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PRINCIPAL INVESTIGATOR: Qing Chen, M.D.

CONTRACTING ORGANIZATION: Roswell Park Cancer Institute
Buffalo, New York 14263

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Qing Chen, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Roswell Park Cancer Institute
Buffalo, New York 14263

E-Mail: Qing.chen@roswellpark.org

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13. ABSTRACT (Maximum 200 Words)
A major challenge is to develop approaches to target delivery of tumor-specific immune effector cells to tumor tissues. Immune cells are frequently excluded from the intratumoral region of primary tumors including breast cancer. Thus, these cells cannot initiate contact-dependent lysis of tumor targets. We have reported that poor infiltration of tumor tissues in murine models correlates with limited expression of critical gatekeeper adhesion molecules (e.g., intercellular adhesion molecule-1, ICAM-1) which control egress of blood-borne lymphocytes into tissues. Our studies demonstrate that fever-range thermal therapy upregulates ICAM-1 expression on intratumoral vessels in transplantable murine breast tumors and other tumor models. ICAM-1 upregulation occurs principally on $CD31^+$ vessels of tumor and lymphoid tissues, but not in extralymphoid organs. Thermal induction of ICAM-1 correlates with enhanced $CD8^+$ T cell adhesion, homing and infiltration in tumor tissues. Neutralization of selected inflammatory cytokines (IL-6, but not TNF-α or IL-1β) suppresses thermal induction of ICAM-1 on vessels. 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 overcome the microvascular barrier to tumor immunity through stimulation of heightened trafficking of lymphocyte subsets to tumor sites.

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Fever-range thermal stress, tumor vasculature, ICAM-1, lymphocyte trafficking

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Table of Contents

INTRODCUTION

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. Moreover, major impediment to successful immunotherapies is the failure of the appropriate immune cells to gain access to tumors. Thus, 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 stress in controlling lymphocyte trafficking. We postulate that stimulation of lymphocyte-endothelial adhesion 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 the grant investigate the testable hypothesis that IL-6 mediates thermal activation of lymphocyte adhesion in the complex microenvironment of breast cancer tissues. 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-α, IL-1β). A major barrier to anti-tumor immunity is the failure of tumor-reactive $CD8^+$ cytolytic lymphocytes to gain access to tumor tissues. This is associated with a poor prognosis in cancer patients. In studies published during the past year we have shown that poor infiltration of tumor tissues in murine models correlates with limited expression of critical gatekeeper adhesion molecules such as ICAM-1 which are known to control egress of blood-borne lymphocytes into tissues (1-3).

In follow-up studies we have determined that elevating the body temperature of EMT6 breast cancer-bearing mice to the range of naturally occurring fever (39.5-40°C) markedly upregulates expression of ICAM-1 in tumor tissues (4, 5). Equivalent ICAM-1 induction on tumor vessels was observed in other murine tumor models including RIP-Tag5 transgenic pancreatic tumors, CT26-tumor, and B16 melanoma (4, 5). Two approaches were taken to establish that ICAM-1 induction occurs on tumor vessels, and not on tumor cells or stromal cells within the tumor microenvironment. Two-color immunofluorescence confocal microscopy demonstrated that fever-range whole body hyperthermia (WBH) induced ICAM-1 expression exclusively on $CD31^+$ vessels within tumor tissues. 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 luminal surface of tumor tissues (i.e., at the site necessary to initiate firm adhesion of lymphocytes to vessel walls). Marked ICAM-1 induction was also observed on HEV of lymphoid organs (lymph nodes, Peyer's patches) whereas no induction of ICAM-1 expression was detected in non-malignant extralymphoid organs (pancreas, liver, lung, heart, kidneys) (4-6).

An important finding is that thermally regulated ICAM-1 is at a sufficient density to support improved lymphocyte-endothelial adhesion (detected in both *in vitro* and *in vivo* assays) and site-specific homing *in vivo* (4-6). Using neutralizing antibody approaches outlined in the application, we demonstrated that lymphocyte-endothelial adhesion and homing was dependent on LFA-1/ICAM-1 interactions. Notably, $CD8^+$ T cells (i.e., murine TK1 cell line) show improved homing to lymphoid organs and tumor tissues following adoptive transfer into mice pretreated with fever-range thermal therapy. Immunohistochemical analysis further established that

fever-range thermal stress causes a transient increase in infiltration of $CD8^+$ T cells in intratumoral regions that temporally correlates with upregulation of ICAM-1 expression and vascular adhesion. Furthermore, we have shown that fever-range thermal stress also promotes ICAM-1/E-selectin-dependent adhesion of neutrophils to tumor tissues, supporting the hypothesis proposed in the grant that therapeutic application of thermal stress increases access of both innate and adaptive immune effector cells to the tumor microenvironment. These results are currently being confirmed by phenotypic analysis of tumor infiltrating cells by flow cytometry.

Studies have been initiated to analyze the effect of thermal stress on ICAM-1-mediated lymphocyte adhesion to tumor vessels by intravital microscopy using techniques described in the grant. To acquire expertise in this specialized technology, Dr. Evans (mentor) and Qing Chen (predoctoral fellow) initiated collaborative studies in Dr. U. von Andrian's laboratory (Harvard; from 3/28 – 4/10, 2004). Dr. von Andrian is a renowned expert in the area of leukocyte trafficking and intravital microscopy. Qing Chen also received training in intravital microscopic imaging of tumors grown in dorsal skin-flap window chambers at Dr. M. Dewhirst's laboratory (Duke; Feb. 2004). During Year 1, we have reported our results regarding blood flow in tumor vessels and baseline lymphocyte-endothelial interactions under normothermal conditions (1). These studies provide the foundation for the series of studies, proposed in the grant, to investigate central questions relating to the complex mechanisms governing leukocyte trafficking in tumor microenvironments.

We have performed studies proposed in the grant to investigate the role of IL-6 in stimulating the expression of homing molecules in tumor microvessels. For these studies, tumor bearing mice were injected intravenously with neutralizing antibodies specific for IL-6, TNF-α or IL-1β, prior to initiation of fever-range WBH treatment. Neutralization of IL-6, but not TNF-α or IL-1β, fully blocked thermal induction of ICAM-1 expression on tumor vessels as well as thermal enhancement of lymphocyte-endothelial adhesion in frozen-section adherence assays (4, 5). Notably, *IL-6 neutralizing mAb fully prevented thermal stimulation of $CD8^+$ T cell infiltration in tumor tissues*. Thermal enhancement of lymphocyte homing to HEV-bearing organs (lymph nodes, Peyer's patches) was also shown to be dependent on IL-6 while low-level trafficking of lymphocytes to extralymphoid organs was not affected by neutralization of IL-6, TNF-α or IL-1β. To discriminate between the involvement of a membrane (m) versus soluble (s) form of the IL-6 receptor (sIL-6R) binding subunit in thermal responses, mice were injected with a recombinant form of soluble gp130. Our recent published studies established that soluble gp130 functions as a competitive inhibitor of IL-6/sIL-6R signaling *in vitro* and *in vivo*. Blockade of IL-6/sIL-6R trans-signaling effectively prevents thermal induction of ICAM-1 expression, lymphocyte-endothelial adhesion, and homing to tumor tissues or selected lymphoid organs (4-6). These results provide direct evidence that the gp130 signal transducing chain is a major regulator of tumor microvascular adhesion in response to fever-range thermal stress.

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 fever-range thermal effect on intratumoral vessels. Studies are planned to detect local changes in IL-6 levels in tumor tissues after heat treatment according to our schedule described in the grant.

KEY RESEARCH ACCOMPLISHMENTS

- These studies demonstrate for the first time that fever-range thermal stress induces the ICAM-1 expression on tumor vessels.
- Fever-range thermal induction of adhesion molecules occurs selectively in intratumoral vessels and HEV in lymphoid organs.
- Fever-range thermal stress increases adhesion, homing and infiltration of CD8+ lymphocytes in tumors and HEV-bearing lymphoid tissues.
- Fever-range thermal stimulation of adhesion in intratumoral vessels is dependent on IL-6/sIL-6Rα trans-signaling mechanism.

REPORTABLE OUTCOMES

Papers:

1. Appenheimer, M.M., **Chen Q.**, Girard, R.A., Wang, W.-C., and Evans, S.S. Impact of fever-range thermal stress on lymphocyte-endothelial adhesion and lymphocyte trafficking. *Immunol. Invest. (In press).*
2. **Chen, Q.,** Fisher, D. T., Kucinska, S. A., Wang, W. C., Evans, S. S. Dynamic control of lymphocyte trafficking by fever-range thermal stress. *Cancer Immunol. Immunoth. (In press).*
3. **Chen, Q.**, Clancy, K., Wang, W.C., and Evans, S.S. High endothelial venules: master regulators of lymphocyte trafficking and targets of fever-range thermal stress. In: *Endothelial Biomedicine*; William Aird, editor; Cambridge University Press *(In press).*
4. Zhou, L., Fisher, D., **Chen, Q.,** Wang, W.C.,and Evans, S.S. IL-6 trans-signaling and leukocyte trafficking: balance between health and disease. *Arch. Immunol. Therap. Exper. (In preparation, invited review)*
5. **Chen, Q.,** Clancy, K., Unger, E., Passanese, J., Appenheimer, M., Fisher, D., Wang, W. C., Baumann, H., and Evans, S.S. Fever-range thermal stress induces ICAM-1 expression on high endothelial venules (HEV) via an IL-6 trans-signaling mechanism *(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.
2. **Chen, Q.**, Passanese, J., Clancy, K., Kucinska, S., Green, C., Wang, W.C., Dewhirst, M., Hanahan, D., Repasky, E., Baumann, H., and Evans, S.S. Fever-range thermal stress controls vascular endothelial display of ICAM-1 via an IL-6/soluble IL-6 receptor trans-signaling mechanism. Immunology Department Retreat, Roswell Park Cancer Institute, September, 2004, Buffalo, NY. *(Oral presentation)*
3. **Chen, Q.**, Passanese, J., Clancy, K., Kucinska, S., Green, C., Wang, W.C., Dewhirst, M., Hanahan, D., Repasky, E., Baumann, H., and Evans, S.S. Fever-range thermal stress controls vascular endothelial

display of ICAM-1 via an IL-6/soluble IL-6 receptor trans-signaling mechanism. 4th Annual Buffalo Conference on Immunology, October 7-8, 2004, Buffalo, NY.

4. **Chen, Q.,** Unger, E., Passanese, J., Clancy, K., Appenheimer, M., Fisher, D., Wang, W. C., Baumann, H., and Evans, S.S. fever-range thermal stress controls HEV display of ICAM-1 via an IL-6 trans-signaling mechanism. Keystone Symposium, Leukocyte Trafficking: Cellular and Molecular Mechanisms, March 1 - 6, 2005, Taos, New Mexico.
5. **Chen, Q.**, Passanese, J., Fisher, D., Kucinska, S., Clancy, K., Wang, W.-C., Appenheimer, M., Zhou, L., Repasky, E., Baumann, H., Evans, S.S. Fever-range thermal stress controls vascular endothelial display of ICAM-1 via an IL-6/soluble IL-6 receptor trans-signaling mechanism. Meeting of the Society of Thermal Medicine, April 1, 2005, National Institutes of Health, Bethesda, MD. *(Oral presentation)*

Awards:

1. Travel award to attend Keystone Symposium, Leukocyte Trafficking: Cellular and Molecular Mechanisms, March 1 - 6, 2005, Taos, New Mexico.
2. Travel award to attend the Society for Thermal Medicine Annual Meeting 2005, March 30 – April 4, 2005, NIH, Bethesda, M.D.

Training:

1. 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.
2. Participated in collaborative studies involving intravital microscopy techniques in the laboratory of Dr. Ulrich von Andrian, Harvard University, Medical School, Boston, MA.

CONCLUSIONS

These studies provide the first evidence that fever-range thermal stress 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 lymphocyte subsets to tumor sites and tumor-draining lymph nodes.

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1. Chen, Q., Fisher, D. T., Kucinska, S. A., Wang, W. C., Evans, S. S. Dynamic control of lymphocyte trafficking by fever-range thermal stress. *Cancer Immunol. Immunoth. (In press).*
2. Appenheimer, M.M., Chen Q., Girard, R.A., Wang, W.-C., and Evans, S.S. Impact of fever-range thermal stress on lymphocyte-endothelial adhesion and lymphocyte trafficking. *Immunol. Invest. (In press).*

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APPENDICES

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Certificates:

1. Travel award to attend Keystone Symposium, Leukocyte Trafficking: Cellular and Molecular Mechanisms, March 1 - 6, 2005, Taos, New Mexico.
2. Training course of intravital microscopy techniques in the laboratory of Dr. Mark Dewhirst, Duke U. Medical School, Durham, NC.

Biographical Sketch:

Immunological Investigations (in press)

IMPACT OF FEVER-RANGE THERMAL STRESS ON LYMPHOCYTE-ENDOTHELIAL ADHESION AND LYMPHOCYTE TRAFFICKING

Michelle M. Appenheimer[1§], Qing Chen[1§], Rachael A. Girard[1], Wan-Chao Wang[1], and Sharon S. Evans[1*]

[§] These authors contributed equally to this work.

* To whom correspondence should be addressed: Roswell Park Cancer Institute, Buffalo, NY, 14263-0001; Email: sharon.evans@roswellpark.org; Phone: 716-845-3421.

[1]Department of Immunology, Roswell Park Cancer Institute.

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ABSTRACT

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.

1. OVERVIEW: 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 (1). 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 (1-3). Similarly, antipyretic therapy results in increased mortality during bacterial infections in mice and rabbits (4-6). Clinical data in humans have also demonstrated the

benefit of fever and risk of antipyresis in gram negative bacteremia (7), bacterial peritonitis (8), chicken pox (9), and rhinovirus infection (10). In contrast, some studies have shown harmful effects of fever in animal models during bacterial sepsis (3, 11-13). 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. (3, 14, 15). 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 (3, 16, 17).

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 hydrophilia*) 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 (18). This heat-seeking behavior and the survival benefit of elevated body temperature have also been observed in fish and even in insects (1, 2, 19-21). 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 (20, 22).

A powerful argument for the beneficial nature of fever is its presence throughout the subphylum 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 (1). 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 (c. 500 BCE) said, "Give me the power to induce fever and I will cure all diseases" (23), while Hippocrates routinely used heat treatment, including warm baths and burying patients in hot desert sands, to treat many diseases including cancer (24). 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% (25). 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 (23). 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 (23, 26-28).

1.1. 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 (1, 3, 29, 30) 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 (17, 31-33). Similar effects on neutrophil function have been observed in the desert iguana *D. dorsalis* (34). The bactericidal activity as well as FcR expression of macrophages is also reportedly enhanced by fever-range hyperthermia (35, 36). A recent series of studies demonstrate that febrile temperatures regulate migration of dendritic cells *in vivo* (37-40). 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 (41-44). Additionally, heat stimulates T cell helper function, resulting in enhanced antibody synthesis by murine B cells (45). Thermal stress enables some antibodies to irreversibly neutralize specific viruses (i.e., to have viricidal activity) (46, 47). 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) (43, 48-52), 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 and site 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, IFN-α, IFN-γ, RANTES, MCP-1) are observed in leukocytes or endothelial cells exposed to thermal stress *in vitro* (50, 53, 54). Similarly, fever-range temperatures have no appreciable effect on the circulating levels of cytokines (IFN-α, IL-6, TNF-α, IL-1β) when administered to mice *in vivo* (50, 55-57). 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 non-replicating inflammatory agonist), causes a transient increase in pro-inflammatory cytokine levels *in vitro* or *in vivo* (IL-6, TNF-α, IL-1β) (3, 11, 54, 57-59). Heat treatment has also been shown to augment the antiviral and antiproliferative activities of human and mouse interferons (IFN) (32, 41, 60-62).

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 (3, 63-65). Moreover, even moderately elevated temperatures (e.g., 1°C above physiologically normal temperatures) suppress NK cell function in vitro (32, 66) while high temperatures inhibit CTL responses (67).

2. 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 (68). 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.

2.1. 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 lumenal surface to support the continuous recruitment of circulating lymphocytes (69, 70). 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 (70-73).

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) (69-71, 73, 74). Tethering and rolling of naïve/central memory lymphocytes in MLN and PP is mediated by L-selectin and α4β7 integrin-dependent binding to distinct domains within mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (69-71, 73). Once the action of L-selectin or α4β7 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) (69-71, 73, 75). 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 (70, 71, 75-77).

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) (69-74, 78, 79). Through these mechanisms, activated/effector or memory lymphocyte subsets are recruited to sites of inflammation or injury in tertiary organs.

2.2. 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 (50, 80-82). 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 (1). 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 (1, 3, 32), we were able to identify a novel IL-6-dependent trans-signaling mechanism operative in controlling lymphocyte homing in response to thermal stimuli (50).

An experimental approach was used to raise the core temperature of mice to febrile levels using a whole body hyperthermia (WBH) protocol developed by Repasky and colleagues (28). Fever range WBH 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 (40, 50, 80, 82, 83). 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 (84).

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 fluorescent-labeled splenocytes introduced *i.v.* in mice pretreated with WBH for 6 hours (80, 82). 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 (50, 80-82). Moreover, the combined use of mAb-based adhesion-blocking strategies and lymphocyte cell lines expressing defined homing receptors (i.e., L-selectin or α4β7 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 X 10^6 lymphocytes traverse HEV per second in humans under normothermic conditions (69), 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 (80, 82). 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/ α4β7 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 (82).

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. (82). 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) (80-82). 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) (53). 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) (3, 53). 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 α4β7 integrin in lymphocytes (50, 53). 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* (82). 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 (1, 32), as well as the afferent lymph, which delivers factors that support the maintenance of HEV structures (69, 72, 73, 85, 86). 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 HEV occur in the absence of detectable changes in the cell surface density of the vascular addressins PNAd or MAdCAM-1 (82). 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 (50, 87-96). In support of this notion, fever-range thermal stress has been shown to augment actin polymerization in primary endothelial cell cultures *in vitro* (53). 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 (97-100).

2.3. 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 in Section 2.2). 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 (28, 50, 80-82, 96, 101). 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 (50, 81, 82). Moreover, thermal effects on lymphocyte adhesion are fully reversible, returning to baseline levels within 12 hours of

cessation of heat treatment (81). Two major lymphocyte homing receptors, L-selectin and α4β7 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 α4β7 integrin^{lo} LFA-1^{lo} (50, 87, 96, 102, 103); TK1 CD8^{+} T cells are α4β7 integrin^{hi}/L-selectin^{lo}) (50, 82, 96, 101, 102, 104). Notably, similar increases in adhesion are detected when lymphocytes experience heat *in vivo* (28, 50, 80, 82, 101). Thus, splenocytes isolated from WBH-treated (6 hr) mice showed marked increases in both L-selectin and α4β7 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 thermal regulation of lymphocyte adhesion is shown (Figure 1A and B) 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 1A)(50, 81, 96). Notably, the *in vitro* adherence assay (employing mouse LN tissues as substrate) is highly predictive of lymphocyte homing potential *in vivo* (50, 74, 82, 105-107). 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 (108) while sulfation-dependent functional determinants of L-selectin ligands (PNAd) of human and mouse HEV are

recognized by the same mAb (MECA-79) (74, 109, 110). 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 *in vitro* assays (81). 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 vascular endothelial counter-receptor MAdCAM-1, it suppresses α4β7 integrin-mediated binding to the extracellular matrix protein, fibronectin (82, 101). 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 (104, 111-113). 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 (50). 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 (114, 115). 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 (50, 80-82, 101, 116, 117). 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 chicken (*Gallus gallus*) which is 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 (116-118). Chicken-derived splenocytes were cultured for 6 hours under normothermic (40.8°C) or fever-range hyperthermic temperatures (42.7°C) (119, 120) 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 $\alpha4\beta7$ integrin). Since cross-reactive Ab reagents are not available for analysis of homing receptors in avian 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 (106, 121), 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 α4β7 integrin (50, 81, 96, 101). 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) (81). 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 α4β7 integrin-dependent adhesion in responder cells that are maintained at normothermal temperatures (50, 53, 81, 122). 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 (50, 96, 123, 124). The kinetics of L-selectin redistribution to the detergent-insoluble subcellular fraction are very rapid (1-5 seconds) (96), consistent with the time-frame reported for tethering and rolling of lymphocytes along HEV surfaces (70, 73). 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 (50, 96). 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 (50, 87, 88, 96, 102) 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 (50, 96).

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) (50, 53, 81, 96, 122). 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 (125), 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 (50). 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) (126), fully prevents stimulation of adhesion in response to direct heat or conditioned medium from heat-treated cells (50). 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 (50, 127). 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 (50, 126-130).

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

(50). 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) (131, 132), but not p38 or JNK (SB203580, SP600125) (133, 134), prevented thermal stimulation of L-selectin-cytoskeletal interactions andL-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 (126), thermal stress appears to increase the bioactivity or bioavailability of IL-6 without changing the detectable concentrations of IL-6, sIL-6Rα, or sgp130 (50). 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 (50). 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 (135-139). 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) (50, 126) 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.

3. 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 non-mammalian vertebrate species (i.e., human, mouse, and chicken), strongly suggest that this mechanism of amplifying lymphocyte trafficking contributes to host survival.

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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FIGURE LEGENDS

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 (50, 81, 82, 96). 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 $\pm$ 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 2. Fever range thermal stress stimulates human $CD3^+$ T cell binding to 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 (50, 114, 115, 140). 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 $\geq$ 3 independent experiments. Bar, 50 μm.

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 (50, 81, 82). 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 $\pm$ SE of triplicate counts and are representative of $\geq$ 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.

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

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Immunological Investigations (in press)

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Figure 1

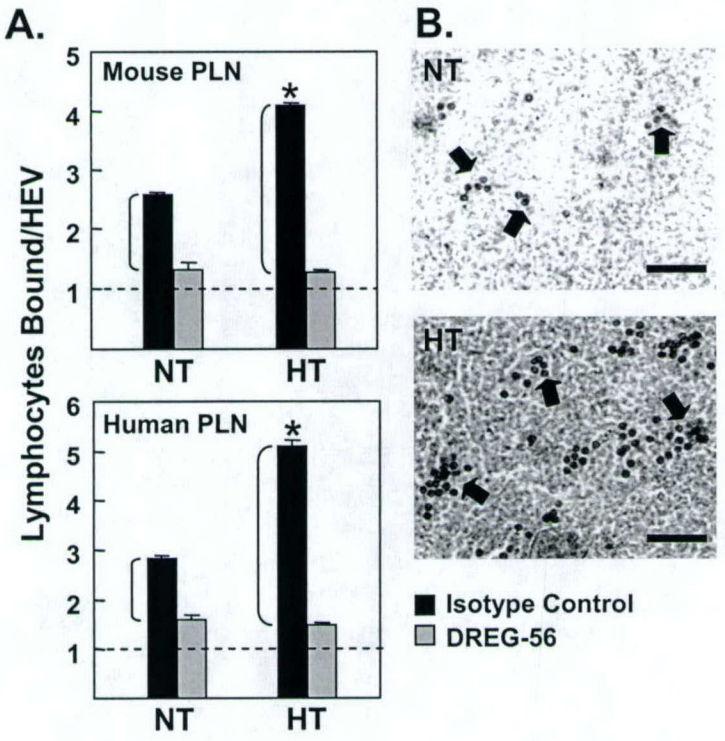

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Figure 2

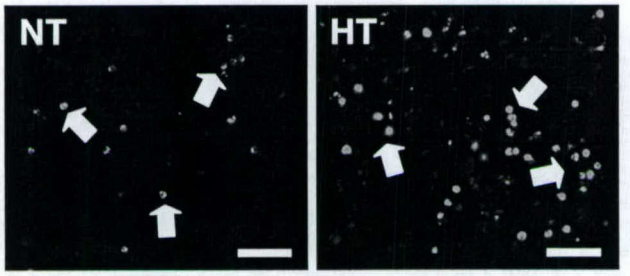

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Figure 3

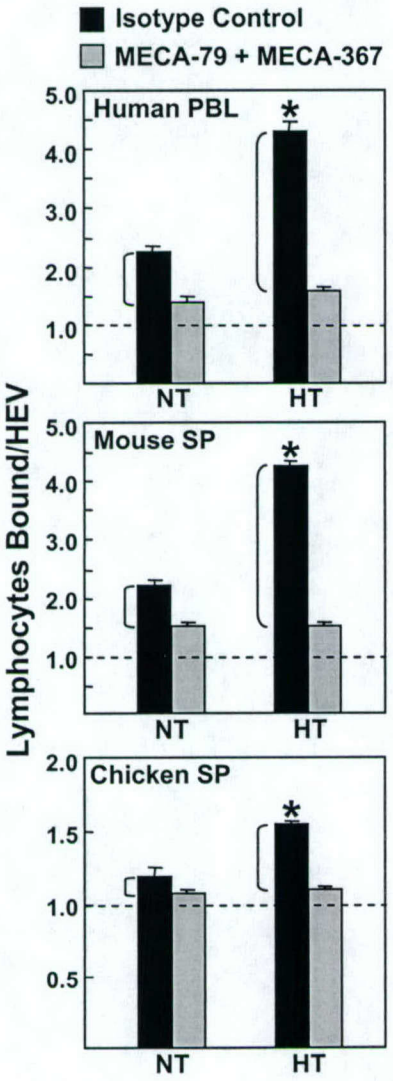

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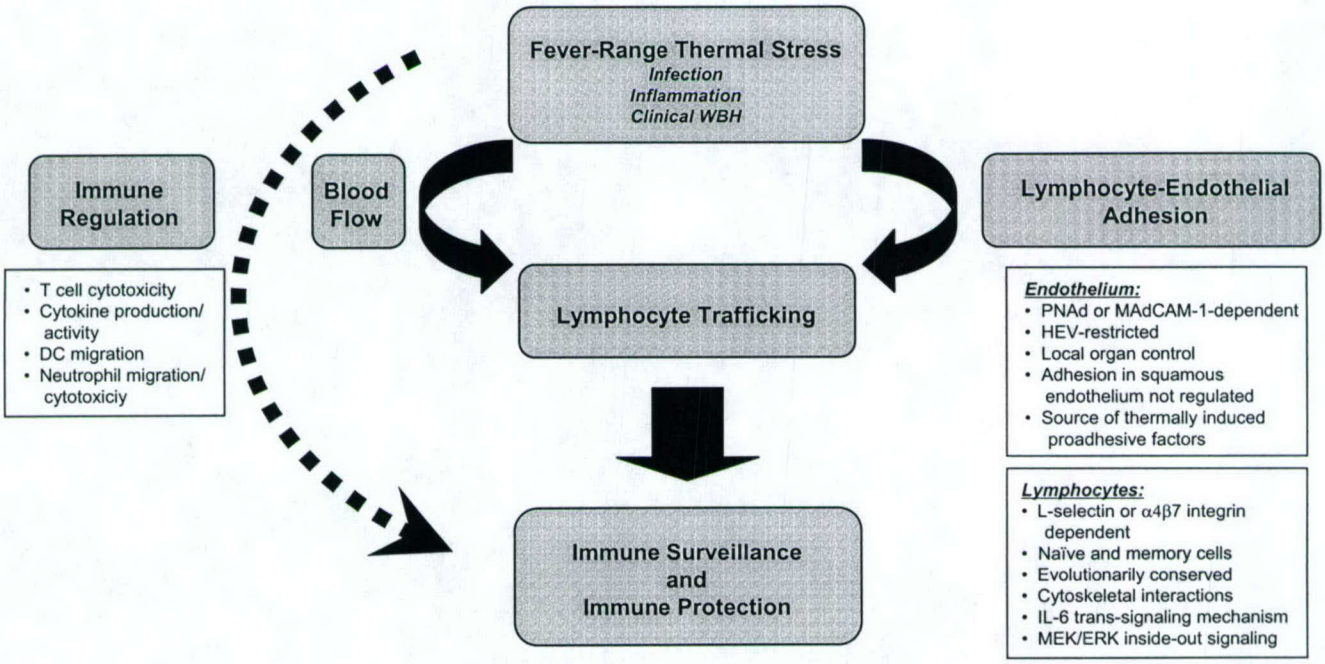
Figure 4



Cancer Immunology, Immunotherapy (in press)

DYNAMIC CONTROL OF LYMPHOCYTE TRAFFICKING BY FEVER-RANGE THERMAL STRESS

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

* To whom correspondence should be addressed: Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY, 14263-0001; Email: sharon.evans@roswellpark.org; Phone: 716-845-3421

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Keywords: lymphocyte homing receptors, adhesion molecules, high endothelial venules, tumor microvessels, fever

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.

High Endothelial Venules: A Locus of Control for Lymphocyte Extravasation

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 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] (Figure 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] (Figure 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/second [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/second [8] (Figure. 1B). The transition from rolling cells to firmly sticking adherent cells (experimentally defined as cells that arrest on vessel walls for ≥ 30 sec) 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 2 β-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] (Figure 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. 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] (Figure 2B) 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].

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, IP-10, RANTES) 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 Chron'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, 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 hours) 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 Figure 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 [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 lymphocyte homing receptor function is regulated by thermal stress in 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 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 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., Chron'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 hours whereas marked increases in HEV adhesion are detected after 6-8 hours. 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] (Figure 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. Based on the estimate that the rate of extravasation across HEV under normothermal conditions in humans is ~ 5 $X10^6$ per second [1], it is predicted that the 2-5 fold increase in trafficking observed in response to fever-range WBH (Figure 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.

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 suggests that regulatory T cells have a negative impact on anti-tumor immune responses in lymphoid organs and tumor tissues [59, 60]. One critical determinant to 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] (Figure 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 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 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 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 numerous vessels 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 (Figure 2). Moreover, there is limited intratumoral expression of hallmark adhesion molecules (ICAM-1, VCAM-1) or chemokines (MIG, IP-10) that 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-ODN) 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-ODN 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-ODN treatment has been shown to induce adhesion molecule expression on endothelial cells in the liver microenvironment and subsequent liver damage in an 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 antitumor response while simultaneously promoting inappropriate contact with normal bystander tissues.

An alternative strategy has been 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 is 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 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 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 extralymphoid 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 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 a 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 $\alpha4\beta7$ 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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Figure 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 (20X) in the same field following injection of fluorescent-labeled LN cells (~2.5 x 10^7 cells/ mouse; labeled with calcein [1 μg/ml, Molecular Probe, Eugene, OR]) 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 2 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 medium 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 Vedio1, available at http://www.....

Figure 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 high endothelial cells by rat anti-mouse PNAd primary mAb [BD Bioscience, San Diego, CA] 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] and FITC-conjugated mouse anti-goat secondary Ab [Jackson ImmunoResearch, West Grove, PA]).

Figure 3. Fever-range thermal stress activates lymphocyte adhesion to PLN HEV *in vitro*. Lymphocytes were isolated from spleen (SP) or LN organs (pooled PLN and MLN) of BALB/c mice and then cultured *in vitro* at 37°C or 40°C for 6 hours. Lymphocyte adherence to HEV in cryosections of BALB/c PLN was evaluated under mechanical shear as described [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., Webser, NY) in a total of 300-500 HEV per PLN cryosection. For consistency in double-blind evaluation, HEV were quantified only if they contained $\geq$ 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]). Data are the mean $\pm$ SE of triplicate samples in two experiments. Results are representative of $\geq$ 3 experiments. The differences between adhesion of untreated cells and hyperthermia-treated cells were significant, * $p < 0.0001$, using an unpaired two-tailed Student *t*-test.

Figure 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 hours) and allowed to resume normothermal temperatures, as described [38, 41]. After 1 hour, 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 $\pm$ SE (n=2 mice per group; data are representative of 4 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 *t*-test.

Figure 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 [92, 93]. In brief, a 12-mm-diameter hole was dissected through one layer of dorsal skin-fold to expose the fascial plane in the other layer of skin fold. 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) via the tail vein (right panel). See also Vedio2, available at http://www.....

Figure 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] and biotin-conjugated goat anti-rat secondary Ab [BD Bioscience, San Diego, CA]) 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. Bars, 50 μm.

Figure 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.

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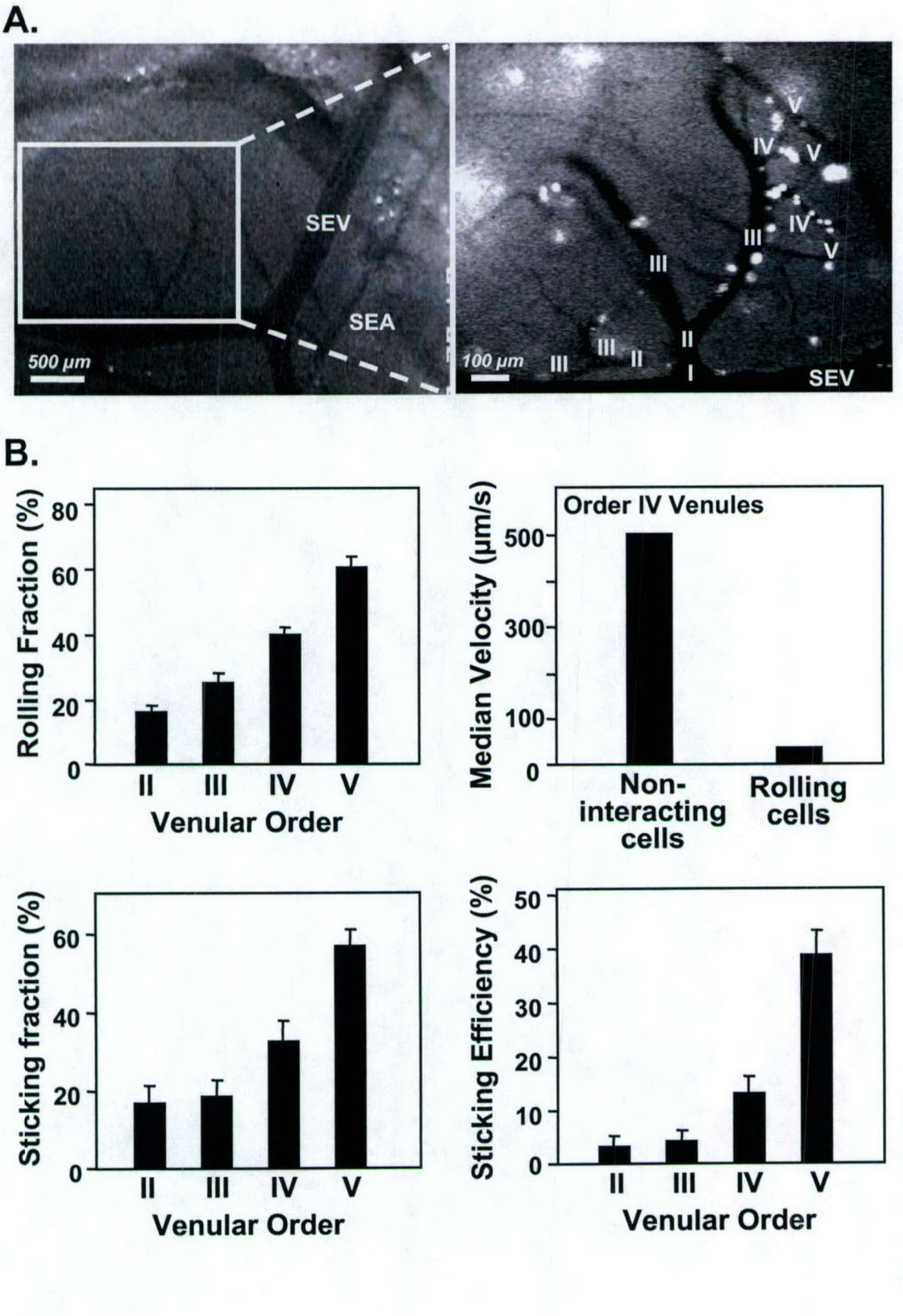
Figure 1



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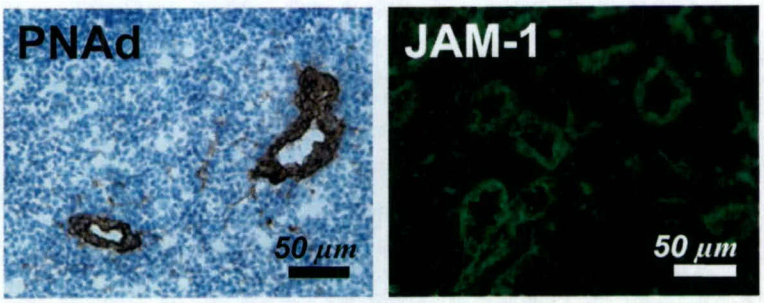
Chen et al.

Figure 2



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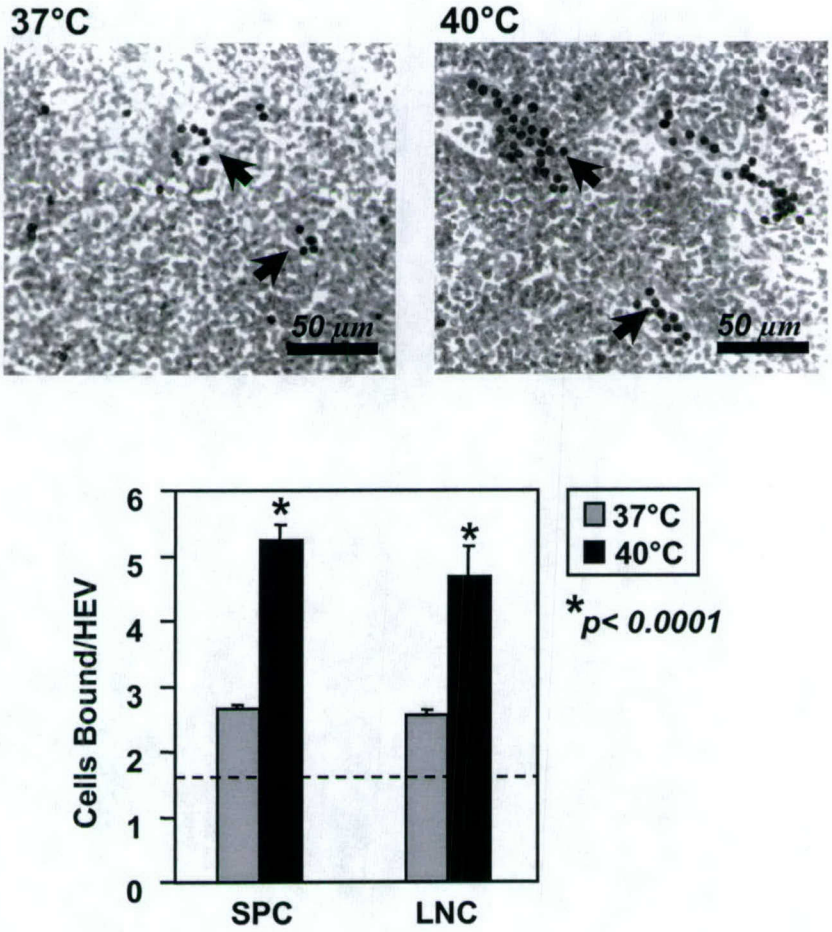
Chen et al.

Figure 3



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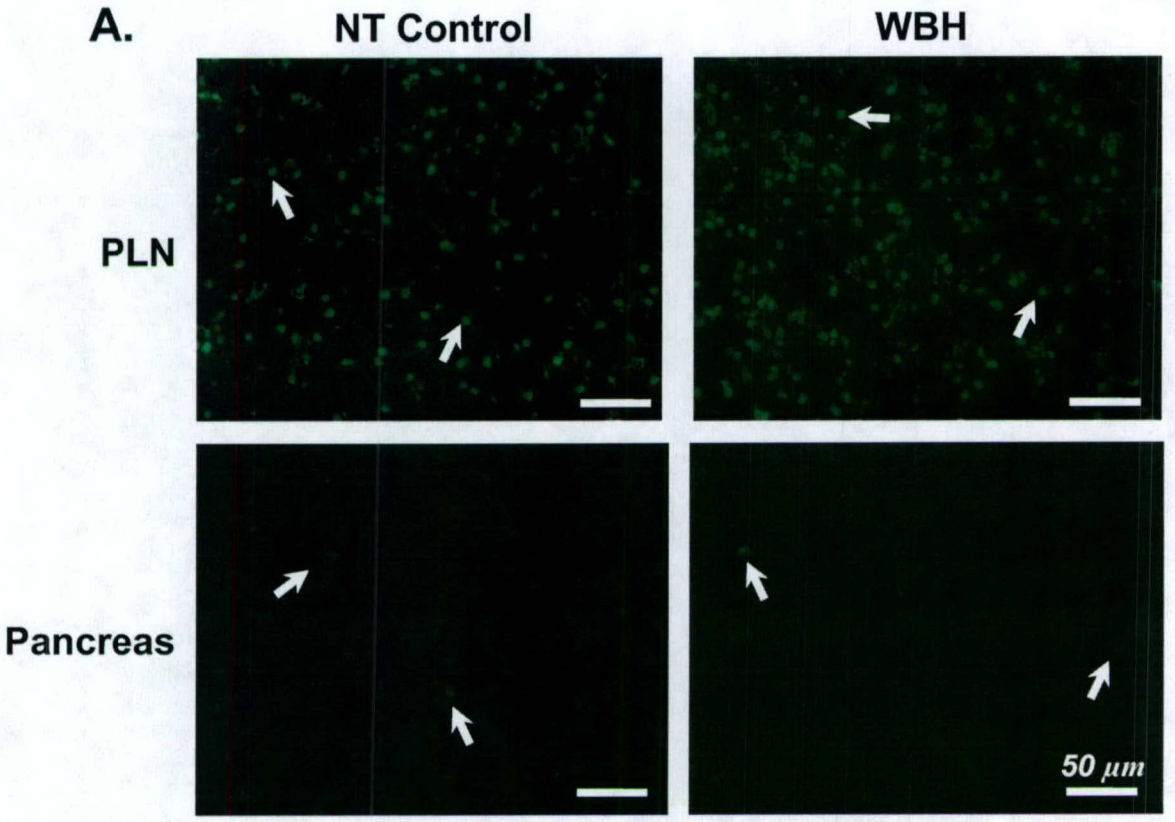
Chen et al.

Figure 4



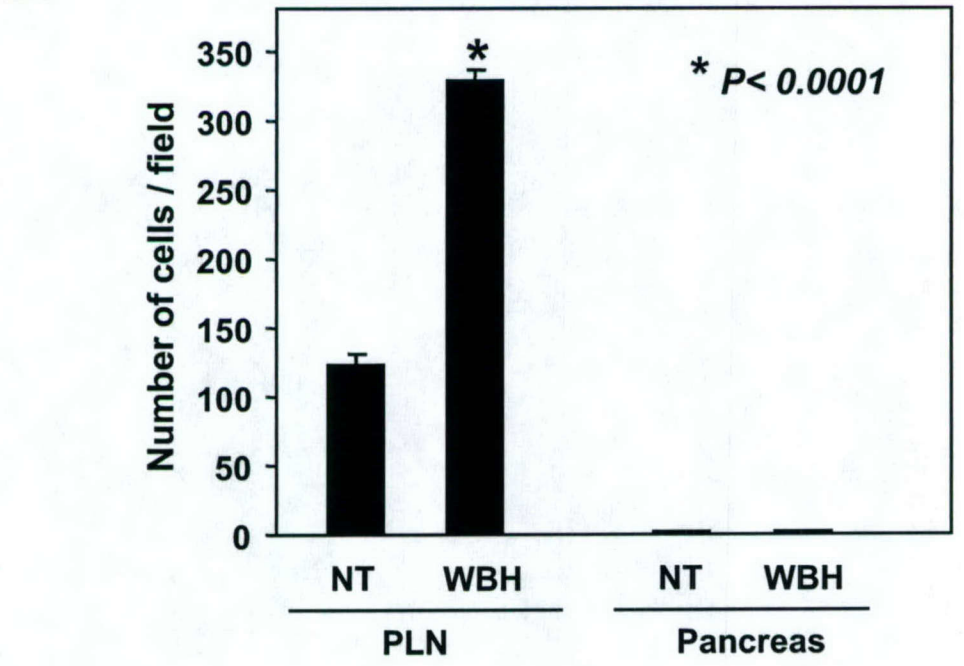


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Figure 5

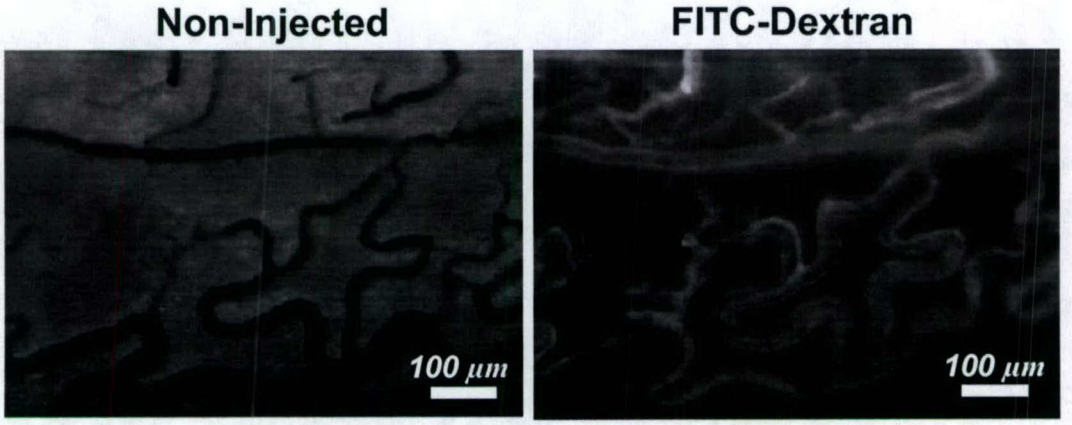

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Figure 6

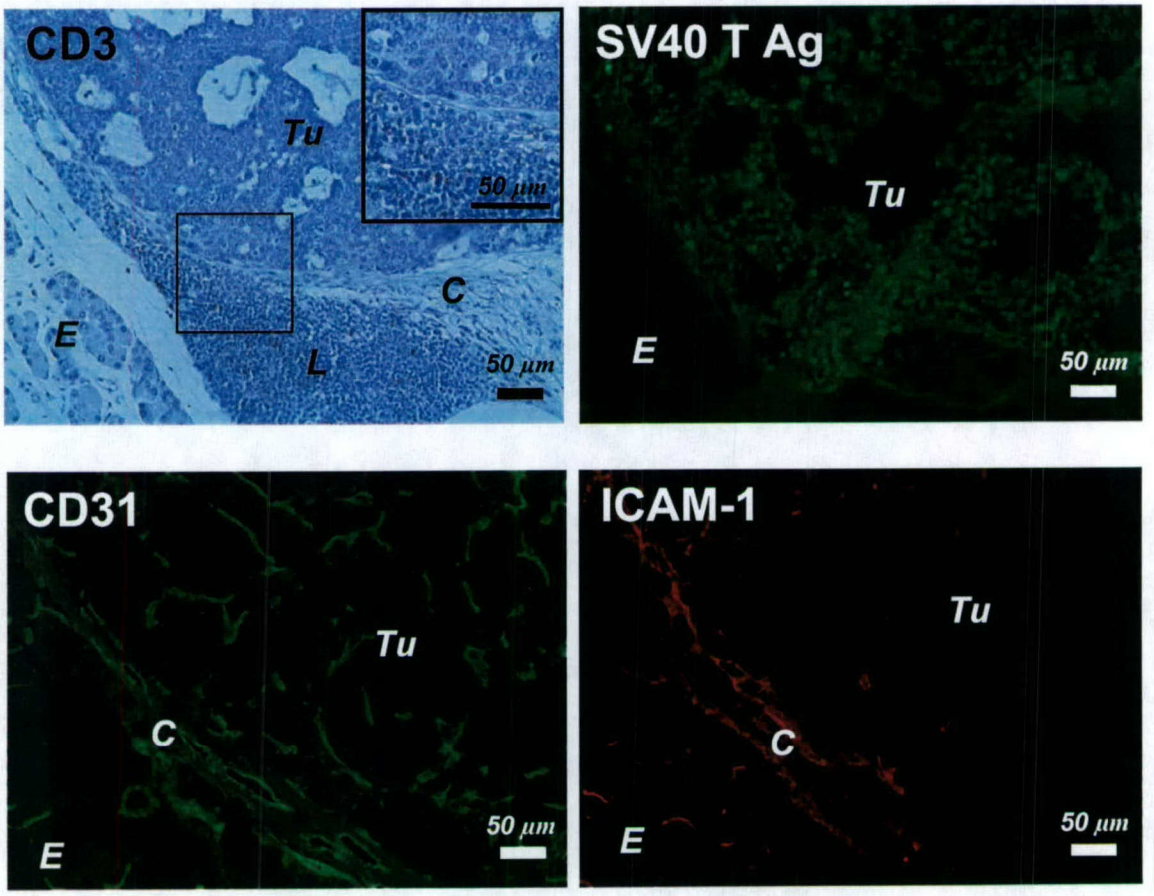

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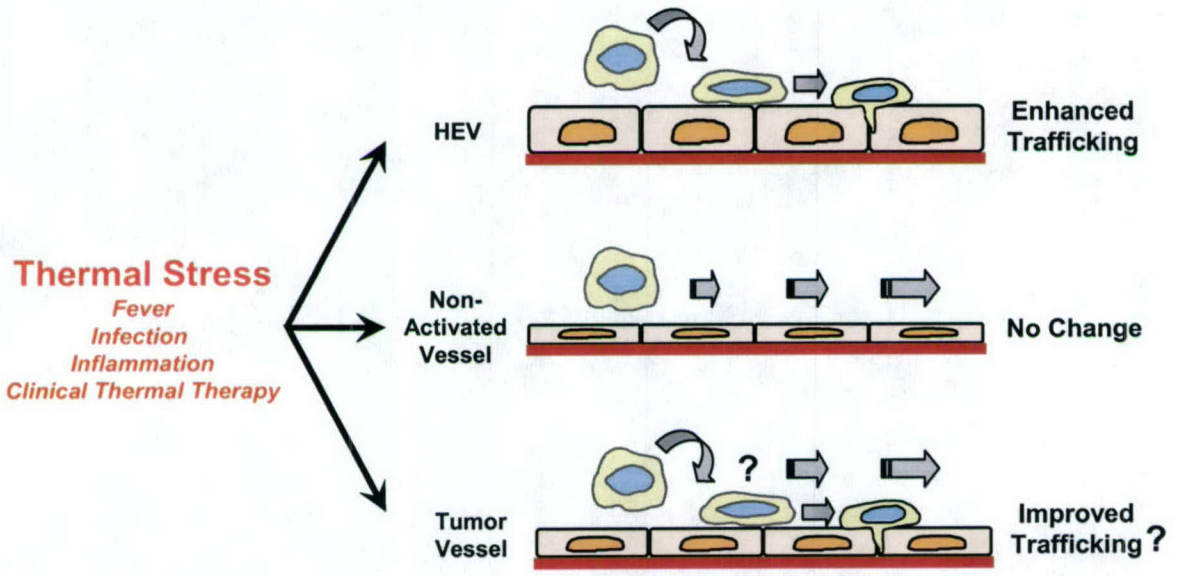
Figure 7



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*

* To whom correspondence should be addressed: Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY, 14263-0001; Email: sharon.evans@roswellpark.org; Phone: 716-845-3421

INTRODUCTION

A longstanding question in immunology resolves 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 review focuses on emerging evidence for a proactive role of fever-range thermal stress in regulating the molecular events that support lymphocyte adhesion in high endothelial venules (HEV). HEV are major sites of lymphocyte extravasation, serving as a locus for recirculation of blood-borne lymphocytes through peripheral lymphoid organs. Entry of naïve and central memory lymphocytes across HEV is crucial for immune homeostasis and immune surveillance. Notably, the mechanisms by which fever-range thermal stress promotes 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 HEV while squamous, non-activated endothelial cells are non-responsive. These observations support the concept that HEV 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 HEV

HEV 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 post-capillary venules are localized exclusively in the T

cell-enriched zones of all secondary lymphoid organs except spleen. The endothelial cells lining HEV are morphologically and biochemically differentiated from the flat, squamous endothelial cells of the majority of the vessels throughout the body. High endothelial cells (HEC) exhibit a cuboidal morphology, allowing them to extrude into the lumen of vessels and provide an irregular surface topography. These biophysical parameters likely 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 lymphocyte extravasation occurs preferentially across HEV which comprise a relatively small percentage of the total vasculature.

Lymphocyte migration across HEV is coordinated by adhesion molecules and chemokine/chemokine receptor partners. These molecules participate in a step-wise 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 LN (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 HEV. L-selectin binds transiently to counter-receptors on HEV collectively termed PLN addressins (PNAd) that include CD34, GLYCAM-1, podocalyxin, endomucin and sgp200 (3). The N-terminal 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 luminal surface of Peyer's Patches (PP) HEV. Instead, L-selectin binds to the mucin stalk of

mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in PP HEV (5). The efficiency of lymphocyte tethering and rolling in PP HEV is enhanced by binding of a second leukocyte homing receptor, $\alpha4\beta7$ integrin, to a negatively charged aspartic acid residue in the N-terminal immunoglobulin domain of MAdCAM-1 (5). Lymphocyte homing in mesenteric LN (MLN) is mediated by combined interactions of L-selectin with PNAd and MAdCAM-1 as well as $\alpha4\beta7$ 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 HEV (5, 6). This is most evident in higher order venules that are proximal to the capillary bed. Here, free-flowing lymphocytes, moving at a rate of > 400-500 μm/sec, transition to slow rolling cells with a speed of ≤ 50 μm/sec (7, 8). The increased transit time allows lymphocytes to sample the chemokine microenvironment on the surface of HEV. A single chemokine synthesized by HEC 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 HEV (4, 6).

Engagement of CCL21 by CCR7, a seven-transmembrane-spanning chemokine receptor on naïve and central memory lymphocytes, triggers G-protein-dependent conformational changes in the β2 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 HEV (2, 4, 6). In PLN HEV, firm sticking of lymphocytes is primarily mediated by LFA-1/ICAM-1-2 interactions whereas, in MLN and PP, both LFA-1/ICAM-1-2 and $\alpha4\beta7$ integrin/MAdCAM-1 contribute to firm adherence (5). LFA-1/ICAM-1-2 have also been implicated in supporting transendothelial migration although the molecular mechanisms controlling this process in HEV are not well understood (4-6). At tertiary

sites of injury or inflammation, ICAM-1 is highly induced by inflammatory cytokines on cuboidal, HEV-like vessels where it predominates over ICAM-2 in recruitment of neutrophils, macrophages, and lymphocyte subsets (9).

Fever-Range Thermal Stress Amplifies Lymphocyte-HEV Adhesion and Lymphocyte Homing

Local increases in temperature at sites of inflammation and 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 Repasky *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 advanced cancer patients 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 HEV (spleen) (10). Investigation of the underlying mechanisms has revealed that fever-range thermal stress influences trafficking by independently stimulating adhesion in two distinct cellular targets, i.e., lymphocytes and HEC (Fig. 1). Collectively, these findings support the notion that fever-range thermal stress provides a danger signal during inflammation to proactively regulate lymphocyte egress across HEV in secondary lymphoid organs (1, 8).

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 1) were cultured *in vitro* under fever-range 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 HEV serve as substrates. Alternatively, lymphocytes treated *in vitro* with heat were evaluated for their ability to traffic to various tissues in short-term (1 h) *in vivo* homing assays. These studies established that marked increases in lymphocyte adhesion to HEV and homing to HEV-bearing organs (i.e., PLN, MLN, PP) are observed following lymphocyte culture at physiologic fever-range temperatures (i.e., 2-4°C above normal temperature for 6 h) (Table 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 HEV such as the spleen (Table 1) (1, 8, 10, 14).

Evidence for organ-specific trafficking of heat-treated lymphocytes raised the possibility that thermal stress targets the function of known lymphocyte homing receptors. This hypothesis was confirmed using L-selectin and $\alpha4\beta7$ integrin-specific function-blocking mAb or cell lines expressing a non-functional form of L-selectin (i.e., 300.19/Δcyto transfectants that lacks the C-terminal 11 amino acid cytoplasmic tail (19)) (Table 1). Fever-range thermal stress increases L-selectin-mediated adhesion and homing of lymphocytes via engagement of PNAd on PLN and MLN HEV as well as MAdCAM-1 on PP HEV (Table 1, Fig. 1) (14-18). Parallel increases in $\alpha4\beta7$ integrin-mediated adhesion to MAdCAM-1 on PP and MLN HEV were demonstrated (Table 1, Fig. 1) (10, 20). Exquisite selectivity in thermal regulation of integrin binding activity

is suggested by results that heat treatment of lymphocytes fails to increase α4β7 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 HEV 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 (i.e., 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 α4β7 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 studies 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 luminal surface of HEV. Remarkably, treatment of lymphocytes with fever-range thermal stress causes L-selectin to pre-associate 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 HEV (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 proadhesive 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 (endothelial cells, fibroblasts) are all sources of heat-induced trans-activating proadhesive 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-6 (IL-6), in regulating lymphocyte homing (Fig. 1) (1, 8, 17). Functional blockade of IL-6 and its receptor components, i.e., 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-α, IL-1β, interferon-α (IFN-α), IFN-γ, IL-8, IL-11, leukemia inhibitory factor, or oncostatin M do not contribute to the proadhesive 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 MEK1/ERK1-2, but not p38 MAPK or JNK, in the IL-6/sIL-6Rα signaling pathway upstream of activation of L-selectin-cytoskeletal interactions and L-selectin avidity/affinity (Fig. 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 HEV adhesion

An independent line of investigation revealed that fever-range thermal stress enhances adhesion in HEC that are the major portals governing lymphocyte extravasation (Fig. 1). These studies employed frozen-section *in vitro* adherence assays to compare the binding activity of HEV in lymphoid 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 HEV to support lymphocyte adhesion under shear in this *in vitro* assay (Fig. 2A) (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 HEV in PLN cryosections is inhibited by the MECA-79 mAb, which recognizes the sulfation determinant on PNAd (Fig. 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 HEV of heat-treated animals. Parallel increases in adhesion are detected in PP HEV where binding of $\alpha4\beta7^{hi}$ L-selectin^{lo} TK1 cells to HEV is blocked by mAb specific for $\alpha4\beta7$ 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 lymphocytes and enumeration of labeled cells in various organs. Since fluorescent-tagged lymphocytes are not subjected directly to thermal stress, this experimental design allows for the analysis of vascular

responses to elevated temperatures. An example of this type of study is shown in Figure 2B. Enhanced interactions of TRITC (red)-labeled lymphocytes with HEV (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 (Fig. 2B) (8, 10). Notably, lymphocyte interactions with HEV temporally precede infiltration of fluorescent-labeled lymphocytes in the stroma of PLN organs of heat-treated animals (Fig. 2B). These observations are consistent with the notion that HEV 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 h whereas markedly elevated responses are observed following sustained exposure to thermal stress for 6-8 hours (8, 10). The 2-5 fold 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 HEV, 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 a 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 HEV of LN and PP whereas no increase in adhesion is detected by *in vitro* adherence assays in squamous, non-activated endothelial cells of extralymphoid organs (e.g., pancreas). Moreover, WBH does not increase the localization of fluorescent-labeled lymphocytes in non-HEV bearing organs (i.e., 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 HEV in lymphoid organs, while sparing tertiary sites.

Studies have been initiated to define the specific adhesion events targeted by thermal stress in HEV. The murine 300.19/ transfectant cell line engineered to express human L-selectin (19) has been useful in segregating the relative contributions of primary (tethering/rolling) and secondary (firm adhesion) interactions since it does not express detectable levels of LFA-1 molecules (1, 8, 10, 17). 300.19/L-selectin cells exhibit enhanced adhesion to HEV 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 non-functional form of L-selectin (19) fail to adhere to HEV or traffic to LN or PP in response to WBH (10). These results strongly suggest that fever-range thermal stress promotes primary, L-selectin-dependent tethering and rolling interactions in LN and PP HEV. Enhanced HEV adhesion is not accompanied by detectable increases in expression of PNAd (Fig. 2B) or MAdCAM-1 molecules (1, 10).

Recent studies suggest that fever-range thermal stress also regulates secondary molecular events that control firm adhesion of lymphocytes in HEV. Thus, fever-range WBH treatment of mice markedly upregulates the luminal expression of ICAM-1 on HEV of LN and PP whereas no induction of ICAM-1 is observed in vessels of extralymphoid organs.[1] Expression of ICAM-1 at

high density on HEV would be predicted to promote firm adhesion via interactions with LFA-1 on opposing 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 HEV, 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 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 (HUVEC) and microvascular (HMVEC) endothelium (20, 25). This is in sharp contrast to physiologic fever-range temperatures (39.5-40°C) that have no effect on adhesion molecule expression (ICAM-1, E-selectin, VCAM-1, P-selectin, 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, MCP-1, MIG) in cultured endothelial cells (20, 26). Non-activated endothelium are not entirely refractory to fever-range thermal stress, however, since proadhesive factors can be recovered from the conditioned medium of HUVEC and HMVEC following culture at 40°C (1, 8, 17, 20). These soluble factors act in *trans* to stimulate the binding function of L-selectin and α4β7 integrin on lymphocytes. Based on these findings, it is tempting to speculate that the vast vascular beds indirectly contribute to lymphocyte delivery to LN and PP during febrile inflammatory responses by providing factors that stimulate lymphocyte homing receptor function.

Conclusions and Perspectives on Future Directions

Recent studies support the concept that febrile temperatures function as a rheostat to amplify lymphocyte trafficking and thereby, the efficacy of immune surveillance (Fig. 3). Selective targeting of primary and secondary adhesion events in specialized HEV focuses the delivery of immune effector cells to peripheral lymphoid organs where there is the opportunity for optimal sensitization or restimulation of naïve or central memory lymphocytes, respectively. In the absence of such tight control of lymphocyte-endothelial adhesion, inappropriate trafficking of lymphocytes could lead to extensive damage in tertiary tissues.

- 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 HEC contribute to the specificity of vascular responses.
- Major questions remain regarding the molecular mechanisms underlying thermal control of adhesion in HEV. Inflammatory cytokines such as TNF-α, IL-1β, IFN-γ, or IL-6 are potential candidates since 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 HEV 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 lymphocytes as well as in HEV (17).[1] An important issue that awaits further investigation is how pleiotropic cytokines, such as IL-6, selectively target lymphocyte-HEV adhesion, while maintaining the tightly regulated balance between physiological and pathological responses during febrile inflammatory responses.

Acknowledgements: We thank Elizabeth Repasky and Heinz Baumann for insightful discussions relating to this work and Jennifer Black and Michelle Appenheimer for the critical review of the manuscript. This work was supported by grants from the NIH, the Department of Defense, and the Roswell Park Alliance Foundation.

[1]Chen, Q., Unger, E., Passanese, J., Clancy, K., Appenheimer, M., Fisher, D., Wang, W. C., Baumann, H., and Evans, S.S. Fever-range thermal stress induces ICAM-1 expression on high endothelial venules (HEV) via an IL-6 trans-signaling mechanism *(in preparation).*

Figure 1. Fever-range thermal stress proactively stimulates lymphocyte-HEV adhesion.

Figure 2. Fever-range thermal stress enhances lymphocyte adhesion to PLN HEV *(A)* and homing to PLN organs *(B)*. BALB/c mice were treated with fever-range WBH for 6 h (core temperature, 39.5 ± 0.5C°) (10). The core temperature of normothermal control mice (NT) was 36.8 ± 0.2C°. In ***(A)***, adherence of mouse splenocytes to HEV in PLN cryosections was evaluated *in vitro* under shear as described (10). The level of L-selectin/PNAd-specific adhesion (indicated by brackets) was determined by treating PLN cryosections with MECA-79 mAb (6.25 μg/ml, BD Bioscience). Photomicrographs show typical images of lymphocytes (black arrows) bound to HEV in toluidine-stained PLN tissues. The number of adherent lymphocytes was quantified by light microscopy in a total of 300-500 HEV. For consistency in double-blind evaluation, HEV were quantified only if they contained ≥ 1 adherent cell. In ***(B)***, short-term homing studies were performed essentially as described (8, 10). Mouse splenocytes labeled with TRITC (3.6 μg/ml, Sigma) 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) HEV or infiltrated into the stroma of PLN tissue at 60 min. The number of TRITC-labeled cells associated with HEV (> 40 HEV) 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 min. (not shown) (10).

Figure 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 HEV in PLN, MLN, and PP. In contrast, lymphocyte-endothelial adhesion and trafficking across non-activated squamous endothelium is not enhanced in spleen or tertiary tissues.

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Table 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 h 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 non-functional 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

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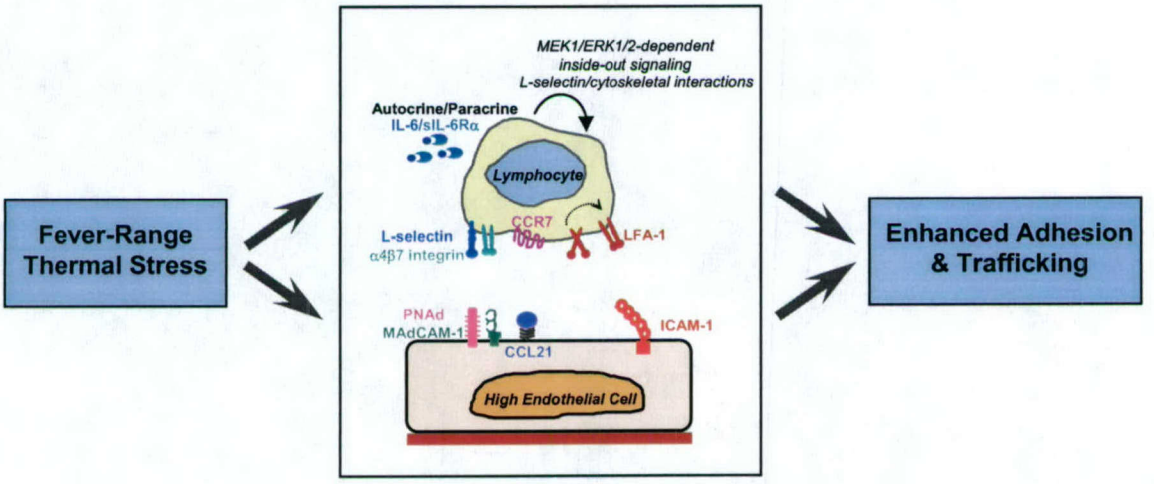
Figure 1



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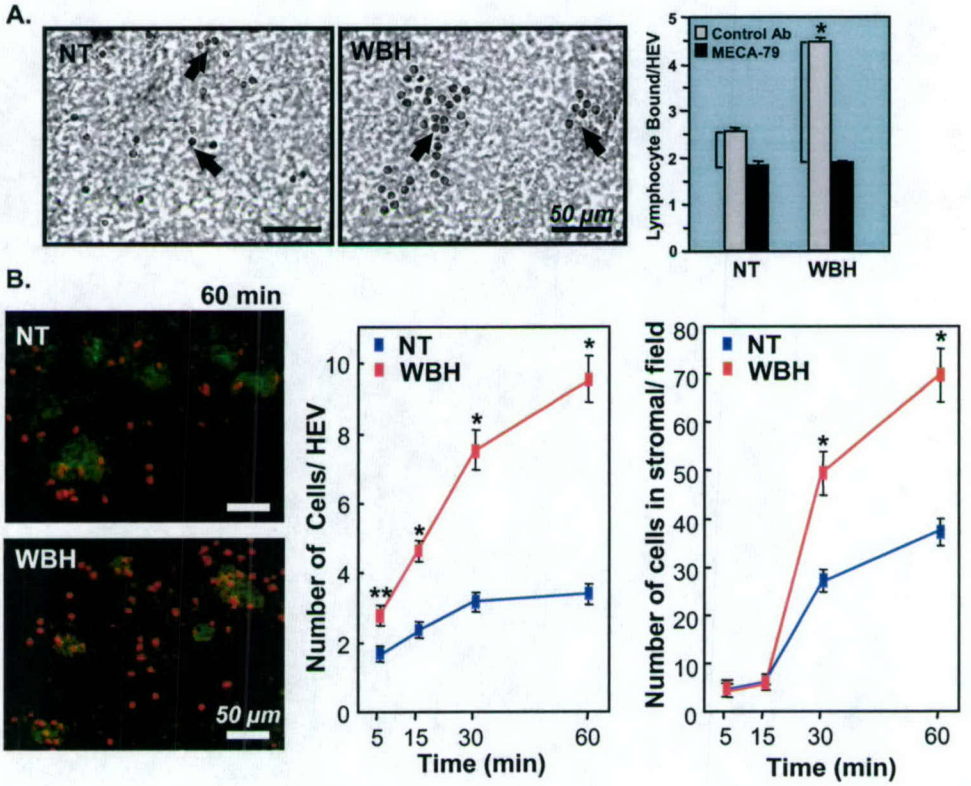

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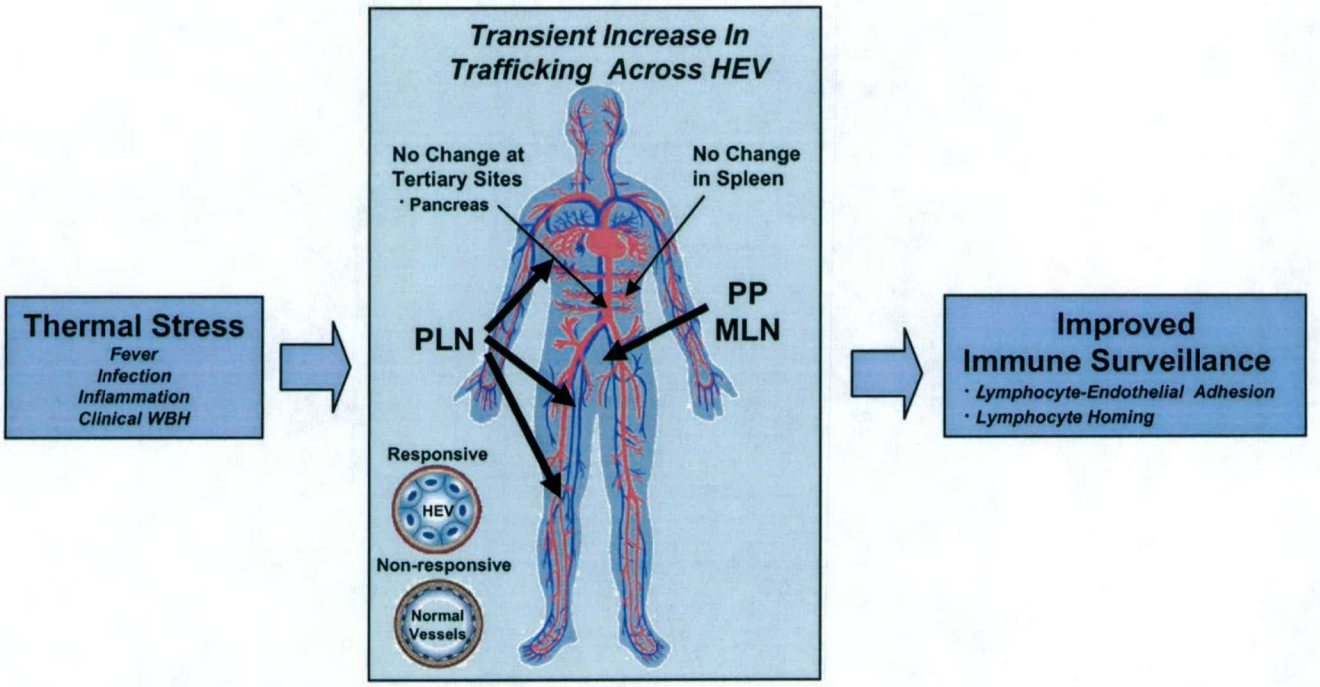

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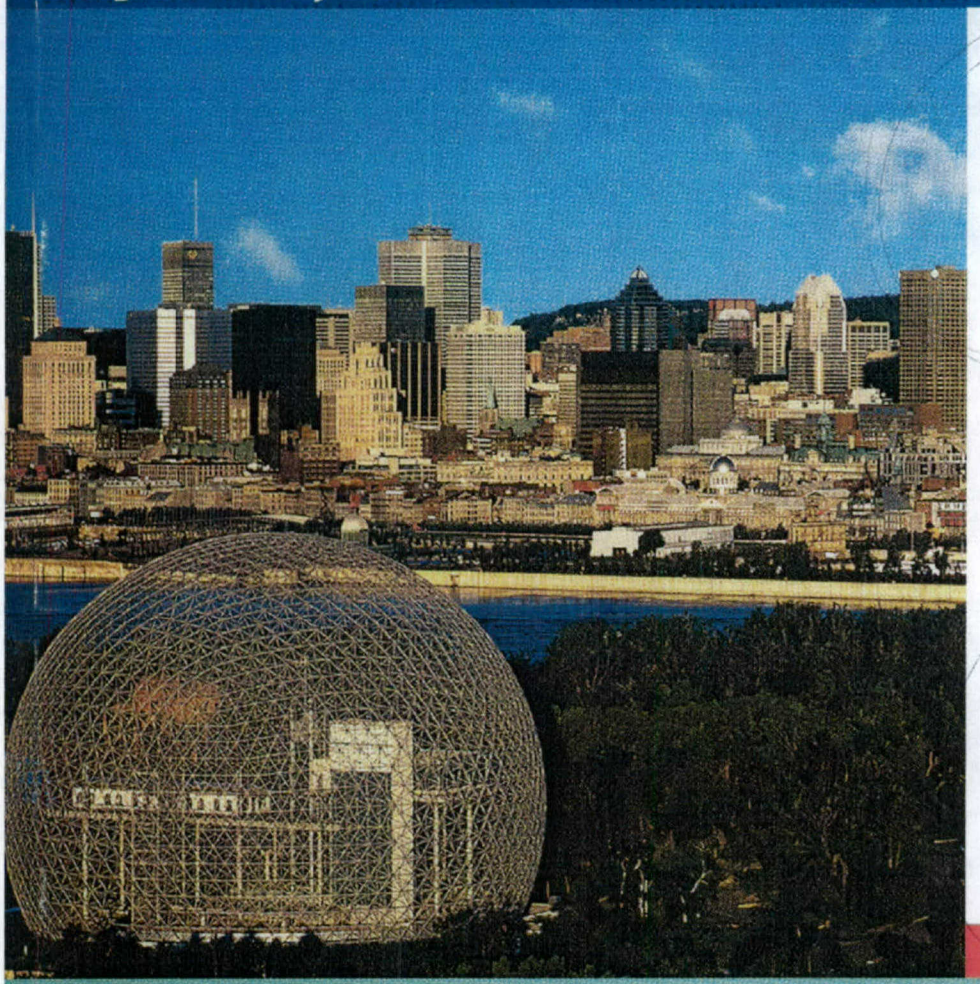

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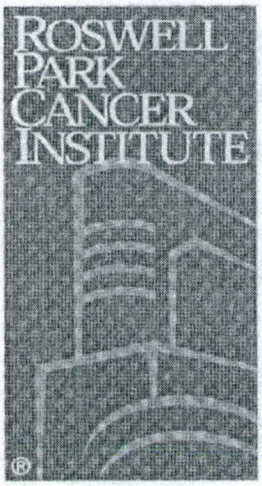
Fever-Range Thermal Stress Stimulates Lymphocyte Homing Receptor Function through an Interleukin-6-Dependent Trans-Signaling Mechanism.

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 $\alpha 4\beta 7$ 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 $\alpha 4\beta 7$ 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 $\alpha 4\beta 7$ 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 $\alpha 4\beta 7$ 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)



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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

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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).

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

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Qing Chen, M.D.	**Predoctoral Graduate Fellow**

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Capital University of Medical Sciences, Beijing, China	**M.D.**	**1992-1997**	**Clinical Medicine**
Beijing Institute of Neuroscience, Beijing, China	**M.S.**	**1997-2000**	**Molecular Neurobiology**
University at Buffalo, State University of New York, NY, U.S.A.		**2000-2001**	**Biomedical Sciences**
Roswell Park Cancer Institute, NY, U.S.A.		**2001-**	**Immunology**

A. POSITIONS & HONORS

1995-1997	Intern in Beijing Friendship Hospital, Capital University of Medical Sciences, Beijing, China
1997-2000	Graduate student with Dr. QunYuan Xu, Department of Neurobiology, Capital University of Medical Sciences, Beijing, China
2000-2001	Predoctoral student, Interdisciplinary Graduate Program of Biomedical Sciences, University at Buffalo, State University of New York, Buffalo, NY
2001-	Predoctoral student with Dr. Sharon Evans, Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY

B. PUBLICATIONS

1. Chantakru, S., Wang, W.-C., van den Heuvel, M., **Chen, Q.,** Croy, B.A. and Evans, S.S. Coordinate regulation of lymphocyte-endothelial interactions by pregnancy-associated hormones. *J. Immunol.* 171:4011-4019, 2003.
2. **Chen, Q.**, Wang, W.-C., and Evans, S.S. Tumor microvasculature as a barrier to anti-tumor immunity. *Cancer Immunol. Immunother.* 52:670-679, 2003.
3. **Chen, Q.**, Wang, W.-C., Bruce, R., Li, H., Schleider, D.M., Mulbury, M.J., Bain, M.D., Wallace, P.K., Baumann, H., and Evans, S.S. Central role of IL-6-receptor signal transducing chain gp130 in activation of L-selectin adhesion by fever-range thermal stress. Immunity, 20:59-70, 2004.*[Highlighted in preview article "IL-6 trans-signaling: the heat is on", Immunity 20:2-4, 2004].*

4. Appenheimer. M.M., **Chen Q.**, Girard, R.A., Wang, W.-C., and Evans, S.S. Role of the evolutionarily conserved fever response in controlling lymphocyte trafficking. *Immunol. Invest. (in press).*
5. **Chen, Q.**, Kucinska, S.A., Appenheimer, M.M., Fisher, D.T., Wang, W.-C., and Evans, S.S. Dynamic control of lymphocyte trafficking by fever-range thermal stress. Cancer Immunol. Immunother. *(in press).*
6. **Chen, Q.**, Clancy, K.A., Wang, W.-C., and Evans, S.S. High endothelial venules: master regulators of lymphocyte trafficking and targets of fever-range thermal stress. In: *Endothelial Biomedicine*; William Aird, editor; Cambridge University Press *(in press).*
7. Zhou, L., Fisher, D.T., **Chen, Q.**, Wang, W.-C., Baumman, H., and Evans, S.S. IL-6 Trans-Signaling and Leukocyte Trafficking: Balance Between Health and Disease. *Arch. Immunol. Therap. Exper. (In preparation, invited review)*
8. **Chen, Q.,** Clancy, K., Unger, E., Passanese, J., Appenheimer, M., Fisher, D., Wang, W. C., Baumann, H., and Evans, S.S. Fever-range thermal stress induces ICAM-1 expression on high endothelial venules (HEV) via an IL-6 trans-signaling mechanism *(in preparation).*

C. MEETING ABSTACTS

1. Evans,S.S., Wang,W.C., Bain, M.D., Burd,R., Ostberg, J.R., Bruce,R.A., Mulbury,M.J., Li,H., **Chen,Q.**, Baumann,H., and Repasky, E.A.. Dynamic regulation of lymphocyte delivery to high endothelial venules by fever-range hyperthermia. 9th International Conference on Lymphocyte Activation and Immune Regulation: Lymphocyte Traffic and Homeostasis. February, 2002; Newport Beach, CA
2. **Chen, Q.,** Li, H., Bruce, R., Mulbury, M., Wang,W.-C., Ostberg, J.R., Kraybill,W.G., Baumann,H. Repasky, E.A. and Evans, S.S. Novel role of IL-6 signaling in controlling lymphocyte homing. 5th Annual Regional Cancer Center Consortium for Biological Treatment of Cancer, Feburary, 2002; Cleveland Ohio.
3. **Chen, Q.,** Ginnetti,J., Baumann,H., Kraybill,W.G., Ostberg,J.R., Repasky,E.A., Wang,W.-C., and Evans, S.S. Dynamic control of lymphocyte trafficking by fever-range thermal stress. Radiation Research Society Annual Meeting. April, 2002; Reno, NV.
4. **Chen, Q.,** Unger, E., Passanese, J., Bangia, P., Pritchard, M., Wang, W.C., Repasky, E.A., and Evans, S.S. Fever-range thermal stress augments lymphocyte-endothelial adhesion in the tumor microvasculature. 94th Annual American Association of Cancer Research Meeting. April, 2003.
5. **Chen, Q.,** Kucinska, S., Wang, W.C., Baumann, H., Ostberg, J., Repasky, E., and Evans, S.S. IL-6/soluble IL-6 receptor trans-signaling activates tumor microvascular adhesion in response to fever-range thermal stress. Joint meeting of the 1st International Congress on Stress Responses in Biology and Medicine and 21st Annual Meeting of the North American Hyperthermia Society. Sept. 2003; Quebec, Canada
6. **Chen, Q.**, Kucinska, S., Wang, W.C., Wallace, P., Baumann, H., and Evans, S.S. Fever-range thermal stress stimulates lymphocyte homing receptor function through an interleukin-6-dependent trans-signaling mechanism. 7th Annual Regional Cancer Center Consortium for Biological Treatment of Cancer. Feburary, 2004; Buffalo, NY.
7. **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.
8. **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. 6th Annual Symposium on Oncology Sciences. September, 2004; Buffalo, NY.

trans-signaling mechanism. 6th Annual Symposium on Oncology Sciences. September, 2004; Buffalo, NY.

9. **Chen, Q.**, Unger, E., Passanese, J., Appenheimer, M.M., Fisher, D.T., Wang, W.-C., Baumann, H., and Evans., S.S. Fever-range thermal stress controls HEV display of ICAM-1 via an IL-6 trans-signaling mechanism. Keystone Symposia (Leukocyte Trafficking: Cellular and Molecular Mechanisms). March, 2005; Taos, NM.

D. SELECTED LECTURES AND ORAL PRESENTATIONS

1. **Chen, Q.**, Dynamic control of lymphocyte trafficking by fever-range thermal stress. Graduate Student Seminar, Roswell Park Cancer Institute, April, 2002
2. **Chen, Q.**, Mechanism of activation of lymphocyte/endothelial cell adhesion by fever-range thermal stress. Department Retreat, June, 2002
3. **Chen, Q.**, Thermal control of lymphocyte trafficking. Graduate Student Seminar in Roswell Park Cancer Institute, September, 2002
4. **Chen, Q.**, Fever-range thermal regulation of lymphocyte trafficking. 2nd Immunology Meeting for Graduate Student. September, 2002; Ithaca, NY
5. **Chen, Q.**, Kucinska, S., Passanese, J., Wang,W.C., Baumann, H., and Evans. S.S. The role of IL-6 signaling in Activation of $\alpha 4\beta 7$ integrin dependent lymphocyte/endothelial cell adhesion by fever-range thermal stress. Immunology Department Retreat, Roswell Park Cancer Institute, August, 2003
6. **Chen, Q.**, Central role of IL-6 receptor signal transducing chain gp130 in activation of L-selectin adhesion by fever-range thermal stress, Graduate Student Seminar, Roswell Park Cancer Institute, November, 2003
7. **Chen, Q.**, Passanese, J., Clancy, K., Kucinska, S., Green, C., Wang, W.C., Dewhirst, M., Hanahan, D., Repasky, E., Baumann, H., and Evans, S.S. Fever-range thermal stress controls vascular endothelial display of ICAM-1 via an IL-6/soluble IL-6 receptor trans-signaling mechanism. Immunology Department Retreat, Roswell Park Cancer Institute, September, 2004.
8. **Chen, Q**., Passanese, J., Fisher, D.T., Kucinska, S.A., Clancy, K.A., Wang, W.-C., Appenheimer, M.M., Zhou, L., Repasky, E.A., Baumann, H. and Evans, S.S. Fever-range thermal stress controls vascular endothelial display of ICAM-1 via an IL-6/soluble IL-6 receptor trans-signaling mechanism. Society for Thermal Medicine 2005 Annual Meeting. April, 2005; Bethesda, MD.

E. AWARDS

Department of Defense — 2/04 – 1/08
Predoctoral Traineeship BC032139 — $ 89,993 (for three years)
(**Chen, Q., P.I.;** Evans, S.S., Mentor)
Role of Proinflammatory Cytokines in Thermal Activation of Lymphocyte Recruitment to Breast Cancer Tumor Microvessels
The goal of this project is to determine if fever-range thermal stress, acting through an IL-6-dependent mechanism, promotes leukocyte homing to breast tumors.

Susan Komen Breast Cancer Foundation

Dissertation Research Award — This grant was approved for funding in May 2004; the award was declined because of overlap with DOD predoctoral Traineeship BC032139

(Evans, S.S., Dissertation Supervisor; **Chen, Q., Dissertation Candidate**)
Role of Proinflammatory Cytokines in Thermal Activation of Lymphocyte Recruitment to Breast Cancer Tumor Microvessels

Keystone Symposia scholarship
Travel award to attend Keystone Symposia (Leukocyte Trafficking: Cellular and Molecular Mechanisms). March, 2005; Taos, NM.

Society of Thermal Medicine
Travel award to attend the Society for Thermal Medicine Annual Meeting 2005, March 30 – April 4, 2005, NIH, Bethesda, M.D.

F. SPECIALIZED 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.