treated in vitro with heat were evaluated for their ability to traffic to various tissues in short-term (1 hour) in vivo homing assays. These studies established that marked increases in lymphocyte adhesion to HEVs and homing to HEVs-bearing organs (e.g., PLN, MLN, and PP) are observed following lymphocyte culture at physiologic fever-range temperatures (i.e., 2–4°C above normal temperature for 6 hours) (Table 52-1) (1,8,10,14–18). Notably, fever-range thermal stress does not improve short-term homing of lymphocytes to organs that do not express HEVs such as the spleen (Table 52-1) (1,8,10,14).

Table 52.1

Evidence for organ-specific trafficking of heat-treated lymphocytes raised the possibility that thermal stress targets

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Table 52-1: Fever-Range Thermal Stress Improves Lymphocyte–HEV Adhesion and Lymphocyte Trafficking to LN and PP Organs

	Adhesion Molecule Expression			*HEV Adhesion after HT*		*Homing after HT*		
Cells	*L-Selectin*	*α4β7*	*LFA-1*	*LN*	*PP*	*LN*	*PP*	*Spleen*
Primary Lymphocytes								
Human PBL	+	+	+	↑	↑	ND	ND	ND
Mouse splenocytes	+	+	+	↑	↑	↑	↑	↔
Mouse LN–derived cells	+	+	+	↑	ND	↑*	↑*	↔*
Cell Lines								
300.19/L-selectin transfectant†	+	−	−	↑	↑	↑	↑	↔
300.19/Δcyto transfectant††	+	−	−	↔	↔	↔	↔	↔
TK1 cells‡	−	+	+	↔	↑	↔	↑	↔

HT, hyperthermia treatment (6 hours at 40°C); ND, not determined; + indicates positive expression of adhesion molecules; – indicates lack of expression of adhesion molecules; ↑ indicates the increased adhesion or homing to the indicated tissues after heat treatment; ↔ indicates no change in adhesion or homing after heat treatment.
*Personal observations, Q. Chen and S. Evans.
†Murine B lymphoma cell line transfected with full-length human L-selectin (300.19/L-selectin).
††Murine B lymphoma cell line transfected with a nonfunctional form of human L-selectin (300.19/Δcyto; lacking C-terminal 11 amino acids).
‡Murine $CD8^+$ T lymphoma cell line; L-selectin is expressed at levels below the threshold to support adhesion.
References: 14,16–20.

the function of known lymphocyte homing receptors. This hypothesis was confirmed using L-selectin and $\alpha 4\beta 7$ integrin-specific function-blocking mAb or cell lines expressing a nonfunctional form of L-selectin (i.e., murine B lymphoma 300.19/Δcyto transfectants that lack the C-terminal 11 amino acid cytoplasmic tail) (Table 52-1) (19). Fever-range thermal stress increases L-selectin-mediated adhesion and homing of lymphocytes via engagement of PNAd on PLN and MLN HEVs as well as MAdCAM-1 on PP HEVs (Table 52-1, Figure 52.1) (14–18). Parallel increases in $\alpha 4\beta 7$ integrin-mediated adhesion to MAdCAM-1 on PP and MLN HEVs were demonstrated (Table 52-1, Figure 52.1) (10,20). Exquisite selectivity in thermal regulation of integrin-binding activity is suggested by results that heat treatment of lymphocytes fails to

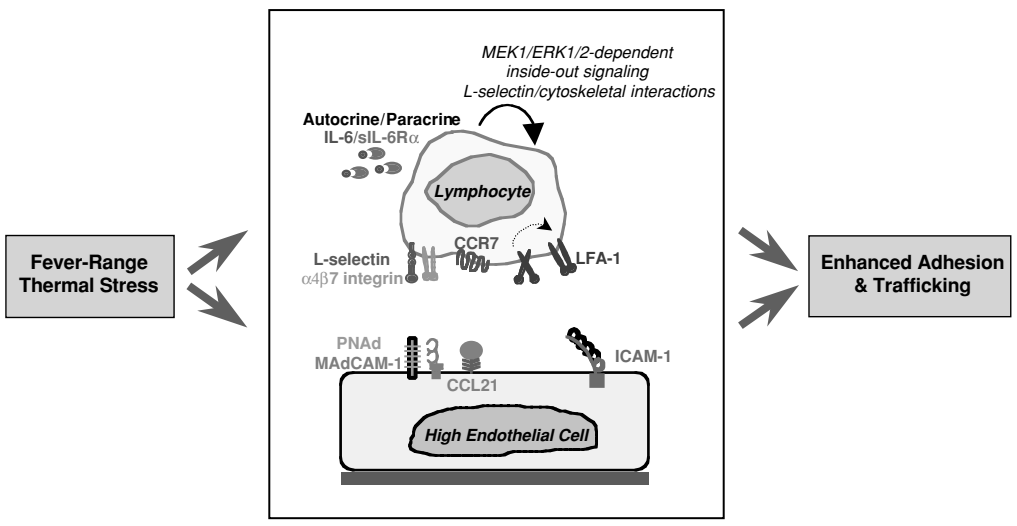


Figure 52.1. Fever-range thermal stress proactively stimulates lymphocyte–HEV adhesion. Fever-range temperatures enhance lymphocyte trafficking to lymphoid organs by exerting independent effects on the molecular events culminating in enhanced adhesion in lymphocytes and ECs of HEVs.

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increase $\alpha 4\beta 7$ integrin-mediated adhesion to the extracellular matrix protein, fibronectin, or LFA-1-dependent binding to ICAM-1 (14,18). An important finding is that equivalent increases in L-selectin-dependent adhesion are observed in lymphocytes whether they are exposed to fever-range thermal stress in vitro or in vivo (i.e., during heat treatment of cultured cells or WBH treatment, respectively) (10,13,17). Multiple lymphocyte subsets were found to increase L-selectin-based adhesion to HEVs in response to thermal stress (i.e., $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells, $CD45RA^+$ naïve lymphocytes, and $CD45RO^+$ memory cells) (16–18). Moreover, thermal stress improves adhesion to PNAd or MAdCAM-1 substrates in lymphocyte populations of endothermic species that diverged during evolution 300 million years ago (e.g., mouse, human, chicken) (1). Collectively, these findings support the notion that thermal regulation of lymphocyte homing receptor activity confers a survival benefit that was maintained during the diversification of vertebrate species.

A growing body of evidence indicates that conventional mechanisms are not responsible for thermal control of L-selectin and $\alpha 4\beta 7$ integrin adhesion. Thermal stress does not alter the cell surface density of these homing receptors on lymphocyte subsets (14,16–18). Moreover, fever-range temperatures do not affect the lectin-binding activity of the N-terminal domain of L-selectin (14). Electron microscopy revealed that heat also does not influence the overall distribution of L-selectin on microvillous projections (14). However, these latter results do not exclude the possibility that heat enhances L-selectin clustering in membrane microdomains that cannot be resolved by immunogold-labeled antibody reagents.

Studies were undertaken to determine if thermal stress affects the association of L-selectin with the detergent-insoluble cytoskeletal matrix as a possible mechanism for regulating the binding activity of this homing receptor. In lymphocytes maintained at normothermal temperature (37°C; 98.6°F), L-selectin is fully extracted from the detergent-insoluble subcellular fraction (15–17,21). However, upon L-selectin cross-linking by antibodies or L-selectin ligands (GlyCAM-1), this homing receptor rapidly ($\leq$5 seconds) becomes associated with the detergent-insoluble cytoskeletal matrix (16,21). L-selectin interactions with cytoskeletal elements require the 11-amino acid region within the cytoplasmic tail that contains a binding site for the cytoskeletal linker protein, α-actinin (16,22). These results are consistent with the notion that L-selectin becomes stably associated with the actin-based cytoskeleton during transient tethering and rolling along the lumenal surface of HEVs. Remarkably, treatment of lymphocytes with fever-range thermal stress causes L-selectin to preassociate with the detergent-insoluble matrix without the requirement for L-selectin cross-linking (1,8,15–17). Thus, heat-induced associations between L-selectin and the cytoskeletal scaffolding underlying the lymphocyte plasma membrane are speculated to increase L-selectin tensile strength and thereby its ability to withstand shear within postcapillary HEVs (16,17).

One of the most intriguing findings is that thermal regulation of lymphocyte adhesion can be segregated into a two-step process, thereby excluding a role of heat per se in directly altering the organization or conformation of adhesion molecules in the lipid bilayer of the plasma membrane. In this regard, conditioned medium from cells treated in vitro with fever-range thermal stress (i.e., the "initiation phase") can be used in the "effector phase" to stimulate L-selectin/cytoskeletal interactions as well as the binding activity of L-selectin or $\alpha 4\beta 7$ integrin in lymphocytes maintained at normothermal temperatures (1,8,14,15,17,20). These experiments provide unequivocal evidence that soluble factors are responsible for mediating the pro-adhesive effects of thermal stress. Moreover, the source of the soluble factor appears to be remarkably cell-type specific. Hematopoietic cells (T cells, B cells, monocytes) and stromal cells (ECs, fibroblasts) are all sources of heat-induced transactivating pro-adhesive factors, whereas no activity is detected in culture supernatants of cell lines representing parenchymal cells of various organs (lung, liver, breast, brain, skin) (17,20).

These observations led to the discovery of a novel role for a well-known immunomodulatory cytokine, interleukin (IL)-6, in regulating lymphocyte homing (Figure 52.1) (1,8,17). Functional blockade of IL-6 and its receptor components – IL-6 receptor α (IL-6Rα) and the gp130 signal transducing chain – prevent fever-range thermal stimulation of L-selectin adhesion in vitro and in vivo. In contrast, other cytokines – including tumor necrosis factor (TNF)-α, IL-1β, interferon (IFN)-α, IFN-γ, IL-8, IL-11, leukemia inhibitory factor, or oncostatin M – do not contribute to the pro-adhesive activity of thermal stress in lymphocytes under normal conditions. Thermal stimulation of lymphocyte adhesion was further found to depend on a trans-signaling mechanism that involves not only IL-6 but also a soluble form of the IL-6Rα (1,8,17). The requirement for these two components to function together as a heterodimeric cytokine provides a sophisticated level of control in this system. Combined biochemical and pharmacological inhibitor approaches positioned extracellular signal regulated kinase (ERK)1/2, but not p38 mitogen-activated protein kinase (MAPK) or c-Jun N-terminal kinase (JNK), in the IL-6/sIL-6Rα signaling pathway upstream of activation of L-selectin–cytoskeletal interactions and L-selectin avidity/affinity (Figure 52.1) (17). Taken together, these data enlarge on the concept that IL-6/sIL-6Rα trans-signaling actively contributes to immune responses by regulating leukocyte trafficking during both acute and chronic inflammation (23).

FEVER-RANGE THERMAL STRESS AMPLIFIES HIGH-ENDOTHIAL VENULE ADHESION

An independent line of investigation revealed that fever-range thermal stress enhances adhesion in HEVs that are the major portals governing lymphocyte extravasation (Figure 52.1). These studies employed frozen-section in vitro adherence assays to compare the binding activity of HEVs in lymphoid

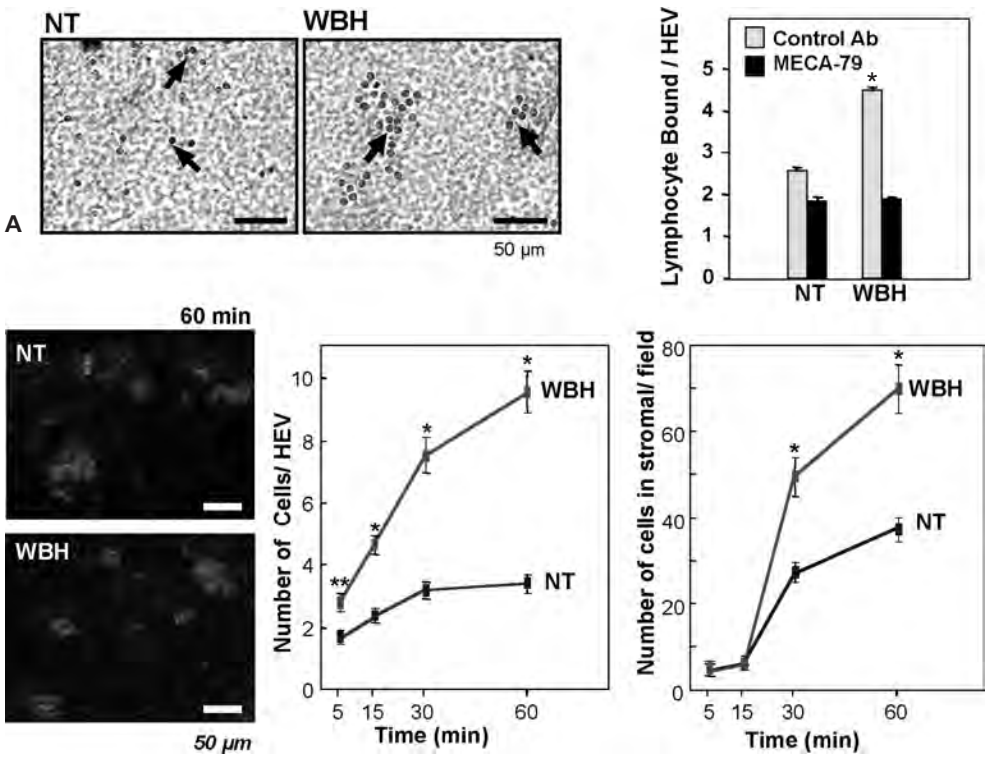


Figure 52.2. Fever-range thermal stress enhances lymphocyte adhesion to PLN HEVs (**A**) and homing to PLN organs (**B**). BALB/c mice were treated with fever-range whole body hyperthermia (WBH) for 6 hours (core temperature, 39.5 ± 0.5C°). The core temperature of normothermal control mice (NT) was 36.8 ± 0.2C°. (**A**) Adherence of mouse splenocytes to HEVs in PLN cryosections was evaluated in vitro under shear as described. The level of L-selectin/PNAd-specific adhesion (*brackets*) was determined by treating PLN cryosections with MECA-79 mAb (6.25 μg/mL). Photomicrographs show typical images of lymphocytes (*black arrows*) bound to HEVs in toluidine-stained PLN tissues. The number of adherent lymphocytes was quantified by light microscopy in a total of 300–500 HEVs. For consistency in double-blind evaluation, HEVs were quantified only if they contained ≥1 adherent cell. (**B**) Short-term homing studies were performed essentially as described. Mouse splenocytes labeled with TRITC (3.6 μg/mL) were injected intravenously (3×10^7 cells/mouse) into NT control or WBH-treated mice and PLN were isolated at the indicated time points. Cryosections of PLN were stained with PNAd-specific MECA-79 mAb (6.25 μg/mL) and FITC-labeled goat anti-rat IgMμ (PIERCE). Photomicrographs show typical images of TRITC-labeled (*red*) cells associated with PNAd-positive (*green*) HEVs or infiltrated into the stroma of PLN tissue at 60 minutes. The number of TRITC-labeled cells associated with HEVs (>40 HEVs) or stroma (>10 fields; 0.35 mm^2/field) in each sample was quantified (double-blind) by fluorescence microscopy. Data are the mean ± SE and are representative of ≥3 independent experiments. The differences between NT and WBH-treated mice were significant by unpaired two-tailed Student *t*-test ($^*p < 0.0001$, $^{**}p < 0.001$). Fever-range WBH did not increase the localization of fluorescent-labeled cells in splenic tissues at 60 minutes (not shown). (Reproduced with permission from Chen Q, Fisher DT, Kucinska SA, et al. Dynamic control of lymphocyte trafficking by fever-range thermal stress. *Cancer Immunol Immunother*. 2005;1–13 and Evans SS, Wang WC, Bain MD, et al. Fever-range hyperthermia dynamically regulates lymphocyte delivery to high endothelial venules. *Blood*. 2001;97: 2727–2733.) For color reproduction, see Color Plate 52.2.

Figure 52.2

organs of normothermal (NT) controls or mice treated 6 hours with fever-range WBH (core temperature of 39.5–40°C; 103–104°F). Fever-range thermal stress markedly increases the ability of PLN HEVs to support lymphocyte adhesion under shear in this in vitro assay (Figure 52.2A; for color reproduction, see Color Plate 52.2) (1,8,10). The molecular events regulated by thermal stress appear to be remarkably stable when considering that enhanced adhesion is detected in frozen tissues stored at –20°C. Lymphocyte adhesion to HEVs in PLN cryosections is inhibited by the MECA-79 mAb, which recognizes the sulfation determinant on PNAd (see Figure 52.2A), as well as by mAb that bind to the N-terminal lectin domain of the L-selectin homing receptor (DREG-56, MEL-14) (1,8,10). These results confirm the requirement for L-selectin/PNAd adhesive partners in supporting lymphocyte adhesion to HEVs of heat-treated animals. Parallel increases in $\alpha 4\beta 7$ integrin/MAdCAM-1–dependent adhesion were identified following heat treatment in PP HEVs using $\alpha 4\beta 7^{hi}$

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L-selectin^{lo} TK1 cells and neutralizing mAb specific for $\alpha 4\beta 7$ integrin and MAdCAM-1 (i.e., DATK32 and MECA-367, respectively)(1,8,10). Thermal stimulation of HEV adhesion is also observed in LN and PP organ cultures following incubation at fever-range temperatures in vitro (10). These data suggest that regulation of HEV adhesion occurs under local microenvironmental control and is not dependent on feedback mechanisms provided by neuronal or lymphatic systems or by the hypothalamus/pituitary/adrenal axis that orchestrates physiological responses during febrile episodes.

Thermal stimulation of adhesion at the level of the HEV correlates with improved trafficking of lymphocytes to LN and PP in short-term homing studies (1,8,10). In this series of studies, the core temperature of mice is initially raised to the range of natural fever (38–40°C; 100–104°F) by WBH treatment for 6 hours. Mice are then allowed to revert to their normal basal temperature (∼36.5–37°C) prior to intravenous injection of fluorescent-labeled splenocytes and enumeration of labeled cells in various organs. Because fluorescent-tagged splenocytes are not subjected directly to thermal stress, this experimental design allows for the analysis of vascular responses to elevated temperatures. Flow cytometric analysis has shown that T and B lymphocytes constitute the major population of tetramethylrhodamine B isothiocynate (TRITC)-labeled cells that traffic across HEVs in normothermic mice or in mice pretreated with WBH, whereas entry of monocytes or neutrophils is limited under either temperature condition (Sharon Evans, unpublished data). An example of this type of homing study is shown in Figure 52.2B. Enhanced interactions of TRITC (red)-labeled lymphocytes with HEVs (stained with PNAd-specific MECA-79 mAb and FITC [green]-labeled secondary Ab) are detected in the PLN of WBH-treated mice as early as 5 minutes after lymphocytes injection. Increased lymphocyte–HEV interactions are observed in response to heat treatment at subsequent time points of 15, 30, and 60 minutes (Figure 52.2B) (8,10). Notably, lymphocyte interactions with HEVs temporally precede infiltration of fluorescent-labeled lymphocytes into the stroma of PLN organs of heat-treated animals (Figure 52.2B). These observations are consistent with the notion that HEVs are the major focal point directing lymphocyte trafficking into lymphoid organs in response to thermal stress.

The kinetics for optimal stimulation of HEV adhesion is tightly regulated. Moderate increases in HEV function are detected by in vitro adherence assays or in vivo homing studies following WBH treatment for 2 hours, whereas markedly elevated responses are observed following sustained exposure to thermal stress for 6 to 8 hours (8,10). The approximately twofold increase in lymphocyte–HEV adhesion and trafficking documented in response to fever-range thermal stress appears to represent a biologically significant amplification of lymphocyte access to lymphoid organs. Stimulation of the frequency of lymphocyte extravasation across HEVs, which is estimated to occur at a rate of 5×10^6 cells per second in humans under normothermal temperatures (2), would be expected to profoundly enhance the potential for immune surveillance. HEV adhesion returns to normal basal levels within 12 hours of cessation of thermal stress (10). Transient regulation of vascular adhesion is in line with the sequence of events in natural fever where physiological feedback loops are designed to restore biological systems to steady-state equilibrium after resolution of an infection.

A notable finding relates to the selectivity of thermal regulation of endothelial adhesion (1,8,10). Fever-range WBH preferentially amplifies adhesion in HEVs of LN and PP, whereas no increase in adhesion is detected by in vitro adherence assays in squamous ECs of noninflamed extralymphoid organs (e.g., pancreas). Moreover, WBH does not increase the localization of fluorescent-labeled lymphocytes in non–HEV-bearing organs (e.g., spleen, pancreas) in short-term homing studies (1,8,10). These findings suggest that febrile temperatures associated with infection or inflammation proactively focus the delivery of lymphocytes across HEVs in lymphoid organs, while sparing noninflamed extralymphoid sites.

The specific adhesion events targeted by thermal stress in HEVs have not been fully characterized. The murine 300.19/transfectant cell line engineered to express human L-selectin has been useful in segregating the relative contributions of primary (tethering/rolling) and secondary (firm adhesion) interactions because it does not express detectable levels of LFA-1 molecules (1,8,10,17,19). 300.19/L-selectin cells exhibit enhanced adhesion to HEVs of lymphoid organs of WBH-treated mice in vitro as well as increased localization in PLN, MLN, and PP organs in vivo (1,8,10). In contrast, 300.19/Δcyto cells that express a nonfunctional form of L-selectin fail to adhere to HEVs or traffic to LN or PP in response to WBH (10,19). These results suggest that fever-range thermal stress promotes primary, L-selectin-dependent tethering and rolling interactions in LN and PP HEVs. Enhanced HEV adhesion is not accompanied by detectable increases in expression of PNAd (Figure 52.2B) or MAdCAM-1 molecules (1,10). Recent findings suggest that fever-range thermal stress also regulates secondary molecular events that control firm adhesion of lymphocytes in HEVs. Thus, fever-range WBH treatment of mice markedly upregulates the lumenal expression of ICAM-1 on HEVs of LN and PP, whereas no induction of ICAM-1 is observed in noninflamed vessels of extralymphoid organs (unpublished data). Expression of ICAM-1 at high density on HEVs would be predicted to promote firm adhesion via interactions with LFA-1 on apposing lymphocyte surface membranes. In addition, expression of ICAM-1 above a threshold level might enable this molecule to cooperate with PNAd or MAdCAM-1 to stabilize primary adhesion events in HEVs, as suggested by recent studies in non-HEV systems that demonstrate overlapping roles of selectins and ICAM-1 in supporting rolling interactions during inflammation in vivo (24). Resolution of this issue will require direct visualization of lymphocyte–HEV interactions in WBH-treated mice by intravital microscopy.

Temperatures that exceed the range of natural fever appear to override the selectivity of vascular targeting. Heat

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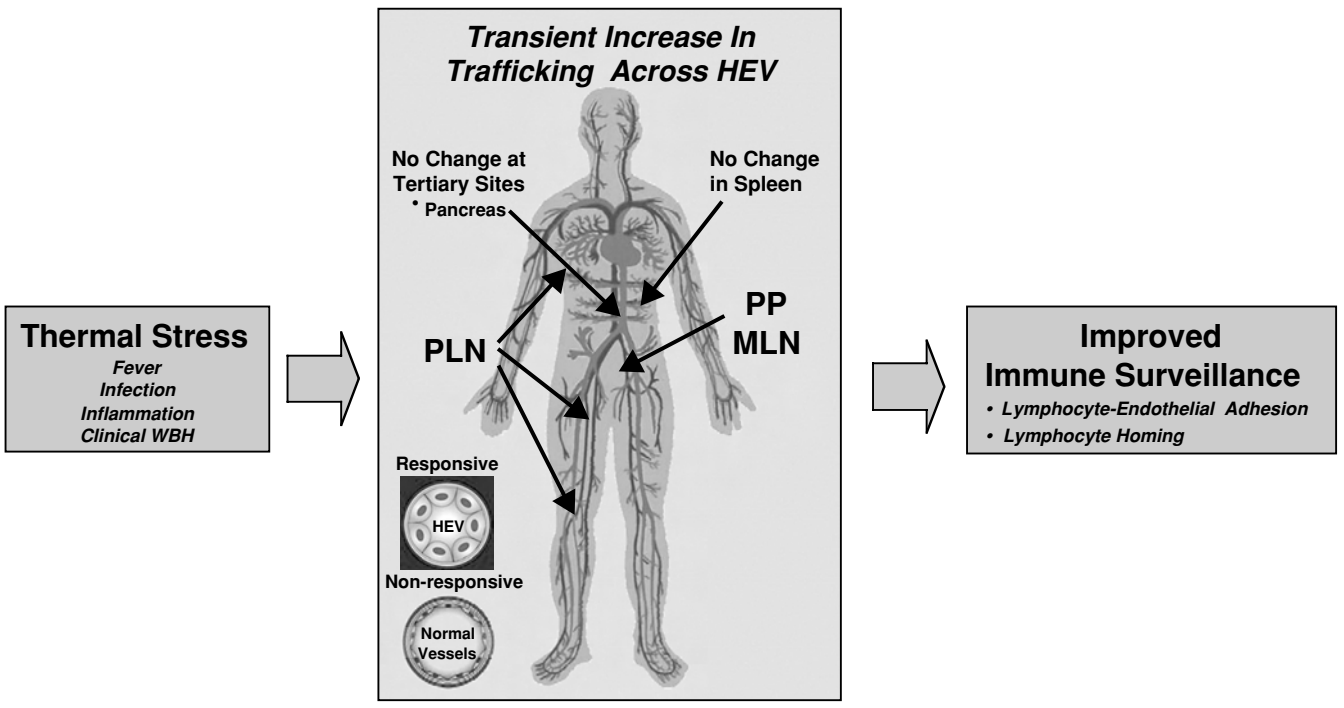


Figure 52.3. Model for fever-range thermal regulation of lymphocytes trafficking across selected vascular beds. Fever-range thermal stress in the context of inflammation or clinical thermal therapy promotes lymphocyte extravasation across cuboidal HEVs in peripheral lymph nodes (PLN), mesenteric LN (MLN), and Peyer's patches (PP). In contrast, lymphocyte–endothelial adhesion and trafficking across nonactivated squamous endothelium is not enhanced in spleen or noninflamed extralymphoid tissues.

shock (43°C; 109°F) upregulates ICAM-1 expression as well as the ability to support lymphocyte adhesion in primary human endothelial cultures that model resting macrovascular endothelium (human umbilical vein ECs [HUVECs]) and microvascular endothelium (human dermal microvascular ECs [HMVECs]) (20,25,26). Moreover, heat shock proteins (HSPs) including HSP70, HSP60, and HSP65, which are major intracellular proteins synthesized in response to high-temperature heat shock or cellular stress, have been shown to act extracellularly to induce the expression of ICAM-1 in nonactivated primary ECs (HUVECs) in vitro (26–29). In sharp contrast, physiologic fever-range temperatures (39.5–40°C) have no effect on adhesion molecule expression (ICAM-1, E-selectin, vascular cell adhesion molecule 1 [VCAM-1], P-selectin, platelet-endothelial cell adhesion molecule 1 (PECAM-1), PNAd, MAdCAM-1), cytokines release (IL-1β, TNF-α, IFN-γ, IL-6, IL-11, IL-12, IL-13), or chemokine secretion (IL-8, RANTES, monocyte chemoattractant protein [MCP]-1, monokine induced by IFN-γ [MIG]) in cultured ECs (20,30). Nonactivated endothelium is not entirely refractory to fever-range thermal stress, however, because proadhesive factors can be recovered from the conditioned medium of HUVECs and HMVECs following culture at 40°C (1,8,17,20). These soluble factors act in trans to stimulate the binding function of L-selectin and $\alpha 4\beta 7$ integrin on lymphocytes. Based on these findings, it is tempting to speculate that the vast majority of vascular beds indirectly contribute to lymphocyte delivery to LN and PP during febrile inflammatory responses by providing factors that stimulate lymphocyte homing receptor function.

KEY POINTS

- Recent studies support the concept that febrile temperatures function as a rheostat to amplify lymphocyte trafficking to secondary lymphoid organs and thereby the efficacy of immune surveillance (Figure 52.3).
- Fever-range thermal stress increases L-selectin and $\alpha 4\beta 7$ integrin-dependent adhesion in B and T lymphocytes, thereby promoting trafficking via HEVs in LN and PP. Thermal regulation of adhesion in lymphocytes involves a trans-signaling mechanism mediated by engagement of membrane-anchored gp130 signal transduction molecules by IL-6 and a soluble form of the IL-6Rα.
- Fever-range hyperthermia transiently promotes the binding activity of gatekeeper HEVs in lymphoid organs. As a result, thermal stress induces a substantial increase in lymphocyte binding to HEVs under shear as well as transmigration across HEVs into the parenchyma of lymphoid organs. Thermal stress

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does not promote adhesion in squamous ECs of non-inflamed extralymphoid organs either in vitro or in vivo.
- Selective targeting of primary and secondary adhesion events in specialized HEVs focuses the delivery of immune effector cells to peripheral lymphoid organs where the opportunity exists for optimal sensitization or restimulation of naïve or central memory lymphocytes, respectively (see Figure 52.3). In the absence of such tight control of lymphocyte–endothelial adhesion, inappropriate trafficking of lymphocytes could lead to extensive damage in non-inflamed extralymphoid tissues.

Future Goals

- An unresolved issue relates to how site-specific vascular targeting is maintained by fever-range thermal stress. It is probable that the unique microenvironment of lymphoid organs as well as the differentiation/activation status of HECs contribute to the specificity of vascular responses. It will be of interest in follow-up studies to determine if adhesion of HEV-like vessels at extralymphoid sites of acute or chronic inflammation is similarly regulated by febrile temperatures.
- Future studies are required to examine whether fever-range thermal stress initiates additional signaling events in ECs of different vascular beds, such as induction endothelial nitric oxide synthase), hemostatic factors, or apoptotic cascades.
- Major questions remain regarding the molecular mechanisms underlying thermal control of adhesion in HEVs. Inflammatory cytokines such as TNF-α, IL-1β, IFN-γ, or IL-6 are potential candidates because they are known to stimulate vascular expression of ICAM-1 in extralymphoid organs in the context of acute or chronic inflammation (2). Although the role of cytokines in controlling HEV adhesion has not been extensively studied, increased adhesion is detected in HEVs during fever responses induced by LPS or turpentine that are associated with high systemic levels of inflammatory cytokines (10). Notably, recent studies have implicated IL-6 as the central mediator of fever-range thermal stimulation of adhesion in HEVs as well as in lymphocytes (17). These findings support the emergence of IL-6 as a molecular switch controlling lymphocyte trafficking across vascular barriers during acute and chronic inflammation (23,31). An important issue that awaits further investigation is how pleiotropic cytokines, such as IL-6, selectively target lymphocyte-HEVs adhesion while maintaining the tightly regulated balance between physiological and pathological responses during febrile inflammatory responses.

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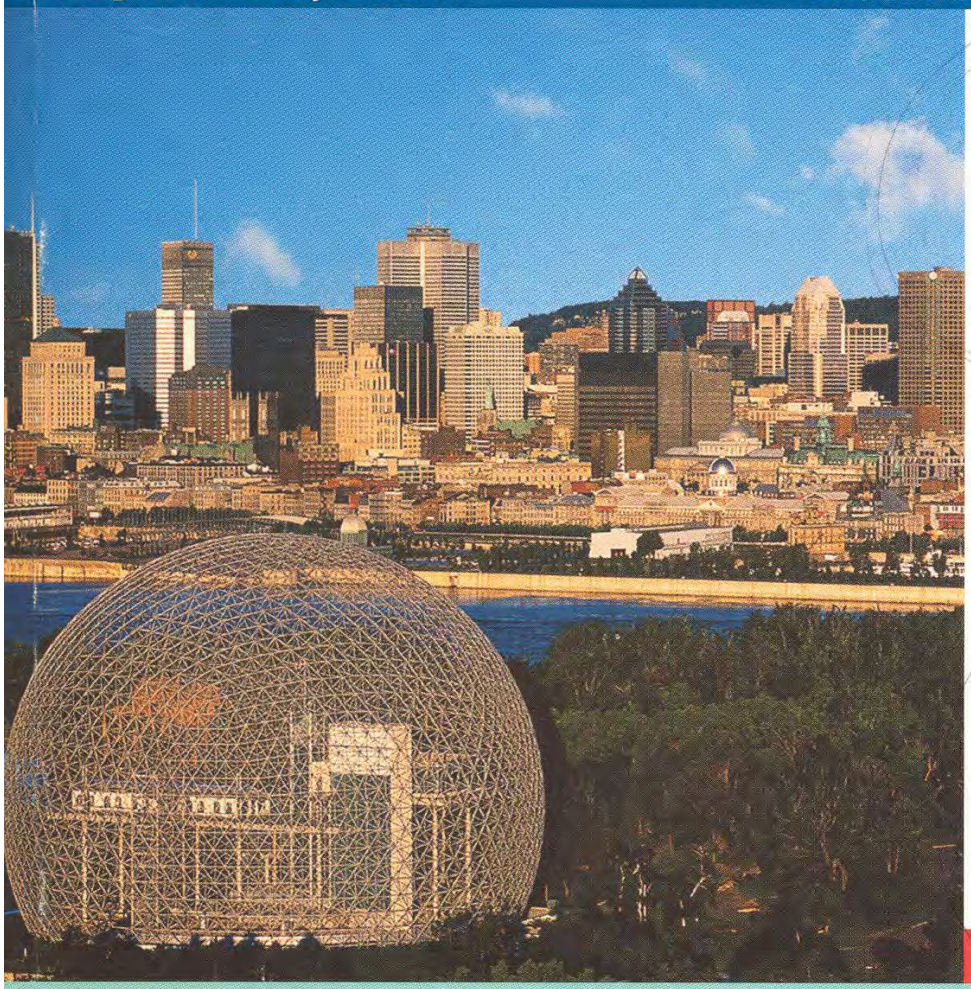
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ADHESION MOLECULES

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Integration and Independent Acquisition of Specialized Skin- Versus Gut-Homing and Th1 Versus Th2 Cytokine Synthesis Phenotypes in Human CD4$^+$ T Cells.

Lucia Colantonio, Barbara Rossi, Gabriela Constantin, Graziella Cimino-Reale, Ettore D'Ambrosio, Daniele D'Ambrosio. Biology, BioXell, Milano, Italy; Molecular Medicine, CNR, Roma, Italy; Dept. Pathology, University of Verona, School of Medicine, Verona, Italy.

CD4$^+$ T helper cells are heterogeneous in terms of tissue-specific homing and cytokine synthesis phenotypes. Mechanisms for the acquisition of tissue-specific homing phenotypes and their relationship with the attainment of polarized cytokine synthesis profiles of T cells are critically important but poorly understood. Here, we analyze the co-ordinate acquisition of Th1 versus Th2 (IFN-g vs IL-4) cytokine synthesis profiles and skin- versus gut-homing (CLA vs integrin b7) phenotypes in human CD4$^+$ T cells. Our data show that acquisition of skin- versus gut-homing T cell phenotypes is independent of Th1 versus Th2 cell fate determination. Acquisition of tissue-specific T cell homing phenotypes occurs in relation to cell cycle progression and follows instructive mechanisms and distinct kinetics. Expression of chemokine receptors CXCR3 and CCR4 correlates with the acquisition of Th1 versus Th2 rather than skin- versus gut-homing phenotypes. These findings, together with the overlap observed *in vivo* between CLA vs integrin b7 and IFN-g vs IL-4 expression, suggest a novel explanation to the complex patterns of chemokine receptor expression on memory T cells. This study paves the way to understanding the molecular basis for acquisition and integration of distinct tissue-specific homing phenotypes and cytokine synthesis profiles in human T cells.

M0.8

Serum Soluble Intercellular-1 and Vascular Cell-1 Adhesion Molecules before and after Treatment of Diseases with Immunological Involvement.

Doru Dejica, Valeria Dejica, Elena Maria Manea, Daniela Neculoiu. Immunopathology, University of Medicine and Pharmacy, 3rd Medical Clinic, Cluj Napoca, Romania.

Introduction. Serum levels of intercellular-1 and vascular cell-1 adhesion molecules (s-ICAM-1, sVCAM-1) reflect the tissue expressing and may constitute a way to assess the local inflammation in different diseases. The aim of our study was to evaluate the serum levels of sICAM-1 and sVCAM-1, before and after treatment of diseases with immunologic involvement, searching for a sensitive tool for activity appraisement and therapy monitoring.

Methods. 20 patients (pts) with chronic hepatitis C (CHC), 12 with ulcerative colitis (UC), 9 with systemic lupus erythematosus (SLE), and 7 with Wegener's granulomatosis (WG), were studied. sICAM-1 and sVCAM-1 were measured using an ELISA kit (R&D Systems, UK).

Results. *CHC pts* comprised 2 subgroups: 10 responders (R) and 10 non-responders (NR) to interferon alpha2b and ribavirin therapy. Significantly higher serum mean levels of sICAM-1 and sVCAM-1, compared to the healthy control group, were demonstrated ($p<0.001$). sVCAM-1 was also significantly elevated, but at a lower degree ($p<0.01$). Significant reduction of sICAM-1 compared to baseline was apparent after treatment, with the normalisation of levels in all but one of pts, the values of R being significantly lower than those of NR. sVCAM-1 did not change significantly with therapy in NR as well as in R.

UC pts were investigated during active disease and after 10 days of dexametasone (Dex) (6 pts) or unfractionated heparin (UH) (6 pts) treatment. sICAM-1 and sVCAM-1 were significantly higher than in controls ($p<0.001$ and 0.01, respectively). After therapy, they decreased in all patients with UH and in half of the pts treated with Dex.

SLE pts were studied at the time of maximal disease activity. Unlike sICAM-1, sVCAM-1 mean level was higher than that of controls ($p<0.001$). In SLE nephritis and SLE with recurrent thrombosis-related anti phospholipid antibody syndrome sVCAM-1 was significantly higher than in pts without these complications.

WG pts had significantly raised levels of sICAM-1 and sVCAM-1, compared to controls ($p<0.001$), but only in pts without kidney involvement sICAM-1 normalized after cyclophosphamide and metilprednisolone therapy.

Conclusions. Serum sICAM-1 and sVCAM-1 reflect the disease activity in pts with CHC, UC, SLE, WG and may be used as sensitive guide of therapeutic monitoring.

M0.9

Fever-Range Thermal Stress Stimulates Lymphocyte Homing Receptor Function through an Interleukin-6-Dependent Trans-Signaling Mechanism.

Qing Chen, Sylvia A Kucinska, Wan-Chao Wang, Paul K Wallace, Heinz Baumann, Sharon S Evans. Immunology, Roswell Park Cancer Institute, Buffalo, NY, USA.

The evolutionarily conserved fever response is closely linked with survival although the physiologic benefit is poorly understood. Prior studies have shown that the thermal component of fever stimulates the binding function of two lymphocyte homing receptors, L-selectin and $\alpha4\beta7$ integrin. These receptors control extravasation across high endothelial venules in lymphoid tissues and at sites of inflammation. Here we report that fever-range thermal stress enhances L-selectin and $\alpha4\beta7$ integrin adhesion through an interleukin-6 (IL-6)-dependent *trans*-signaling mechanism. Thermal stimulation of adhesion *in vitro* and *in vivo* is mediated by engagement of the gp130 signal transducing chain by IL-6 and the soluble IL-6 receptor-α (sIL-6Rα) binding subunit. This mechanism was revealed by evidence that recombinant soluble gp130, a competitive inhibitor of *trans*-signaling via sIL-6Rα, prevents thermal activation of adhesion. The role of L-selectin and $\alpha4\beta7$ integrin in the thermal response was confirmed using adhesion-blocking mAb, lymphocytes from L-selectin$^{-/-}$ mice, or cells expressing a truncated L-selectin lacking the cytoplasmic domain. Multiple lymphocyte subsets are responsive to thermal stress including CD4 and CD8 T cells, CD19 B cells, CD56 NK cells, CD45RA naive cells, and CD45RO memory cells. While monocytes and lymphocytes synthesize IL-6, thermal stress increases the bioactivity of IL-6 without changing the detectable concentrations of IL-6 or sIL-6Rα. Thermal control of adhesion is maintained in IL-6$^{-/-}$ mice through a gp130-dependent compensatory mechanism mediated by the IL-6-related cytokines, oncostatin M, leukemia inhibitory factor, and IL-11. These data suggest the physiological importance of maintaining gp130-dependent signaling for protection against pathogenic challenges. Combined biochemical and pharmacological inhibitor (PD98059, U0126, SB203580, SP600125) approaches positioned MEK1/ERK1-2, but not p38 MAPK or JNK, in the IL-6/sIL-6Rα signaling pathway upstream of activation of L-selectin and $\alpha4\beta7$ integrin. These results provide insight into a highly integrated gp130-linked IL-6/sIL-6Rα trans-signaling response initiated by febrile temperatures that promotes lymphocyte trafficking during inflammation. (Supported by NIH CA79765, CA094045, DK33886, and DOD BC032139)

M0.10

Involvement of Distinct Mitogen-Activated Protein Kinases in the Regulation of CD44 Expression and Induction of Hyaluronan-Adhesive CD44 in LPS- and TNF-α-Activated Human Monocytic Cells.

Katrina Gee, Maya Kozlowski, Ashok Kumar. Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, ON, Canada; Biologics and Genetic Therapies Directorate, Centre for Biologic Research, Health Canada, Ottawa, ON, Canada; Department of Pediatrics, Division of Virology and Molecular Immunology, Research Institute Children's Hospital of Eastern Ontario, Ottawa, ON, Canada.

Interaction of CD44, an adhesion molecule, with its ligand, hyaluronan (HA), in monocytic cells plays a critical role in cell migration, inflammation, and immune responses. Most cell types express CD44 but do not bind HA. The biological functions of CD44 have been attributed to the generation of the functionally active, HA-adhesive form of this molecule. In this study, we have investigated the molecular mechanism underlying lipopolysaccharide (LPS)- and cytokine-induced CD44 expression as well as the induction of HA-adhesive CD44. We show that LPS-induced CD44 expression and CD44-mediated HA-binding in monocytes is regulated by endogenously produced TNF-α and IL-10. The role of the mitogen-activated protein kinases (MAPK) was investigated in this process and our results show that JNK MAPK was required for LPS-induced CD44 expression in monocytic cells while p38 MAPK activation was required for LPS- and TNF-α-induced, but not IL-10-induced, HA-binding in human monocytes. The MAPKs were not found to be involved in IL-10-induced CD44 expression or HA-binding. To dissect the signaling pathways regulating CD44 independently of IL-10-mediated effects, IL-10-refractory promonocytic THP-1 cells were employed. Similar results were obtained in this cell line. Furthermore, our results suggest that lysosomal sialidase activation may be required for the acquisition of the HA-binding form of CD44 in LPS- and TNF-α-stimulated monocytic cells. Interestingly, LPS-induced sialidase activity was dependent on p42/44 MAPK-mediated TNF-α production. Blocking TNF-α production by PD98059, a p42/44 inhibitor, significantly reduced LPS-induced sialidase activity and CD44-HA binding. Subsequently, TNF-α-mediated p38 activation induced sialidase activity and CD44-HA binding. Taken together, our results suggest that the induction of CD44 expression and CD44-HA binding are regulated by two independent signaling events. LPS-induced CD44 expression is dependent on JNK signaling while TNF-α-induced p38 activation may regulate the induction of the functionally active HA-binding form of CD44 by activating sialidase in LPS-stimulated human monocytic cells.

M0.11

Flotillin-1 Expression and Redistribution to the Lipid Rafts Post SDF1a Exposure Is Required for CXCR4 Signaling and Function.

Banabihari Giri, Gary D Collins, Ashani Weeraratna, Dennis D Taub. Laboratory of Immunology, National Institute on Aging/NIH, Baltimore, MD, USA.

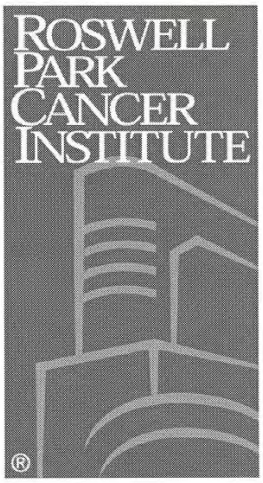
Lipid rafts play an important role in signal integration and cellular activation by the T-cell antigen receptor (TCR). Flotillins are lipid raft-associated proteins, which have been recently implicated in neuronal regeneration, insulin signaling and T-cell receptor signaling. However, a precise functional role for flotillins in chemokine signaling and function remain to be defined. To this end, we have isolated lipid rafts derived from human T-cells and T cell lines pre- and post activation and analyzed both cytoplasmic and raft preparations for flotillin expression. Here, we present evidence that flotillin-1, but not flotillin-2, redistributes to lipid rafts in primary human T cells and Jurkat T cells post SDF1a treatment and associates with several other lipid raft proteins post association with GM1+ lipid rafts including LAT, LCK, CD48 and LFA1a. Similar redistributions were observed post TCR engagement. To functionally analyze the role of flotillin-1 on SDF1a-mediated signaling, we utilized RNAi technology to inhibit expression of this gene and found that loss of flotillin-1 expression resulted in an inhibition of SDF1a-mediated calcium mobilization and functional (chemotaxis and adhesion) activity. Together, these data suggest that the redistribution of flotillin-1 to lipid raft post SDF1a treatment may play a major role in chemokine receptor signaling and function.

M0.12

Interaction of the Skin-Associated Chemokine CCL27 with PSGL-1.

Takako Hirata, Kunio Hieshima, Osamu Yoshie, Masayuki Miyasaka. Laboratory of Molecular and Cellular Recognition, Osaka University Graduate School of Medicine, Suita, Japan; Department of Microbiology, Kinki University School of Medicine, Osaka-Sayama, Japan.

P-selectin glycoprotein-1 (PSGL-1), the major ligand for P-selectin, is expressed on leukocytes and mediates leukocyte rolling on the endothelial surface. PSGL-1 requires tyrosine sulfation and specific O-glycosylation in its amino-terminal region to bind P-selectin. It has been recently shown that some chemokine receptors are modified in the amino-terminal region by tyrosine sulfation and O-glycosylation, which contribute to high affinity binding of chemokines. Based on the similarity in the structures of PSGL-1 and chemokine receptors involved in ligand binding, we studied the interaction of PSGL-1 and chemokines. The binding of PSGL-1 to chemokines was examined in dot blot assays and ELISA-like binding assays using recombinant soluble PSGL-1 (rPSGL-Ig) containing the amino-terminal region of human PSGL-1 and the Fc portion of human IgG1. rPSGL-Ig carries the glycans required for binding to P-selectin. Among various chemokines examined, rPSGL-Ig preferentially bound CCL27 (CTACK/ALP/ILC/ESkine), the skin-associated chemokine that attracts skin-homing T cells. This interaction was abrogated by arylsulfatase treatment of rPSGL-Ig, suggesting that sulfated tyrosines play a critical role. In contrast, removal of either N-glycans or O-glycans by glycosidase treatment of rPSGL-Ig did not affect the interaction. To further define the role of tyrosine sulfation in CCL27-PSGL-1 interaction, we prepared unsulfated rPSGL-Ig and tyrosine-mutated rPSGL-Ig. The binding of CCL27 to unsulfated rPSGL-Ig was significantly reduced. Moreover, the mutations of the tyrosines to phenylalanine abolished the binding, further supporting the role of sulfated tyrosines in CCL27-PSGL-1 interaction. To determine if rPSGL-Ig affects CCL27 function, we examined the chemotaxis of L1.2 cells expressing CCR10, the receptor for CCL27. rPSGL-Ig reduced the CCL27-induced chemotaxis in a dose-dependent manner, suggesting that PSGL-1 binding to CCL27 may regulate the chemokine-mediated responses. Direct interaction of PSGL-1 and CCL27, both of which are critically involved in T cell migration to skin, may confer an additional level of regulation on T cell trafficking.



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TITLE: Fever-Range Thermal Stress Controls Vascular Endothelial Display of ICAM-1 via an IL-6/soluble IL-6 Receptor Trans-Signaling Mechanism

Qing Chen, Jessica Passanese, Kristen Clancy, Sylvia Kucinska, Claudia Green, Wang-Chao Wang, Mark Dewhirst, Douglas Hanahan, Elizabeth Repasky, Heinz Baumann, and Sharon Evans

Intercellular adhesion molecule-1 (ICAM-1) directs lymphocyte recruitment to secondary lymphoid organs and extralymphoid sites of inflammation. We have previously reported that limited lymphocyte infiltration in tumor tissues correlates with low-level expression of ICAM-1 on tumor microvascular endothelium. These observations provide one explanation for immune evasion by tumor cells. Here we report that ICAM-1 expression is markedly upregulated on intratumoral vessels by fever-range whole body hyperthermia (WBH) in a panel of transplantable murine tumors (CT26 colon tumors; B16 melanoma; EMT6, TD40, and R3230 mammary tumors) and in RIP-Tag5 transgenic pancreatic tumors. Two-color confocal immunofluorescence microscopy demonstrated that ICAM-1 upregulation occurs principally on $CD31^+$ vessels rather than on stromal cells or tumor cells within tumor microenvironments. Profound induction of luminal ICAM-1 expression was detected in vessels of tumor tissues and lymphoid organs, but not in extralymphoid tissues (e.g., liver, pancreas), following *i.v.* delivery of ICAM-1-specific mAb in WBH-treated mice. Elevated vascular expression of ICAM-1 correlated with enhanced LFA-1/ICAM-1-dependent lymphocyte adhesion by *in vitro* adherence assays and trafficking in short-term homing studies *in vivo*. Finally, the molecular basis of thermal control of ICAM-1 was examined. Neutralizing mAb to IL-6, but not to TNF-α or IL-1β, fully suppressed thermal induction of ICAM-1 in tumor tissues and lymphoid organs. Recombinant soluble gp130 also prevented ICAM-1 induction, indicating that thermal activities in vascular targets depend on a trans-signaling mechanism whereby IL-6 interacts with a soluble form of the IL-6 receptor. Taken together, these results provide insight into the molecular mechanisms by which febrile temperatures regulate site-specific lymphocyte homing during inflammation or clinical thermal therapy. (Supported by NIH CA79765, CA094045, DK33886, and DOD BC032139).

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FEVER-RANGE THERMAL STRESS CONTROLS VASCULAR ENDOTHELIAL DISPLAY OF ICAM-1 VIA AN IL-6/SOLUBLE IL-6 RECEPTOR TRANS-SIGNALING MECHANISM

Qing Chen, Jessica Passanese, Kristen Clancy, Sylvia Kucinska, Claudia Green, Wang-Chao Wang, Mark Dewhirst, Douglas Hanahan, Elizabeth Repasky, Heinz Baumann, and Sharon Evans

Intercellular adhesion molecule-1 (ICAM-1) directs lymphocyte recruitment to secondary lymphoid organs and extralymphoid sites of inflammation. We have previously reported that limited lymphocyte infiltration in tumor tissues correlates with low-level expression of ICAM-1 on tumor microvascular endothelium. These observations provide one explanation for immune evasion by tumor cells. Here we report that ICAM-1 expression is markedly upregulated on intratumoral vessels by fever-range whole body hyperthermia (WBH) in a panel of transplantable murine tumors (CT26 colon tumors; B16 melanoma; EMT6, TD40, and R3230 mammary tumors) and in RIP-Tag5 transgenic pancreatic tumors. Two-color confocal immunofluorescence microscopy demonstrated that ICAM-1 upregulation occurs principally on $CD31^+$ vessels rather than on stromal cells or tumor cells within tumor microenvironments. Profound induction of luminal ICAM-1 expression was detected in vessels of tumor tissues and lymphoid organs, but not in extralymphoid tissues (e.g., liver, pancreas), following *i.v.* delivery of ICAM-1-specific mAb in WBH-treated mice. Elevated vascular expression of ICAM-1 correlated with enhanced LFA-1/ICAM-1-dependent lymphocyte adhesion by *in vitro* adherence assays and trafficking in short-term homing studies *in vivo*. Finally, the molecular basis of thermal control of ICAM-1 was examined. Neutralizing mAb to IL-6, but not to TNF-□ or IL-1□, fully suppressed thermal induction of ICAM-1 in tumor tissues and lymphoid organs. Recombinant soluble gp130 also prevented ICAM-1 induction, indicating that thermal activities in vascular targets depend on a trans-signaling mechanism whereby IL-6 interacts with a soluble form of the IL-6 receptor. Taken together, these results provide insight into the molecular mechanisms by which febrile temperatures regulate site-specific lymphocyte homing during inflammation or clinical thermal therapy.

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Eugene C. Butcher and Ulrich H. von Andrian

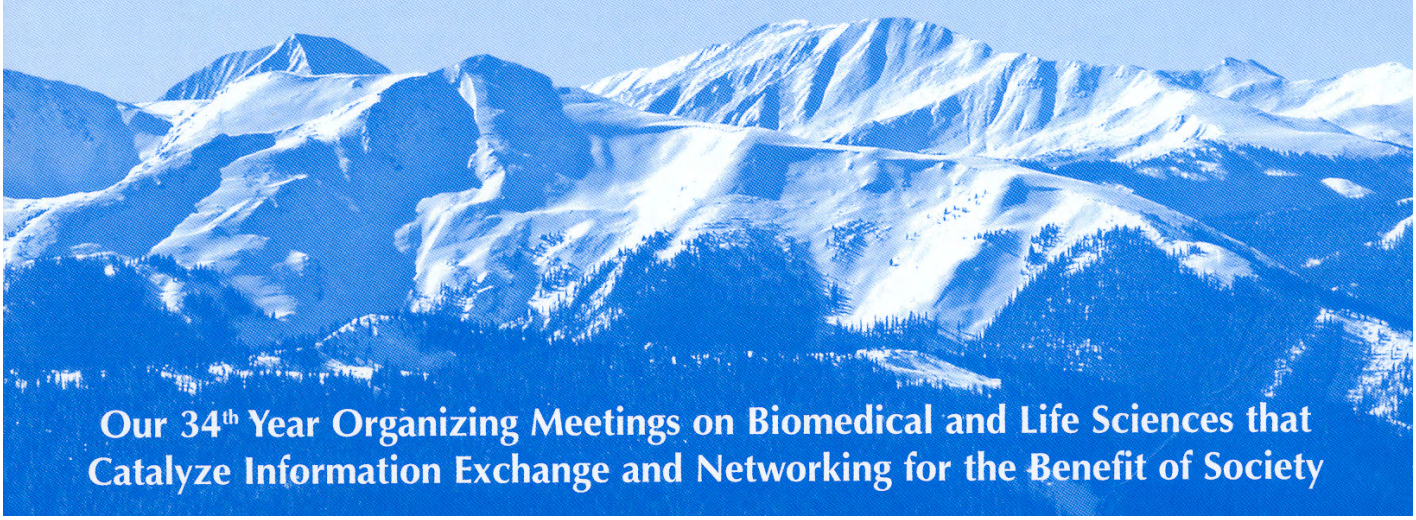
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113 Cybr, a novel cytokine-induced adapter molecule binds cytohesin-1 and regulates lymphocyte functions.

Qian Chen, Alan Coffey, Oonagh T. Lynch, Alyson Kelvin, Wendy T. Watford*, John J. O'Shea*, Massimo Gadina.
Centre for Cancer Research and Cell Biology, Queen's University, Belfast, Northern Ireland; MIIB-NIAMS, NIH Bethesda, MD, USA

Integrin facilitate leukocytes adhesion allowing interactions with the endothelium, extracellular matrix components, and antigen-presenting cells (APCs) ultimately regulating the immune response. The integrin αLβ2 (LFA-1) is involved in these events and it has also been shown to be important for T cells proliferation and IL-2 production and cooperates with CD3 and CD28 enhancing differentiation of Th1 cells. However, little is known about how the adhesion-mediated signaling events occurring through this integrin lead to T cell activation.
Cytohesin-1 is a guanine nucleotide exchange factor (GEF) for ARF GTPases, which also binds to LFA-1 and has been shown to have a role in integrins-dependent lymphocyte adhesion and migration. We have recently identified a new molecule named Cybr (CYtohesin Binder and Regulator) which interacts with cytohesin-1 and influence its enzymatic activity. Cybr is expressed in immune cells including T, B, monocytes, macrophages, and dendritic cells, and its expression is markedly upregulated by cytokine, chemokines stimulation or Toll-like receptor ligation. In the thymus, Cybr is preferentially expressed in single-positive thymocytes. Furthermore, Cybr is preferentially expressed in Th1 cells. Cybr encodes two protein-protein interaction domains: a PDZ domain and a coiled-coil domain and may act as a molecular scaffold. Cybr regulates cytohesin enzymatic functions accelerating the cytohesin-1-dependent binding of GTP by ARF1. We now have evidence that this new protein is also involved in signaling events downstream of the T-cell receptor. In particular, we have found that Cybr expression can augment TCR-dependent transactivation of an NFAT-luciferase reporter construct. Interestingly, co-expression of cytohesin-1 can abolish the increased transactivation. Experiments are now ongoing to dissect the molecular mechanism by which the cytohesin-Cybr pathway can influence lymphocyte functions and the integrin-mediated T cell activation.

114 Fever-Range Thermal Stress Controls HEV Display of ICAM-1 via an IL-6 Trans-Signaling Mechanism

Qing Chen, Emily Unger, Jessica Passanese, Michelle Appenheimer, Daniel Fisher, Wang-Chao Wang, Heinz Baumann, and Sharon Evans

The evolutionarily conserved fever response is associated with survival during acute infection although the protective mechanisms are largely undefined. Previous studies have shown that fever-range thermal stress increases L-selectin and αLβ7 integrin-dependent tethering and rolling events in specialized high endothelial venules (HEV), thereby promoting trafficking to secondary lymphoid organs. Here we examined the effect of fever-range temperatures on the mechanisms controlling firm adhesion in HEV. Marked induction of luminal expression of intercellular adhesion molecule-1 (ICAM-1) occured in $PNAd^+$ or $MAdCAM\text{-}1^+$ HEV of lymph nodes or Peyer's patches, respectively, following elevation of core temperatures of mice by fever-range whole body hyperthermia (6 h at 39.5-40°C). In contrast, febrile temperatures did not alter HEV expression of PNAd, MAdCAM-1, ICAM-2, JAM-1, or CD31. Moreover, thermal stress did not amplify ICAM-1 expression in non-activated vessels of extralymphoid tissues (e.g., liver, pancreas). Elevated vascular expression of ICAM-1 correlated with enhanced LFA-1/ICAM-1-dependent lymphocyte-HEV adhesion *in vitro* and homing to lymphoid organs *in vivo*. Finally, the molecular basis of thermal control of ICAM-1 was examined. Neutralizing mAb to IL-6, but not to TNF-α or IL-1β, fully suppressed thermal induction of ICAM-1 in lymphoid organ HEV. Recombinant soluble gp130 also prevented ICAM-1 upregulation, indicating that thermal activities in vascular targets depend on a trans-signaling mechanism in which IL-6 engages a soluble form of the IL-6 receptor. These findings, together with recent evidence that IL-6 trans-signaling also mediates thermal effects on L-selectin adhesion, identify a highly integrated cytokine signaling network whereby febrile temperatures stimulate site-specific lymphocyte homing and immune surveillance during inflammation. (Supported by NIH CA79765, CA094045, DK33886, and DOD BC032139).

115 Suppression of Tumor Formation in Lymph Nodes by L-Selectin-Mediated Natural Killer (NK) Cell Recruitment

Shihao Chen[1], Hiroto Kawashima[1], John B. Lowe[2], Lewis Lanier[3], and Minoru Fukuda[1]

[1] Glycobiology Program, The Burnham Institute, La Jolla, CA 92037.
[2] Howard Hughes Medical Institute, Department of Pathology, The University of Michigan Medical School, Ann Arbor, Michigan 48109.
[3] Department of Microbiology and Immunology, University of California San Francisco, 513 Parnassus Ave. HSE 1001G, Box 0414, San Francisco, CA 94143-0414.

Natural killer (NK) cells play critical roles in control of virus infection and tumor formation. They largely reside in the spleen and in the peripheral blood. We have recently detected the presence of NK cells in secondary lymph nodes in mouse. We further showed that the NK subsets in lymph nodes are different from those in the blood or the spleen, *i.e.*, less $Ly49C/I^+$ or $Ly49D^+$ NK cells in the nodes. Mouse NK cells express sialyl Lewis x oligosaccharides that are mainly carried on core 2 branched O-glycans. However, the capacity of *in vivo* tumor rejection remained intact in mice defective in C2GnT-I or fucosyltransferase IV and VII; and these two mutant NK cells migrated normally as wild type NK cells when adoptively transferred to wild type host, indicating that sialyl Lewis x oligosaccharides on NK cell surface are not essential for migration to peripheral organs nor cytolysis of tumor cells. When NK cells from wild type and L-selectin deficient mice were simultaneously introduced via tail vein into wild type mouse, we found that L-selectin deficient NK cells were defective in migration to lymph nodes under resting state. Adoptively transferred wild type NK cells were also less efficient in trafficking to lymph nodes in fucosyltransferase IV and VII doubly deficient mice than wild type mice, suggesting that L-selectin ligands generated by fucosyltransferase IV and VII facilitated NK cells migration. Furthermore, a large number of NK cells could be recruited to regional lymph nodes by complete Freund's adjuvant (CFA) stimulation. We showed that this recruitment was also mediated by L-selectin and its ligands. Although L-selectin was not directly involved in NK cell-mediated cytotoxicity, more tumor cells were detected in L-selectin deficient popliteal lymph node than in wild type node 10 days after B16F10 melanoma were subcutaneously inoculated at footpad. The results indicate that NK cell trafficking to lymph nodes is facilitated by L-selectin-mediated adhesion and that lymph node NK cells suppress tumor metastasis and growth.
Supported by grant PO1 CA71932 to M. Fukuda and Cancer Research Institute Postdoctoral Fellowship to S. Chen.

116 Real-time regulation of VLA-4 affinity and conformation under shear: a mechanism for capture of leukocytes by endothelium

A. Chigaev[1], G. Zwartz[1], T. Buranda[1], T. Foutz[1], ER. Prossnitz[2], BS Edwards[1], RS Larson[1], and LA Sklar[1]. [1]CRTC, Depts. of Pathology and [2]Cell Biology, University of New Mexico, Albuquerque, NM 87131

The $\alpha_4\beta_1$ integrin (VLA-4) can exist in multiple conformations defined by varying affinity. The number of VLA-4 molecules on leukocytes is typically less than 5,000. We have used a fluorescent ligand probe to characterize affinity states formed during cell activation and in the presence of different ions. The affinity can be varied over three orders of magnitude by varying divalent cations, but the physiologically active state is only about 6 times higher affinity than the resting state. The affinity changes result primarily from a change in the ligand dissociation rate rather than association rate. The affinity changes for the native ligand, VCAM-1, were also measured and found to vary in parallel with the fluorescent probe. The binding time for the native ligand was estimated to be less than a second for the resting form of the receptor. We determined how affinity changes affected the ability of VLA-4 to mediate cell-cell adhesion. This approach compared cell-cell adhesion times with ligand residence times. The results indicate that the duration of cell-cell adhesion parallels the duration of ligand binding, and that only small numbers of adhesion bonds are required to hold cells together. We used fluorescence resonance energy transfer to show that there is a correspondence between VLA-4 affinity and molecular conformation such that as the head of the VLA-4 molecule extends away from the membrane surface, its affinity increases. Under low shear forces, contrary to expectations, the duration of cell-cell adhesion increases and the increase is dependent on outside-in signaling. Thus, when leukocytes roll on endothelium at low shear, using an interaction between VLA-4 and VCAM-1, both inside-out signaling and force may regulate the conformation of VLA-4 providing a means for VLA-4 to change its affinity and contributing to leukocyte slowing and capture by the endothelium.

Supported by the National Institute of Health grant HL56384, EB02022 and CA88339.

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Poster B/C 01: Thermotolerance Induced at a Mild Temperature of 40°C Protects Cells Against Heat Shock-Induced Apoptosis

Author:

Diana Averill-Bates and Ahmed Bettaieb, Université du Québec à Montréal, Montréal, Québec, Canada

Abstract:

Apoptosis constitutes a response of organisms to various physiological or pathological stimuli, and to different stresses such as heat shock and oxidative stress. The ability of thermotolerance induced at a mild temperature of 40°C to protect against activation of the apoptotic cascade by heat shock was investigated. When Chinese hamster ovary and human adenocarcinoma cervical cells were pretreated at 40°C, they were resistant to subsequent lethal heat shock at 43°C. Induction of thermotolerance at 40°C led to increased expresssion of heat shock proteins 27, 32, 72 and 90. Heat shock induced apoptotic events at the mitochondrial level, involving a decrease in membrane potential, translocation of Bax to mitochondria and liberation of cytochrome c into the cytosol. These events were diminished in thermotolerant cells. Heat shock (42 to 45°C) caused activation of initiator caspase-9 and effector caspases-3, 6 and 7, relative to controls at 37°C. Activation of caspases was decreased in thermotolerant cells. Heat shock caused fragmentation of the caspase substrate, inhibitor of caspase-activated DNase. Fragmentation was diminished in thermotolerant cells. Thermotolerance afforded protection against heat shock-induced nuclear chromatin condensation, but not against necrosis.

Poster B/C 02: Fever-Range Thermal Stress Controls Vascular Endothelial Display of ICAM-1 via an IL-6/soluble IL-6 Receptor Trans-Signaling Mechanism

Author:

Qing Chen, Jessica Passanese, Daniel Fisher, Sylvia Kucinska, Kristen Clancy, Wang-Chao Wang, Michelle Appenheimer, Lei Zhou, Elizabeth Repasky, Heinz Baumann, and Sharon Evans (presenter); Roswell Park Cancer Institute, Buffalo, NY, USA

Abstract:

Intercellular adhesion molecule-1 (ICAM-1) directs lymphocyte recruitment to secondary lymphoid organs and extralymphoid sites of inflammation. We have previously reported that limited lymphocyte infiltration in tumor tissues correlates with low-level expression of ICAM-1 on tumor microvascular endothelium. These observations provide one explanation for immune evasion by tumor cells. Here we report that ICAM-1 expression is markedly upregulated on intratumoral vessels by fever-range whole body hyperthermia (WBH) in a panel of transplantable murine tumors (CT26 colon tumors; B16 melanoma; EMT6, and TD40) and in RIP-Tag5 transgenic pancreatic tumors. Two-color confocal immunofluorescence microscopy demonstrated that ICAM-1 upregulation occurs principally on CD31+ vessels rather than on stromal cells or tumor cells within tumor microenvironments. Profound induction of luminal ICAM-1 expression was detected in vessels of tumor tissues and lymphoid organs, but not in extralymphoid tissues (e.g., liver, pancreas), following i.v. delivery of ICAM-1-specific mAb in WBH-treated mice. Thermal induction of ICAM-1 expression on the tumor vasculature correlated with enhanced LFA-1/ICAM-1-dependent lymphocyte adhesion by in vitro adherence assays and increased lymphocyte trafficking by short-term homing studies in vivo. Enhanced CD8+ T cell infiltration was further observed in response to fever-range WBH in tumor tissues with high vascular ICAM-1 expression. Finally, the molecular basis of thermal control of ICAM-1 was examined. Neutralizing mAb to IL-6, but not to TNF-α or IL-1β, fully suppressed thermal induction of ICAM-1 in tumor tissues and lymphoid organs. Recombinant soluble gp130 also prevented ICAM-1 induction, indicating that thermal activities in vascular targets depend on a trans-signaling mechanism whereby IL-6 interacts with a soluble form of the IL-6 receptor. Taken together, these results provide insight into the molecular mechanisms by which febrile thermal stress regulates site-specific lymphocyte homing during inflammation or clinical thermal therapy. (Supported by NIH grants CA79765 and CA094045 and DOD grant DAMD57-8035-01)

Poster B/C 03: Intracellular Acidification Abrogates the Heat Shock Response and Selectively Sensitizes Human Melanoma DB-8 Cells Grown at pH 6.7 to Thermal Therapy

Author:

RA Coss, CW Storck, DB Leeper, J Reilly, J-S Han, KA Kulp, TC Wells, Dept of Radiation Oncology, Thomas Jefferson University, Philadelphia, PA USA
ML Wahl, Department of Pathology, Duke University Medical Center, Durham, NC USA

Abstract:

SK-Mel-28 and DB-1 human melanoma cells grown at pH 7.3 have a critical intracellular pH (pHi) threshold below which they become sensitized to 42°C, while cells grown at pH 6.7 are already at or near their critical pHi threshold. Consequently, it takes less of a reduction in extracellular pH (pHe) to reduce pHi and selectively sensitize these cells grown at pH 6.7 to thermal therapy. We examined whether or not DB-8 human melanoma cells have pHi thresholds for thermal sensitization. In contrast to SK-Mel-28 and DB-1 cells, the endogenous level of Hsp27 in DB-8 cells cultured at pH 6.7 is 60-fold less than in cells cultured at pH 7.3.

A pHi threshold was found to exist for DB-8 cells cultured at pH 7.3 below which they became sensitized to 42°C. A pHi threshold for heat sensitization did not exist for cells growing at pH 6.7: any reduction in pHi prior to heating resulted in increased cell killing. There was a greater decrease in pHi per unit decrease in pHe for cells grown at pH 6.7. Consequently, DB-8 cells grown at pH 6.7 are sensitized more to 42°C per unit decrease in pHi (and pHe) than cells grown at pH 7.3. Acute acidification abrogated the 42°C-induction of Hsp70 and Hsp27 in the DB-8 cells. The pHi thresholds for abrogation of HSP induction are higher than the pHi thresholds for thermal sensitization of cells grown at pH 7.3, while the thresholds for abrogation of HSP induction and survival were similar for cells cultured at pH 6.7. These findings support the concept for sensitization of human melanoma cells to thermal therapy by strategies that selectively lower the pHi in cells existing in an acidic environment. (Funded by Grant No. PO1 CA56690 from NCI, NIH, DHHS).

Poster B/C 04: Hsp27 Phosphorylation and Resistance to 42°C in Human Melanoma Cells

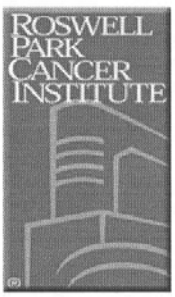
Author:

RA Coss, S Vemuri, CW Storck, TC Wells, KA Kulp, and DB Leeper Department of Radiation Oncology, Thomas Jefferson University, Philadelphia, PA USA

Abstract:

We previously reported that linear relationships did not exist for survival as a function of heat-induced levels of Hsp27 in SK-Mel-28 and DB-8 human melanoma cells. In addition, the levels of phosphorylated Hsp27 (pSer78, antibody from StressGen) were detectable by immunoblot analysis after a 42°C heat shock and the levels increased rather than decreased as total levels of Hsp27 do when cells are acutely acidified prior to and during heating. These findings were not in agreement with the literature that the phosphorylated form of Hsp27 confers heat resistance.

These studies were repeated using an antibody against another Hsp27 phosphorylation site (pSer85, antibody from Affinity Bioreagents) and extended to include 2 additional human melanoma cultures generated from biopsies, DB-10 and DB-20. The endogenous levels of Hsp27 in cells cultured at pH 7.3 ranged between 10x (DB-1), 60x (DB-8) and 240x(DB-10 and -20) the level in SK-Mel-28 cells. The results using the antibody against



RPCI TUMOR IMMUNOLOGY PROGRAM 6TH ANNUAL RETREAT

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Fever-Range Thermal Therapy Induces ICAM-1-Dependent Lymphocyte Trafficking Through an IL-6 Trans-Signaling Mechanism

Qing Chen, Kristen Clancy, Sylvia Kucinska, Daniel Fisher, Wang-Chao Wang, Elizabeth Repasky, Heinz Baumann, and Sharon Evans

Intercellular adhesion molecule-1 (ICAM-1) directs lymphocyte recruitment to secondary lymphoid organs and extralymphoid sites of inflammation. We have previously reported that low-level expression of ICAM-1 on tumor microvascular endothelium is associated with limited lymphocyte infiltration in tumor tissues. These observations provide one explanation for immune evasion by tumor cells. Here we report that ICAM-1 expression is markedly induced on intratumoral vessels by fever-range whole body thermal therapy in a panel of transplantable murine tumors (CT26 colon tumors; B16 melanoma; EMT6, and TD40) and in RIP-Tag5 transgenic pancreatic tumors. Two-color confocal immunofluorescence microscopy demonstrated that ICAM-1 upregulation occurs principally on CD31+ vessels rather than on stromal cells or tumor cells within tumor microenvironments. Intravascular staining revealed that thermal therapy strongly induces ICAM-1 expression on the luminal surface of vessels in tumor tissues and lymphoid organs, but not in extralymphoid tissues (e.g., liver, pancreas). Thermal induction of ICAM-1 expression on the tumor vasculature correlated with enhanced lymphocyte adhesion by in vitro adherence assays and increased CD8+ T cell trafficking by short-term homing studies in vivo. Thermally enhanced intravascular expression of ICAM-1 was functionally linked to improved lymphocyte trafficking across vascular endothelium using ICAM-1 blocking Ab or ICAM-1-deficient mice. Finally, the molecular basis of thermal control of ICAM-1 was examined. Neutralizing mAb to IL-6, but not to TNF-á, IL-1â, or IFN-ã, fully suppressed thermal induction of ICAM-1 in tumor tissues and lymphoid organs. Recombinant soluble gp130 also prevented ICAM-1 induction, indicating that thermal activities in vascular targets depend on a trans-signaling mechanism by which the transmembrane gp130 signal transduction molecules is dually engaged by IL-6 and a soluble form of the IL-6 receptor. Taken together, these results suggest that clinical thermal therapy may be an effective strategy to promote ICAM-1-dependent homing of immune effector cells to the tumor microenvironment. (Supported by NIH grants CA79765 and CA094045 and DOD grant W81XWH-04-1-0354)

Program & Abstracts

Fifth Annual Buffalo Conference on Immunology

"Cancer and Inflammation"

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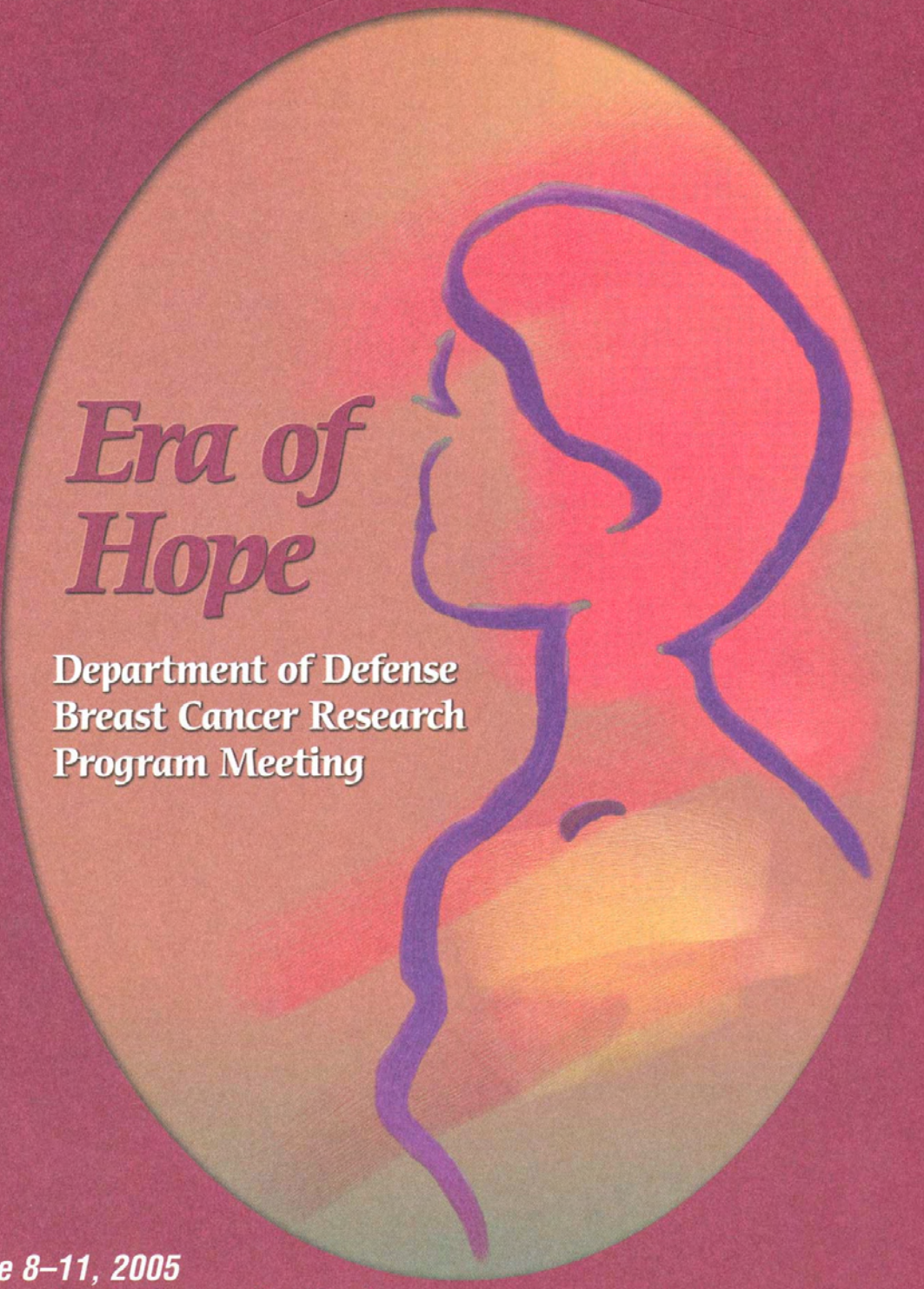
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FEVER-RANGE THERMAL THERAPY INDUCES ICAM-1-DEPENDENT LYMPHOCYTE TRAFFICKING THROUGH AN IL-6 TRANS-SIGNALING MECHANISM

Chen Q, Clancy K, Kucinska S, Fisher D, Wang WC, Repasky E, Baumann H, and Evans S. Department of Immunology, Roswell Park Cancer Institute.

Intercellular adhesion molecule-1 (ICAM-1) directs lymphocyte recruitment to secondary lymphoid organs and extralymphoid sites of inflammation. We have previously reported that low-level expression of ICAM-1 on tumor microvascular endothelium is associated with limited lymphocyte infiltration in tumor tissues. These observations provide one explanation for immune evasion by tumor cells. Here we report that ICAM-1 expression is markedly induced on intratumoral vessels by fever-range whole body thermal therapy in a panel of transplantable murine tumors (CT26 colon tumors; B16 melanoma; EMT6, and TD40) and in RIP-Tag5 transgenic pancreatic tumors. Two-color confocal immunofluorescence microscopy demonstrated that ICAM-1 upregulation occurs principally on $CD31^+$ vessels rather than on stromal cells or tumor cells within tumor microenvironments. Intravascular staining revealed that thermal therapy strongly induces ICAM-1 expression on the luminal surface of vessels in tumor tissues and lymphoid organs, but not in extralymphoid tissues (*e.g.,* liver, pancreas). Thermal induction of ICAM-1 expression on the tumor vasculature correlated with enhanced lymphocyte adhesion by *in vitro* adherence assays and increased $CD8^+$ T cell trafficking by short-term homing studies *in vivo*. Thermally enhanced intravascular expression of ICAM-1 was functionally linked to improved lymphocyte trafficking across vascular endothelium using ICAM-1 blocking Ab or ICAM-1-deficient mice. Finally, the molecular basis of thermal control of ICAM-1 was examined. Neutralizing mAb to IL-6, but not to TNF-α, IL-1β, or IFN-γ, fully suppressed thermal induction of ICAM-1 in tumor tissues and lymphoid organs. Recombinant soluble gp130 also prevented ICAM-1 induction, indicating that thermal activities in vascular targets depend on a trans-signaling mechanism by which the transmembrane gp130 signal transduction molecules is dually engaged by IL-6 and a soluble form of the IL-6 receptor. Taken together, these results suggest that clinical thermal therapy may be an effective strategy to promote ICAM-1-dependent homing of immune effector cells to the tumor microenvironment.
(Supported by NIH grants CA79765 and CA094045 and DOD grant W81XWH-04-1-0354)



Era of Hope

Department of Defense Breast Cancer Research Program Meeting

June 8–11, 2005
Pennsylvania Convention Center
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PROCEEDINGS

P45-4: FEVER-RANGE THERMAL STRESS PROMOTES EXPRESSION OF INTERCELLULAR ADHESION MOLECULE-1 IN TUMOR VESSELS VIA AN INTERLEUKIN-6 TRANS-SIGNALING MECHANISM

Qing Chen, Jessica Passanese, Wan-Chao Wang, Elizabeth Repasky, Heinz Baumann, and Sharon Evans
Roswell Park Cancer Institute, Buffalo, NY
E-mail: qing.chen@roswellpark.org

Significant progress has been achieved in designing vaccine strategies for the treatment of breast cancer. However, a frequently overlooked determinant to successful immunotherapy relates to the efficiency by which cytotoxic $CD8^+$ T lymphocytes egress at tumor sites. Migration of blood-borne T lymphocytes across the vascular endothelial cell barrier involves step-wise adhesive interactions. In murine breast tumors, poor leukocyte infiltration is correlated with low expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) on intratumoral vessels. Thus, inadequate expression of vascular adhesion molecules may be one explanation for immune evasion by tumor cells. Notably, limited $CD8^+$ T cell infiltration is associated with a poor prognosis in advanced breast carcinoma in human patients.

Our previous studies have shown that clinical fever-range thermal therapy stimulates lymphocyte trafficking across specialized blood vessels in lymphoid organs. This finding led to the hypothesis that the molecular mechanisms enacted by fever-range thermal stress can target delivery of lymphocytes to breast tumors. In the present study a multi-pronged approach was taken to address this hypothesis. Mice bearing syngeneic EMT6 transplantable mammary tumors, or other tumor types, were treated with fever-range whole body hyperthermia (WBH; 39.5-40oC for 2-6 h). ICAM-1 expression on the luminal surface of tumor microvessels was detected by fluorescence microscopy following *i.v.* injection of anti-ICAM-1 monoclonal antibodies (mAb). A CD8+ murine T cell line was used to evaluate ICAM-1 function on tumor vessels in frozen-section *in vitro* adherence assays and in short-term (1 h) *in vivo* homing studies. Finally, cytokine-neutralizing mAb were injected prior to WBH treatment to investigate the molecular mechanisms underlying thermal control of ICAM-1 expression.

These studies demonstrate that fever-range WBH selectively induces ICAM-1 expression on intratumoral vessels, but not on vessels of non-involved extralymphoid organs (liver, pancreas, kidney, lungs, and heart). Elevated expression of ICAM-1 correlates with enhanced CD8+ T lymphocyte adhesion to tumor vessels *in vitro* and homing to tumor sites *in vivo*. Moreover, thermal induction of ICAM-1 on intratumoral vessels was shown to depend on interleukin-6 (IL-6), but not IL-1β or tumor necrosis factor-α. Finally, ICAM-1 upregulation was found to involve a trans-signaling mechanism whereby IL-6 and a soluble form of the IL-6 receptor engage the gp130 signal transduction molecule.

This work is the first demonstration for IL-6 trans-signaling in control of ICAM-1 expression on intratumoral vessels and suggests a mechanism to focus delivery of $CD8^+$ T lymphocytes to tumor sites while avoiding deleterious bystander effects in other organs. Further investigation of the molecular mechanisms underlying IL-6 control of tumor microvascular adhesion may provide a framework for developing fever-range thermal therapy as an adjuvant to vaccine strategies to improve the clinical outcome in breast cancer.

The U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0354 and NIH CA79765 and CA094045 supported this work.

P45-5: IMMUNITY TO BREAST CANCER IN MICE IMMUNIZED WITH FIBROBLASTS TRANSFECTED WITH A CDNA EXPRESSION LIBRARY FROM BREAST CANCER CELLS. A NEW VACCINATION STRATEGY

Tae Sung Kim and Edward P. Cohen
Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL
E-mail: epcohen@uic.edu

Breast cancer cells, like neoplasms arising in various other organs and tissues, express an array of tumor associated antigens (TAA). TAA are weakly immunogenic. They fail to provoke anti tumor immune responses, which are capable of controlling tumor growth. Breast cancer cells proliferate without apparent inhibition in immunocompetent mice, and patients. Under appropriate circumstances, however, immunity to TAA expressed by breast cancer cells can be induced, with potential beneficial results for patients with the disease. Many immunotherapeutic strategies, however, are constrained by the need for large amounts of tumor tissue, required to prepare the vaccine. Patients who are at an early stage of the disease when immunization is most likely to be effective may not receive treatment. Here, we report a new vaccination strategy for breast cancer. In a mouse model of the disease, the vaccine was prepared by transfer of a cDNA expression library from a breast neoplasm (SB5b) that arose spontaneously in a C3H/He mouse ($H\text{-}2^k$) into a mouse fibroblast cell line. As the transferred DNA is replicated as the recipient cells divide, the vaccine could be prepared from microgram quantities of tumor tissue. The number of vaccine cells could be expanded as required for multiple rounds of immunization.

cDNA encompasses the array of TAAs that characterize the breast cancer cells. Before DNA-transfer, to augment their nonspecific immunogenic properties, the fibroblasts, also of C3H/He mouse origin, were genetically modified to secrete IL-2 and to express allogeneic class I $H\text{-}2K^b$-determinants. The transfected syngeneic/allogeneic fibroblasts were transfected with the cDNA expression library and then tested for their immunogenic properties against the breast cancer cells in C3H/He mice. Robust immunity toward the breast cancer cells was generated in mice immunized with the vaccine. The immunity, mediated predominantly by $CD8^+$T lymphocytes, was directed toward the breast cancer cells, but not against either of two immunologically non cross-reactive tumors of C3H/He mice (SCCVII squamous carcinoma and C3H/MCA fibrosarcoma). The immunity was sufficient to deter tumor growth in this model system. Immunized C3H/He mice challenged with the breast cancer cells survived significantly longer than mice in various control groups. Among other advantages, a breast cancer vaccine prepared by DNA transfer into a fibroblast cell line enables the cells used as DNA-recipients to be modified in advance of DNA-transfer to augment their immunogenic properties.

Original work supported by the U.S. Army Medical Research and Materiel Command under DAMD17-96-1-6178. Current work supported by NICDR grant number 1 RO1 DEO13970-O1A2 awarded to Dr. Cohen.

P45-6: VACCINE FOR EPITHELIAL NEOPLASMS

Albert B. Deisseroth, M.D., Ph.D.
Sidney Kimmel Cancer Center, San Diego, CA
E-mail: adeisseroth@skcc.org

The immune response ignores breast cancer cells because 1) they are covered by self antigens which are present from birth and therefore are indistinguishable from normal tissue and 2) the surveillance cells of the immune response (dendritic cells (DCs) are most often spatially separated from the breast cancer cells. We have designed a method for in vivo activation and tumor antigen loading of dendritic cells (DCs) so that they can induce a T cell immune response against tumor associated antigens (TAA) in breast cancer.

An adenoviral vector which carries a fusion gene composed of the TAA fused to the extracellular domain (ecd) of the CD40 ligand (CD40L) is injected subcutaneously (sc). The cells infected by the vector are programmed to secrete the TAA/ecdCD40L protein for 10 days. When the CD40L end of the protein binds to the CD40 receptor on the DCs, the DCs are activated to take up the TAA and migrate to the regional lymph nodes. The DCs, which the carry pieces of the TAA on the MHC molecules on their surface, induce an expansion of the T cells which are capable of recognizing and killing TAA positive cancer cells, and making antibodies to the TAA. We found that this vector vaccination can induce immune suppression of the growth of TAA cancer cells for up to one year, even in mice which are unresponsive to the TAA.

We have shown that the Ad-sig-TAA/ecdCD40L vector vaccine breaks tolerance to TAA in two transgenic mouse models in which the mice are immunologically unresponsive to the TAA. Since the Ad-sig-ecdhMUC-1/ecdCD40L vector and ecdhMUC-1/ecdCD40L protein vaccine strategy has been approved by the local IRB, IBC and the NIH RAC, we are now proposing to develop a phase I clinical trial for toxicity evaluation of this vaccine.

Ultimately, our goal is to add this clinical vaccine to standard adjuvant therapy in the post surgical adjuvant setting in patients who are thought to be at high risk of recurrence despite adjuvant therapy.

The U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0554 supported this work.

P45-7: TELOMERASE VACCINATION OF METASTATIC BREAST CANCER PATIENTS INDUCES ANTIGEN-SPECIFIC TUMOR INFILTRATING LYMPHOCYTES AND TUMOR NECROSIS

S. M. Domchek, K. R. Fox, A. Recio, L. M. Schuchter, R. Davidson, A. DeMichele, M. D. Feldman, and R. H. Vonderheide
Abramson Cancer Center, University of Pennsylvania School of Medicine, Philadelphia, PA
E-mail: susan.domchek@uphs.upenn.edu

Background: The telomerase reverse transcriptase hTERT represents a promising target for immunotherapy because of its near universal expression in cancer and its critical role in oncogenesis. CD8+ T lymphocytes recognize peptides derived from hTERT and kill hTERT+ tumors cells of multiple histologies in vitro.

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2006 ABSTRACT BOOK

Chemokines and Chemokines Receptors

Andrew Luster and Philip Murphy

Supported by
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January 15 - 20, 2006
Snowbird Resort • Snowbird, Utah



117 Membrane-bound CC-chemokine inhibitor 'M35K' provides localised inhibition of CC-chemokine activity in vitro and in vivo.

†Christina Bursill, *David Greaves, †Keith Channon, †Department of Cardiovascular Medicine and *Sir William Dunn School of Pathology, University of Oxford, Oxford, UK, OX3 7BN.

The Vaccinia virus expresses a 35kDa soluble protein (35K) that provides broad spectrum blockade of the CC-Chemokine (CC-CK) class. Studies in our laboratory have demonstrated that the CC-CK class is important in atherosclerosis. Adenoviral-mediated delivery of soluble 35K (Ad35K) significantly reduced systemic CC-chemokine activity, inhibited atherosclerosis[1] and reduced vein graft intimal hyperplasia[2] in ApoE knockout mice. We hypothesize that local rather than systemic CC-CK inhibition may be a more useful therapeutic strategy. Accordingly, we generated a membrane-bound form of 35K protein (m35K) incorporating the Fas-ligand trans-membrane domain and an intracellular N-terminal GFP, to aid visualization. In vitro chemotaxis assays showed the migration of 293 cells expressing CCR5 and m35Kwas strikingly attenuated (95%) towards CC-CK RANTES (CCL5) compared to cells expressing CCR5 only. In contrast, co-expression of m35K with the unrelated fractalkine receptor (CX_3CR1) did not inhibit cell migration towards fractalkine. Cells expressing m35K significantly depleted levels of the CC-CKs RANTES and MIP-1 alpha (CCL3) from culture medium, and this sequestration of CC-CKs also prevented the chemotaxis of 'bystander' cells expressing CCR5 but not CX_3C. Infection of mice with adenovirus encoding m35K (Adm35K) produced a striking decrease in hepatic CC-CK activity with only a modest decrease in plasma CC-CK activity, demonstrating predominant organ-specific CC-CK blockade. Broad-spectrum CC-CK inhibition by 'm35K', which is localised to specific cell types via the addition of a trans-membrane domain, may be a useful therapeutic modality for the treatment of chronic inflammatory diseases characterized by monocyte recruitment and macrophage activation such as atherosclerosis, rheumatoid arthritis, asthma and multiple sclerosis.

[1] C. Bursill et al. Circulation, 2004. Oct 19; 110 (16):2460-6.
[2] Z. Ali et al. Circulation. 2005. 112 [suppl I]: I-235 – I-241.

118 IP-10 (CXCL10) oligomerization is important for its in vivo activity

Gabriele S.V. Campanella, Jan Grimm, Benjamin Medoff, Richard Colvin, Ralph Weissleder, Andrew D. Luster, Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, 02114.

The chemokine IP-10 (CXCL10) plays an important role in the recruitment of activated T-cells into sites of inflammation by interacting with the G-protein coupled receptor CXCR3. IP-10, like other chemokines, forms oligomers, the role of which has not yet been explored. In this study, we used a mutant of IP-10 that is an obligate monomer to elucidate the significance of oligomerization for in vitro and in vivo functions.

Monomeric IP-10 had a slightly reduced binding affinity for CXCR3 as well as for heparin, but was able to induce chemotaxis of activated T-cells in vitro and CXCR3 internalization comparable to wildtype IP-10. However, monomeric IP-10 was unable to induce in vivo recruitment of activated CD8 T-cells into the airways of mice following intratracheal instillation. Molecular imaging of mice following intratracheal instillation of indium labeled wildtype and monomeric IP-10 was used to investigate the biodistribution and local retention of monomeric and wildtype IP-10. Additional IP-10 mutants were used to distinguish between the role of glycosaminoglycan binding and oligomerization for its in vivo function. This study will discuss possible mechanisms by which oligomerization of IP-10 is crucial for its in vivo biological functions.

This work was supported by NIH grant RO1-CA69212 to ADL

119 Identification and characterization of novel viral chemokines.

J. Catusse, D.R. Dewin and U.A. Gompels, Department of Infectious & Tropical Diseases, London School of Hygiene and Tropical Medicine, University of London, London, UK, WC1E 7HT.

Human herpesvirus 6 (HHV6) is a lymphotropic herpesvirus highly prevalent in the human population. It has been linked with several inflammatory pathologies including encephalitis and multiple sclerosis. HHV-6 consists of two main variant strain groups. It encodes a chemokine (U83) which is one of the few hypervariable genes between strains. U83 from one laboratory adapted strain has been recently shown to be a low potency highly selective agonist, mediating calcium release and chemotaxis, for human CCR2. Here we investigate effects of strain variation via expression, purification and functional assays. Reactivity with human betachemokine receptors were investigated in transient expression systems as well as on primary leukocyte cell types relevant for the infection etiology. Using radiolabelled chemokines, high affinity interactions with human receptors in transfected cells were demonstrated. This was further investigated using different functional assays. The viral chemokine variants induced responses in calcium release experiments, blocked calcium responses induced by endogenous ligands, and mediated chemotaxis with variable affinities comparable to endogenous ligands. The unique regulation of activation of these receptors allows the virus to interfere with the activity of a broad range of immune cells including macrophages, T-lymphocyte and immature dendritic cells aiding both virus dissemination as well as immune evasion. Implications for cellular tropisms and pathologies are discussed as well as applications for immunomodulation.

Grant support from the Royal Society and Biological and Biochemical Sciences Research Council (UK) are acknowledged.

120 Induction of the Homeostatic CCL21 Chemokine by Fever-Range Thermal Stress

Qing Chen, Kristen Clancy, and Sharon Evans. Roswell Park Cancer Institute, Buffalo, NY 14263 USA

Fever is associated with increased survival during severe inflammatory reactions although the beneficial contribution of the febrile response is poorly understood. Previous studies have shown that fever-range thermal stress promotes lymphocyte trafficking to secondary lymphoid organs by independently regulating adhesion in lymphocytes and specialized high endothelial venules (HEV). The current study examined thermal regulation of the molecular events that support secondary firm adhesion in HEV. Intravascular staining revealed that exposure of BALB/c and C57/BL6 mice to temperatures that simulate the thermal component of fever (6 h at 39.5-40°C) markedly induces the luminal display of the homeostatic chemokine, CCL21, in HEV of peripheral lymph nodes and Peyer's patches. Enhanced expression of ICAM-1 was also detected in HEV upon thermal treatment whereas no change was observed in the density of other endothelial adhesion molecules including PNAd, MAdCAM-1, ICAM-2, JAM-1, JAM-2 or CD31. Fever-range temperature caused ~ a two-fold increase in lymphocyte trafficking across HEV in secondary lymphoid organs. Improved homing was determined to be dependent on CCL21 based on functional blockade of trafficking by (a) disruption of G-protein signaling using the pharmacologic inhibitor, pertussis toxin, (b) CCR7 desensitization by treatment of lymphocytes with high concentrations of CCL21 *in vitro*, and (c) and application of CCL21 neutralizing monoclonal antibody (mAb) *in vivo*. Function-blocking mAb established that enhanced lymphocyte trafficking under hyperthermic conditions depends on LFA-1 engagement by ICAM-1, downstream of CCL21 activation. In contrast, ICAM-2 blockade did not prevent mobilization of lymphocytes to HEV-bearing organs in response to thermal stress. Moreover, ICAM-2 cannot substitute as a binding partner for LFA-1 in ICAM-1-deficient mice despite the high CCL21 expression induced thermal stress. These data suggest that a physiologically important consequence of fever is to promote immune surveillance by enhancing intravascular availability of CCL21 and ICAM-1 during lymphocyte extravasation across HEV. (Supported by NIH grants CA79765 and CA094045 and DOD grant W81XWH-04-1-0354)

Society for Thermal Medicine



2006 Annual Meeting Scientific Program and Abstracts

April 6–8, 2006
Bethesda, Maryland

1:15 pm – 2:00 pm New Investigator Award Presentations

1:15 pm – 1:30 pm Fever-Range Thermal Therapy Promotes Lymphocyte Trafficking Through an IL-6 Trans-Signaling Mechanism

Author:

Qing Chen, Kristen Clancy, Wan-Chao Wang, Daniel Fisher, Emily Unger, Jessica Passanese, Heinz Baumann and Sharon S. Evans
Roswell Park Cancer Institute, Buffalo, NY, USA

Abstract:

The tumor microvasculature is a barrier to immunotherapy because of its failure to express adhesion molecules and chemokines necessary for recruitment of tumor-reactive lymphocytes. These observations provide one explanation for immune evasion by tumor cells. To investigate the role of thermal therapy in regulating lymphocyte trafficking, we employed high endothelial venules (HEV) of lymphoid organs as a model system since the adhesion mechanisms governing extravasation are well defined. Extravasation involves a multi-step sequence of events including: 1) primary tethering and rolling of lymphocytes along vessel walls; 2) chemokine activation; 3) secondary firm sticking; and 4) transendothelial migration. Fever-range whole body hyperthermia treatment (6 h; 39.5-40°C) in mice increased trafficking of naïve and central memory CD4 and CD8 T lymphocytes exclusively across HEV. Intravital microscopy demonstrated that heat treatment increased the frequency of sticking interactions between lymphocytes and HEV without affecting initial tethering or rolling events. Consistent with these observations, thermal stress enhanced intravascular display of two key molecules that support firm adhesion and/or transendothelial migration, i.e., the homeostatic chemokine CCL21 and intercellular adhesion molecule-1 (ICAM-1). Moreover, enhanced lymphocyte trafficking under hyperthermic conditions depended on CCL21 and ICAM-1. Conversely, hyperthermia did not alter HEV expression of PNAd, MAdCAM-1, ICAM-2, JAM-1/2, VCAM-1, or CD31. Finally, a bifurcation in the mechanisms controlling HEV adhesion was revealed by evidence that thermal induction of ICAM-1, but not CCL21, involved a trans-signaling pathway initiated by engagement of the gp130 molecule by interleukin-6 (IL-6) and a soluble form of the IL-6 receptor. These results highlight novel mechanisms by which lymphocyte trafficking can be amplified during physiologic febrile responses and clinical thermal therapy. Taken together, these findings suggest that clinical thermal therapy may be an effective strategy to promote recruitment of immune effector cells to the tumor microenvironment. (Supported by NIH grants [CA79765, CA094045] and DOD grant W81XWH-04-1-0354

1:30 pm – 1:45 pm Hyperthermic Regulation of HIF-1α in Macrophages

Author:

Isabel L. Jackson, Mark W. Dewhirst, Zeljko Vujaskovic
Dept. Radiation Oncology, Duke University Medical Center, Durham, NC, USA

Abstract:

Macrophages play an important role in tumor angiogenesis by generating reactive oxygen species (ROS), VEGF and other tumor promoting factors. Newly formed vessels are generally immature and non-functional resulting in tumor hypoxia, which is associated with treatment resistance and more aggressive tumor phenotype. Under hypoxia, transcriptional regulation of VEGF is controlled primarily by HIF-1α. Recent studies have demonstrated HIF-1α stabilization and transcriptional activity can be modulated by ROS signaling. Hyperthermia (HT) is known to



Program and Abstracts

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FEVER-RANGE THERMAL STRESS PROMOTES LYMPHOCYTE TRAFFICKING ACROSS HIGH ENDOTHELIAL VENULES THROUGH AN INTERLEUKIN-6 TRANS-SIGNALING MECHANISM

Qing Chen[1], Daniel T. Fisher[1], Kristen A. Clancy[1], Jean-Marc M. Gauguet[2], Wan-Chao Wang[1], Emily Unger[1], Stefan Rose-John[3], Ulrich H. von Andrian[2], Heinz Baumann[4] & Sharon S. Evans[1]

[1]Departments of Immunology and [4]Molecular and Cellular Biology, Roswell Park Cancer Institute, Carlton & Elm Streets, Buffalo, New York 14263, USA; [2] The CBR Institute for Biomedical Research and Department of Pathology, Harvard Medical School, Boston, Massachusetts, 02115, USA; [3]Department of Biochemistry, Christian Albrechts University Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany

Fever is an evolutionarily conserved response during acute inflammation although the physiological benefit is poorly understood. Previous studies have shown that fever-range thermal stress increases L-selectin and α4β7 integrin-dependent tethering and rolling events in specialized high endothelial venules (HEV), thereby promoting trafficking to secondary lymphoid organs. Here we examined the effect of fever-range thermal stress on the mechanisms controlling firm adhesion in HEV. Fever-range whole body hyperthermia treatment (6 h; 39.5-40 °C) in mice increased recruitment of naïve and central memory CD4 and CD8 T lymphocytes exclusively across HEV. Intravital microscopy demonstrated that heat treatment increased the frequency of sticking interactions between lymphocytes and HEV without affecting intial tethering or rolling events. Consistent with these observations, thermal stress enhanced intravascular display of two key molecules that support firm adhesion and/or transendothelial migration, *i.e.*, the homeostatic chemokine CCL21 and intercellular adhesion molecule-1 (ICAM-1). Enhanced lymphocyte trafficking under hyperthermic conditions was further proved to be dependent on CCL21 and ICAM-1. Conversely, hyperthermia did not alter HEV expression of PNAd, MAdCAM-1, ICAM-2, JAM-1/2, VCAM-1, or CD31. Moreover, thermal stress did not amplify lymphocyte homing or ICAM-1 expression in extralymphoid tissues (*e.g.*, liver, pancreas). A bifurcation in the mechanisms controlling HEV adhesion was revealed by evidence that thermal induction of ICAM-1, but not CCL21, involved a trans-signaling pathway initiated by engagement of the gp130 molecule by interleukin-6 (IL-6) and a soluble form of the IL-6 receptor. These findings, together with evidence that IL-6 trans-signaling also mediates thermal effects on L-selectin adhesion, identify a highly integrated cytokine signaling network whereby febrile temperatures stimulate site-specific lymphocyte homing and immune surveillance during inflammation. (Supported by NIH grants [CA79765, CA094045] and DOD grant W81XWH-04-1-0354)

9th Annual Upstate New York Immunology Conference

October 15-18, 2006

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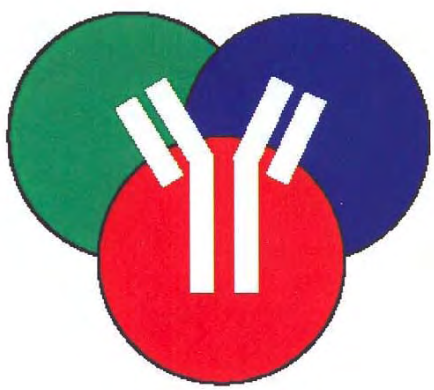
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Mechanisms Linking Inflammation and Cancer

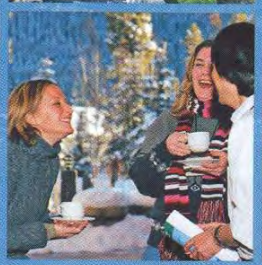
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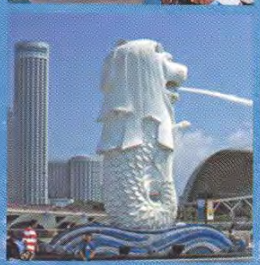


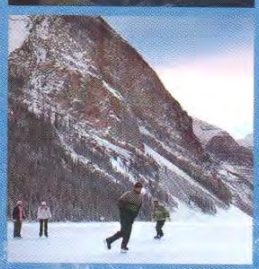






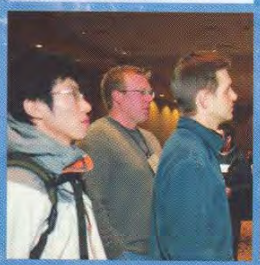


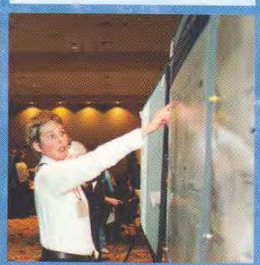






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117 **A novel anti-cancer effect of thalidomide: inhibition of ICAM-1-mediated cell invasion and metastasis through suppression of nuclear factor κB**

Yi-Chu Lin, Chia-Tung Shun, Ming-Shiang Wu and Ching-Chow Chen
Department of Pharmacology, College of Medicine, National Taiwan University, Taipei 10018, Taiwan

Purpose: Thalidomide has been reported to have anti-angiogenic and anti-metastatic effects. Intercellular adhesion molecule-1 (ICAM-1) was demonstrated to be involved in monocyte adherence to epithelial cells and cancer cell invasion. In this study, we further investigated the role of ICAM-1 in tumorigenesis including tumor formation and metastasis. ICAM-1 as a molecular target for cancer and the anti-cancer effect of thalidomide were investigated.

Experimental Design: Expression of ICAM-1 protein in human lung cancer specimens was assessed by immunohistochemistry. ICAM-1 overexpressing A549 cells (A549/ICAM-1) were established to investigate the direct effect of ICAM-1 on *in vitro* cell invasion and *in vivo* tumor metastasis. Transient transfection and luciferase assay, electrophoretic mobility shift assay and chromatin immunoprecipitation were performed to assess the activity and binding of NF-κB to the ICAM-1 promoter. A xenograft model in nude mice was conducted to evaluate the anti-cancer effect of thalidomide.

Results: High expression of ICAM-1 in human lung cancer specimens was correlated with a greater risk of advanced cancers (stage III and IV). A549/ICAM-1 cells were demonstrated to induce *in vitro* cell invasion and *in vivo* tumor metastasis. Anti-ICAM-1 antibody and thalidomide had inhibitory effect on these events. Thalidomide also suppressed TNF-α-induced ICAM-1 expression through inhibition of NF-κB binding to the ICAM-1 promoter. The *in vivo* xenograft model showed the effectiveness of thalidomide on tumor formation.

Conclusion: These studies provide a framework for targeting ICAM-1 as a biologically based therapy for cancer, and thalidomide might be effective in human lung caner.

This work was supported by a research grant from the National Science Council of Taiwan, NSC 94-2320-B002-098.

118 **Role of Interleukin-6 Trans-Signaling in Overcoming Hurdles to T Lymphocyte Trafficking in the Tumor Microenvironment**

Qing Chen[1], Lei Zhou[1], Daniel T. Fisher[1], Kristen A. Clancy[1], Jessica Passanese[1], Wan-Chao Wang[1], Heinz Baumann[2], Elizabeth A. Repasky[1], and Sharon S. Evans[1]. [1]Departments of Immunology and [2]Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY 14263 USA

Successful cancer immunotherapy based on vaccines or adoptive T cell transfer depends on the ability of cytolytic effector T lymphocytes to traffic to the tumor microenvironment. The mechanisms limiting the rate of entry of blood-borne $CD8^+$ effector T cells across the vascular endothelial cell barrier at tumor sites are poorly understood. Here, we report on the role of a hallmark vascular gatekeeper, intercellular adhesion molecule-1 (ICAM-1), in governing T cell trafficking at three distinct sites: (1) specialized high endothelial venules (HEV) of secondary lymphoid organs (lymph nodes, Peyer patches); (2) non-activated endothelium of normal extralymphoid tissues; and (3) tumor vessels of spontaneous and transplantable murine tumors. ICAM-1 dependent firm adhesion and extravasation of T cells occurred at high frequency in HEV of lymphoid organs which are the major portals for entry of naive and central memory lymphocytes during routine immune surveillance. Conversely, tumor vessels and normal vessels of non-inflamed extralymphoid organs expressed low levels of ICAM-1, consistent with observations that these vessels did not efficiently support $CD8^+$ T cell interactions or extravasation. Comparative analysis of vascular responses to systemic thermal therapy (core temperature elevated to 39.5-40 °C for 2-6 h) revealed that selected tissue microenvironments predispose vessels to respond to stress cues. In this regard, systemic thermal therapy strongly upregulated the intravascular density of ICAM-1 on tumor vessels and HEV, causing a 5-fold and 2-fold increase in ICAM-1 dependent homing of $CD8^+$ T cells at these respective vascular sites. In sharp contrast, normal vessels of extralymphoid organs (heart, kidney, liver, pancreas) were not responsive to thermal therapy as indicated by a failure to increase ICAM-1 intravascular display or improve ICAM-1-dependent $CD8^+$ T cell trafficking. Our studies further identified a non-redundant role for the inflammatory cytokine, interleukin-6 (IL-6), in promoting ICAM-1-dependent trafficking of $CD8^+$ T cells in response to thermal stress. Regionalized control of vascular ICAM-1 expression in lymphoid organ HEV or tumor vessels involved an IL-6 trans-signaling mechanism whereby IL-6 and a soluble form of the IL-6 receptor engaged membrane-anchored gp130 transduction molecules. These findings provide the first evidence for an IL-6 trans-signaling mechanism that improves ICAM-1 dependent trafficking of $CD8^+$ T cells through vascular checkpoints in lymphoid organs or tumor tissues. The results further demonstrate the feasibility of therapeutically exploiting IL-6 trans-signaling responses within the tumor microenvironment to mobilize $CD8^+$ T recruitment to malignant lesions. This work was supported by NIH grants CA79765, CA85580 and CA094045 and DOD grant W81XWH-04-1-0354.

119 **Upregulation of the antimicrobial, pro-inflammatory peptide LL-37 in ovarian tumors potentiates cancer progression through recruitment of stem cell populations**

Seth B. Coffelt, Ruth S. Waterman, Luisa Florez, Kerstin Höner zu Bentrup, Kevin J. Zwezdaryk, Suzanne L. Tomchuck, Elizabeth S. Danka, and Aline B. Scandurro
Department of Microbiology & Immunology
Tulane University Health Sciences Center,
New Orleans, LA, USA, 70112

Human cationic peptide 18 (hCAP-18) secretion is increased at sites of tissue damage and inflammation by epithelial and immune cells. Once secreted, hCAP-18 is enzymatically cleaved releasing its bioactive, C-terminal peptide, termed LL-37 that functions as a chemoattractant, mitogen, and pro-angiogenic factor. Given LL-37's established role as an effector of tissue repair and immune responses, it was hypothesized that over-expression of hCAP-18/LL-37 in ovarian cancers leads to promotion of tumor survival and recruitment of mesenchymal stem cells (MSC). Tumor tissue lysate and biopsy arrays revealed the over-expression of hCAP-18/LL-37 in ovarian tumors when compared to normal ovary. Ovarian cancer cell lines expressed both hCAP-18 and LL-37's receptor, FPRL-1. LL-37 treatment of ovarian cancer cell lines resulted in increased proliferation, migration, invasion, and MMP secretion. Stimulation of MSC by LL-37 induced the secretion of several angiogenic and inflammatory cytokines, chemotaxis, invasion, and differentiation of these cells. Moreover, treatment of ovarian cancer xenografts with a neutralizing LL-37 antibody reduced the proliferation rate of ovarian tumors and inhibited MSC recruitment. These data suggest that the antimicrobial peptide LL-37 is a critical mediator of tumorigenesis in the ovary.

120 **Inflammatory cell infiltrate and tumor immune tolerance in the BK5.COX-2 mouse model of pancreatitis and pancreatic tumorigenesis**

J.K.L. Colby[1], R.D. Klein[2], K. Kiguchi[1], P. Riggs[1], M.J. McArthur[3], J. Rundhaug[1], A. Pavone[1], and S.M. Fischer[1], [1]University of Texas M.D. Anderson Cancer Center Science Park, Research Division, Smithville, TX, [2]The Ohio State University, Columbus Ohio, [3]University of Texas M.D. Anderson Cancer Center Science Park Veterinary Division, Bastrop, TX

The significance of tumor-infiltrating immune cells is a complex and controversial topic; data suggest beneficial and detrimental roles, depending upon tissue/tumor type as well as numbers and behavior of specific immunocytes. Altered immune function is one of many events associated with prostaglandin (PG) signaling in inflammation-driven tumorigenesis. The BK5.COX-2 mouse is a model in which PGE2 overexpression leads to chronic pancreatitis that ultimately progresses to pancreatic adenocarcinoma. In pre-tumor transgenic pancreata, PGE2 levels were elevated ~15 fold vs. normal wild-type FVB pancreata; levels in transgenic tumors were elevated ~120 fold compared to wild-type. We determined that PGE2 produced by transgene-positive cells stimulates autocrine/paracrine expression of endogenous COX-2, initiating a persistent, proinflammatory environment in the tissue. Sequelae include acinar-ductal transdifferentiation, proliferation, activation of stellate cells and deposition of stroma, and chemoattraction of inflammatory cells. Inflammatory cells appearing early in the process include granulocytes and macrophages, which persist as lesions progress; infiltration of large numbers of B and T lymphocytes occur in more advanced lesions and tumors, often forming distinct "lymphoid" structures. Of interest is a subset of T cells positive for Foxp3, a marker for regulatory T cells (Tregs), which are critical for immunologic self-tolerance. We believe that Tregs represent one level of suppression of antitumor responses mediated by PGE2 signaling. Elevated mRNAs for a number of factors (e.g., IL-18, granzyme B, perforin, Fas ligand) measured via a Taqman® Mouse Immune Panel add further support to our hypothesis that PGE2 promotes a tumor immune tolerant microenvironment in the pancreata of transgenic mice. Supported by T32 ES07247 from NIEHS and CA100140 from NIH.

Duke University Medical School

Department of Radiation Oncology

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Mark W. Dewhirst, DVM, PhD

February 20th, 2004

Director of Tumor Microcirculation Laboratory

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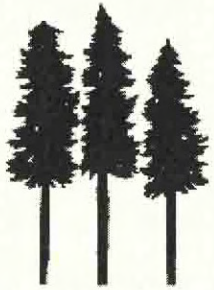
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