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Award Number: W81XWH-09-1-0742

TITLE: Safe Gene Therapy for Type 1 Diabetes

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REPORT DATE: October 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-10-2011		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 28 SEP 2010 - 27 SEP 2011	
4. TITLE AND SUBTITLE Safe Gene Therapy for Type 1 Diabetes				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-09-1-0742	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Massimo Trucco E-Mail: mnt@pitt.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Pittsburgh Pittsburgh, PA 15213				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT In light of accumulating evidence that the endocrine pancreas has regenerative properties, that hematopoietic chimerism can abrogate destruction of beta cells in autoimmune diabetes, and that in this way physiologically-sufficient endogenous insulin production can be restored in clinically-diabetic NOD mice, recapitulating what has also been sporadically seen in humans, we originally proposed to test reliable and clinically translatable alternatives able to re-establish euglycemia in diabetic patients. Instead of relying on the risky allogeneic BM transplantation to obliterate the autoimmune process that causes type 1 diabetes, we originally proposed to reconstitute, by gene supplantation, susceptible (non-Asp57+) NOD mice with their own BM genetically engineered ex vivo to also express a resistance (Asp57+) MHC class II molecule. The thymus of the reconstituted mice -- carrying BM-derived cells that co-expressed both their own diabetogenic (non-Asp57) and the transfected Asp57 beta chain -- repopulated by the engineered BM cells, can restore an efficient negative selection and consequently the ability to delete T cells potentially auto-reactive to pancreatic beta cells. These diabetics will then be disease-free.					
15. SUBJECT TERMS Type 1 diabetes; autoimmunity; bone marrow; stem cells; histocompatibility					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 48	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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 Pediatric Diabetes 11:292, 2010.	
 BioTechniques, In press, 2011.	

Our first quarterly scientific progress report for the second year of our project (09/28/10 – 12/27/10) described the following:

Background and Significance

Insulin expression was found in both lymph node (LN)-resident stromal cells of non-hematopoietic origin and bone marrow (BM)-derived antigen-presenting cells (APCs)¹⁻³. Although little is known about the function of insulin expression in LNs, recent studies have shown that TSA-expressing stromal cells can effectively deplete TSA-specific autoreactive CD8⁺ T cells from the peripheral repertoire⁴⁻⁶. As for insulin expression in BM-derived APCs, discordant results have been reported regarding the specific cell subsets. Both (pro)insulin transcripts and proteins were found in human CD11c⁺ dendritic cells (DCs) in the thymus and the peripheral lymphoid tissues⁷. In contrast, Hansenne *et al.* found neither *Ins1* nor *Ins2* transcripts in mouse CD11c^{high} DCs, regardless of their maturation status⁸. Transplantation of BM cells harvested from NOD.*Ins2*^{+/+} mice failed to slow down diabetes progression in NOD.*Ins2*^{-/-} recipients, suggesting that endogenous levels of *Ins2* expression in BM-derived cells of NOD mice cannot restore peripheral tolerance to insulin⁹. However, these data should be interpreted with caution as the authors pointed out that the levels of *Ins2*-expression in spleen and pancreatic LN decrease significantly after weaning (3-4 weeks) in NOD mice¹⁰. The failure of restoring insulin tolerance might be attributed to the low levels of *Ins2*-expression in the transplanted BM cells. Thus, the role of insulin expression in secondary lymphoid tissues in regulating peripheral tolerance of beta-cells remains elusive.

As reported in the previous quarters, we have generated a number of animal models to investigate the potential role of APC-insulin expression in mediating peripheral tolerance of beta-cells. Recently, we have focused our effort on the CD11c-ΔIns/H2g7 model, as we have shown that insulin expression is restricted to Aire⁺CD11c^{int} cells of BM-origin in the spleen. In this quarter, we have further characterized this population of cells, and identified them as tolerogenic plasmacytoid dendritic cells (pDCs). In addition, we investigated further the anti-insulin autoimmunity in CD11c-ΔIns/H2g7 mice, and found that although these animals can effectively maintain homeostasis of blood glucose, elevated levels of lymphocyte infiltration into the pancreata were observed, suggesting a partial break down of peripheral tolerogenic mechanisms to beta-cells.

Results of Experiments in Progress

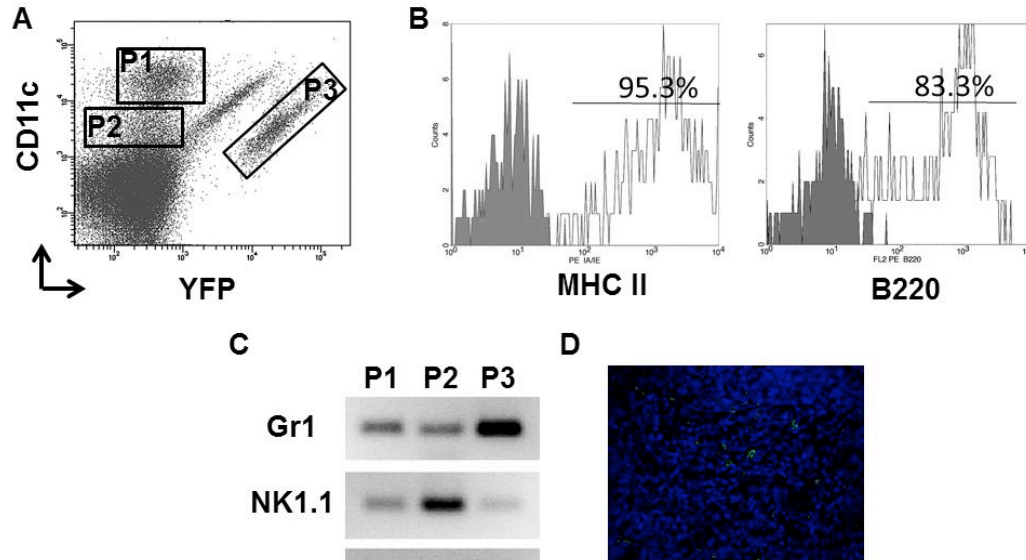
1) Characterization of insulin-expressing antigen presenting cells

The absence of anti-islet autoimmunity in ID-BMC mice prompted us to identify the subset(s) of BM-derived APCs which express insulin. Since it has been shown that *Ins2* expression within the thymus is regulated by the autoimmune regulator (Aire) gene, and that low levels of Aire gene expression are found in BM-derived CD11c⁺ dendritic cells (DCs), we setup to examine whether insulin is also expressed in Aire-expressing cells of BM-origin. Taking advantage of our previous findings that the Aire-Cre transgene can faithfully recapitulate the endogenous Aire gene expression pattern, we crossed the Aire-Cre transgenic mice to reporter RosaYFP mice to generate the Aire-Cre:RosaYFP line, in which all the Aire-expressing cells were positively marked with YFP. FACS analyses showed that about 1-2% of the CD45⁺ splenocytes were YFP-positive (Figure 1A). Of interest, these YFP⁺ cells expressed intermediate levels of DC marker CD11c, as well as high levels of MHCII and B220 molecules. Combined, this is the molecular signature of the previously described tolerogenic plasmacytoid DCs (pDCs)⁵⁶ (Figure 1B).

In addition, another recently identified DC subset, the interferon-γ (IFN-γ)-secreting natural killer DCs (NKDCs) was also found to express the B220 marker on the surface. To further determine the identity of the CD11c^{int}YFP⁺ population, we separated the CD11c⁺ cells into three populations via FACS: the CD11c^{high}YFP⁻ “conventional” DCs (P1), the CD11c^{int}YFP⁻ DCs (P2) and the CD11c^{int}YFP⁺ “tolerogenic” DCs (P3). RT-qPCRs were performed to examine the expression levels of genes specific to different DC subsets. It was previously shown that Gr-1 is highly expressed in pDCs, whereas NK cell marker NK1.1 is predominantly found in NKDCs. As shown in Figure 1C, high levels of Gr-1 transcripts

were found in CD11c^{int}YFP⁺ P3 cells, whereas NK1.1 signal was barely detectable, suggesting that these cells belong to the pDC subset. In contrast, cells in the CD11c^{int}YFP⁻ P2 population expressed higher levels of NK1.1 but lower levels of Gr-1, suggesting that the P2 population was comprised primarily of NKDCs (Figure 1C).

Figure 1. Insulin expression in the spleen is largely restricted to Aire⁺CD11c^{int}B220⁺ tolerogenic pDCs. A.



FACS analysis of splenocytes isolated from Aire-Cre:RosaYFP mice. Cells were positively selected by anti-CD45 conjugated magnetic beads, followed by staining with anti-CD45 and anti-CD11c antibodies. As shown, CD11c⁺ cells (gated on CD45⁺) were separated into three populations: P1, CD11c^{high}YFP⁻; P2, CD11c^{int}YFP⁻; P3, CD11c^{int}YFP⁺. Shown is a representative FACS result from three independent experiments of spleens pooled from 3 animals. **B.** Cells of P3 population was sorted and stained with anti-MHCII and anti-B220 antibodies (solid lines), or IgG controls (filled grey lines). **C.** RT-PCR analysis of expression of genes specific to subsets of DCs. Gr1, pDCs; NK1.1, NKDCs. **D.** Immunohistochemical analysis of spleen sections of Aire-Cre:RosaYFP mice. 5um cryosections were probed with anti-YFP antibody (green).

Histologic examination revealed that YFP⁺ cells were primarily located in the marginal zone, consistent with previous reports (Figure 1D). These findings, in conjunction with our previous RT-qPCR results showing that insulin transcripts were essentially undetectable in either the conventional DCs (P1) or the CD11c^{int}YFP⁻ NKDCs (P2), but were predominantly present in Aire⁺ pDCs (P3). These data indicate that, unlike thymus and lymph nodes, insulin expressions in the spleen are largely restricted to Aire⁺ tolerogenic pDCs of BM origin.

2) Characterization of anti-insulin autoimmunity in CD11c-ΔIns/H2g7 mice

As reported previously, CD11c-ΔIns/H2g7 mice can effectively maintain normal blood glucose levels. When challenged with intraperitoneal glucose injections, no difference of glucose metabolism was observed between and littermate controls, suggesting the existence of sufficient islet function. However, consistent with previous reports, the introduction of the diabetes-prone H2g7 MHC alleles to B6 mice (B6/H2g7 congenic) was sufficient to induce low degrees of insulitis in control littermates (Figure 2, top panels), while both the severity and the percentage of islets infiltrated by T cells were significantly increased in pancreata harvested from CD11c-ΔIns/H2g7 mice (Figure 2, lower panels). Specifically, about 50% of islets were infiltrated with CD4⁺ T cells, as compared with less than 20% in controls (53±15% vs. 14±4%, p<0.05). Similar levels of islet infiltration were observed for CD8⁺ T cells

(46%±6% vs. 16±4%, $p<0.01$). Elevated levels of CD4⁺ and CD8⁺ T cell infiltration in CD11c-ΔIns/H2g7 suggest that insulin-expressing, Aire+CD11c^{int} tolerogenic pDCs might play a role in controlling the activation and expansion of islet-specific CD4⁺ effector and CD8⁺ cytotoxic T cells which have escaped thymic negative selection.

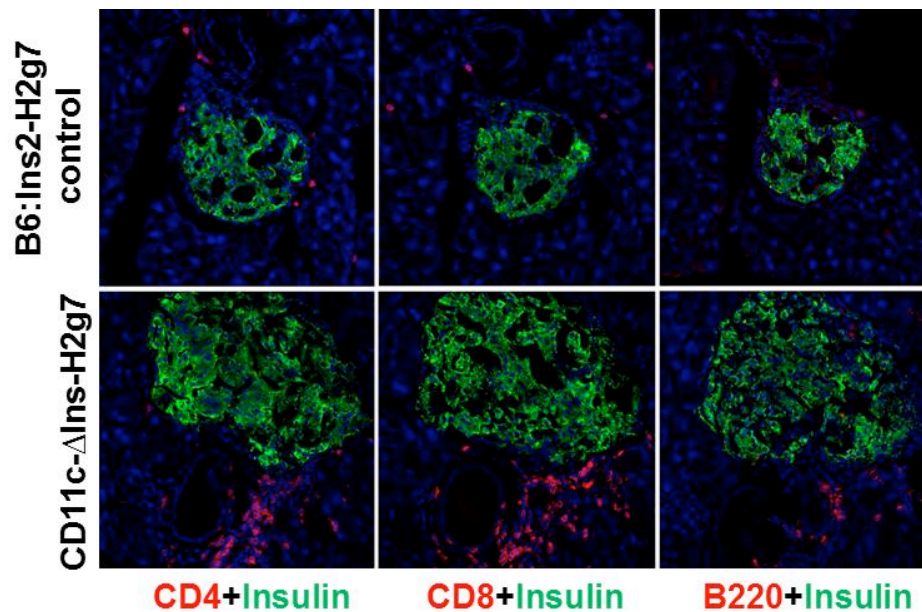


Figure 2. CD11c-ΔIns-H2g7 mice displayed increased levels of islet autoimmunity. Immunohistochemical analyses of pancreata harvested from CD11c-ΔIns/H2g7 mice and non-Cre littermate controls (B6:Ins2/H2g7). Shown are representative consecutive cryosections stained with anti-insulin antibody (green), in conjunction with anti-CD4 (left panels), anti-CD8 (middle panels) and anti-B220 (right panels) antibodies (red).

As one of the potential mechanisms of controlling autoreactive T cell expansion is mediated by the suppressive action of T regulatory (T_{reg}) cells, we examined the presence of T_{reg} cells in both the pancreatic lymph nodes and spleens of CD11c-ΔIns/H2g7 mice. Compared to the littermate controls, no decrease of either percentage or absolute number of T_{reg} cells was observed, suggesting that pDC-insulin mediates peripheral b-cell tolerance primarily through other immune tolerogenic mechanisms (data not shown).

3) CD11c- Δ Ins/H2g7 mice developed autoimmune sialadenitis.

Of interest, lymphocyte infiltration was found in salivary glands of CD11c- Δ Ins/H2g7 mice, but not in littermate B6/H2g7 controls (Figure 3). Both CD4⁺ effector T cells and CD8⁺ cytotoxic T cells were present, indicating the presence of ongoing autoimmune responses targeting salivary glands. We also observed elevated levels of B220⁺ cells. Further characterization revealed that these cells were negative for B-cell marker CD19, suggesting that they most likely belonged to the B220⁺ DC subsets. As it was reported previously that insulin transcripts were found at low levels in salivary glands, the unexpected observation of T-cell infiltration into salivary glands suggested that insulin deletion in Aire⁺ pDCs might also induce tolerance breakdown of salivary glands. These data demonstrated further the essential role of insulin-expression in Aire⁺ pDCs in mediating peripheral tolerance of insulin-expressing tissues.

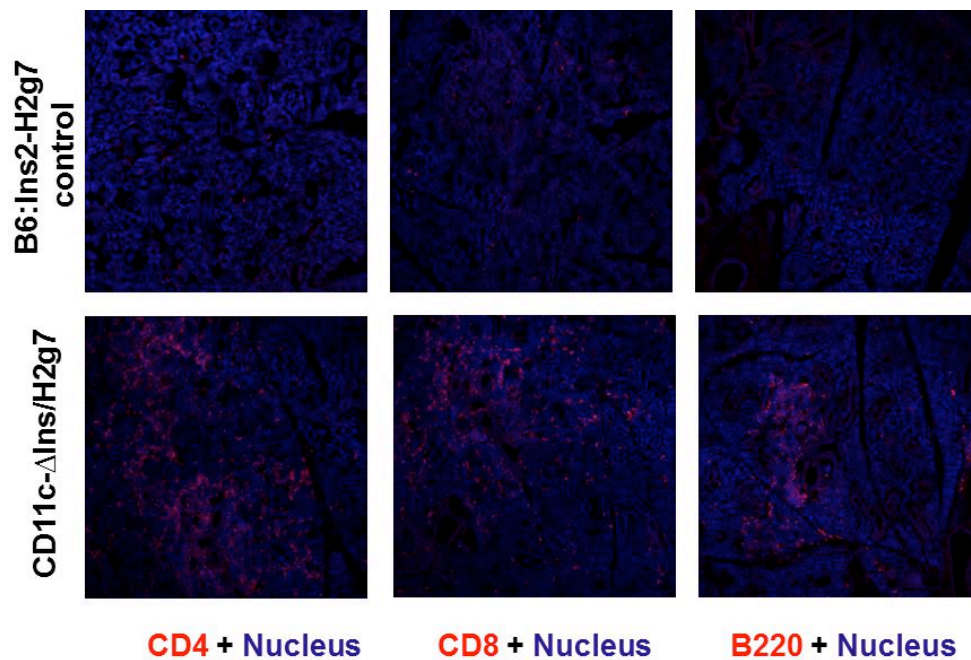


Figure 3. CD11c- Δ Ins-H2g7 mice developed autoimmune sialadenitis. Immunohistochemical analyses of salivary glands harvested from CD11c- Δ Ins/H2g7 mice and non-Cre littermate controls (B6:Ins2/H2g7). Shown are representative consecutive cryosections stained with anti-CD4 (left panels), anti-CD8 (middle panels) and anti-B220 (right panels) antibodies (red).

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

- 1) We have identified the insulin-expressing cells as a population of pDCs with the Aire⁺CD11c^{int}B220⁺MHCII⁺Gr1^{high}NK1.1^{low} phenotype. We will further characterize these cells in the next quarter. The ultimate goal is to explore the possibility of using these cells to treat T1D.
- 2) We found more severe levels of insulinitis in pancreata of CD11c-ΔIns/H2g7 mice, compared to B6/H2g7 littermate controls. Our data suggested an essential role of insulin-expression in Aire⁺ pDCs in mediating the maintenance of peripheral tolerance towards pancreatic b-cells.
- 3) In addition to insulinitis, CD11c-ΔIns/H2g7 mice also developed autoimmune sialadenitis, similar to the type 1 Sjogren syndrome in human. Of note, 20-30% of T1D patients also developed other forms of autoimmune disorders. Further characterization of the autoimmune responses to salivary glands in CD11c-ΔIns/H2g7 mice will help us to delineate the underlying mechanisms of autoimmune disorders associated with T1D.

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Our second quarterly scientific progress report for the second year of our project (12/28/10 – 03/27/11) described the following:

Background and Significance

The essence of adaptive immunity is its capability to effectively distinguish self from non-self and take actions accordingly: eliciting protective immune response to invading pathogens while maintaining immune unresponsiveness to self-antigens under steady state. To establish a self-tolerant, functional T-cell repertoire, developing T-cells must survive both the positive and the negative selections mediated by self-antigen-presenting thymic stromal cells. T-cells fail to establish stable contacts with thymic epithelial cells of the thymic cortex will receive no survival signal and perish by negligence (positive selection), whereas T-cells bearing T-cell receptors (TCRs) with high affinity to self-antigens are considered to be autoreactive and undergo apoptosis within the thymic medulla (negative selection). While it is conceivable that blood-borne self-antigens can be captured and engulfed by the BM-derived APCs and be trafficked back to the thymus, the mechanism of thymic presentation of autoantigens of tissue-specific nature remained elusive until about a decade ago when above-the-noise levels of transcripts of tissue-specific antigens (TSA) were found in mTECs. One of the master regulators of TSA ectopic expression in mTECs is the autoimmune regulator (Aire) gene. Patients with mutations in the Aire gene develop autoimmune polyglandular syndrome I (APS-I), a rare autoimmune disorder affecting multiple organs and tissues, which is also known as autoimmune polyendocrinopathy, candidiasis, and ectodermal dysplasia (APECED). Targeted mutagenesis of the mouse Aire gene partially recapitulated the human disease symptoms – autoimmune responses, manifested as the presence of autoantibodies targeting multiple organs and tissues as well as lymphocyte infiltration, were observed in these animals. Of note, expressions of more than 300 TSAs are significantly down-regulated in mTECs, providing, for the first time, direct experimental evidence linking defective thymic TSA expression with peripheral multiple organ autoimmunity.

However, in most human autoimmune disorders of organ/tissue-specific nature, such as autoimmune diabetes type 1A (T1D) and autoimmune thyroid disease (ATD), one specific organ or tissue is primarily targeted. In addition, autoimmune responses targeting to one (or more) specific TSA(s), such as insulin and thyroglobulin, were often found to be the pivotal driving forces for disease progression. Thus, the broad effects of Aire inactivation on thymic TSA expression and its suggestive roles in mTEC differentiation and maturation render it difficult to study the impact of individual TSA in establishing immunologic tolerance of a specific organ with the Aire-KO animal model.

As reported previously, we have successfully established the ID-TEC animal model in which insulin expression is specifically abrogated in mTECs, but remains intact in pancreatic beta-cells (designated as ID-TEC mouse for insulin-deletion in mTECs). Our results demonstrated conclusively the essential roles of mTEC-insulin expression in mediating central negative selection of insulin-reactive T-cells to establish immune tolerance of insulin-secreting islet beta-cells of the pancreas. However, whether the similar thymic imaging/islet tolerance principle of insulin can be applied to other TSAs remains unanswered.

In this quarter, we succeeded in establishing the Aire-Cre:iDTR model, in which the human diphtheria toxin receptor is expressed exclusively in Aire-expressing mTECs in the thymus. This novel animal model enables us to further evaluate the immunomodulatory roles of mTECs in establishing immune self-tolerance of peripheral organs. Also in this quarter, we have successfully overcome one of the technical obstacles in our attempts to identify and characterize fluorescent molecule labeled T-regulatory cells in transgenic models.

Results

1. Efficient detection of T-regulatory cell-specific FoxP3 proteins in YFP-expressing cells.

As described in our previous Quarterly report, we have identified a population of Aire-expressing APCs in the spleen which display surface markers of tolerogenic plasmacytoid dendritic cells (pDCs, CD11c^{low}, MHCII⁺, Gr1⁺, B220⁺). Consistently, high levels of insulinitis were observed in pancreata of

animals in which the mouse *Ins2* gene is deleted in CD11c-expressing cells under the H2^{g7} and *Ins1*-knockout genetic backgrounds, suggesting that insulin-expression in CD11c cells is essential for maintenance of peripheral tolerance of islet beta-cells. Of note, one of the potential peripheral tolerogenic mechanisms mediated through pDCs is to promote the proliferation and survival of antigen-specific T-regulatory cells. Thus, the ability to track and characterize a specific population of T-regulatory cells, which are fluorescently labeled *in vivo*, is important to unravel the mechanism of insulin-expressing pDCs in exerting their protective roles of islet autoimmunity.

However, FACS detection of cytosolic or nuclear proteins in fluorescent protein (FP)-expressing cells has been shown to be technically challenging as loss of FP signal is frequently observed when the intracellular staining procedure is used. The common approach to overcome this obstacle is to FACS sort the FP-expressing cells first, followed by intracellular characterization. However such an approach is not only time-consuming, but also impractical when studying rare cells. We evaluated and modified the intracellular staining procedure for simultaneous detection of nuclear proteins and cytosolic YFP molecules, using the nuclear *Foxp3* molecule expressed in T-regulatory cells of *Vav-Cre:Rosa-YFP* reporter mice as a model target.

To identify *Foxp3*⁺ T-regulatory cells in *Vav-Cre: Rosa-YFP* mice, we first stained YFP⁺ splenocytes with antibodies specific to surface markers of T cells (i.e., CD3 and CD4), and subsequently subjected the cells to the intracellular staining procedure outlined in Figure 1A. To our disappointment, although we were able to stain nuclear *Foxp3* proteins efficiently, the YFP signal became essentially undetectable (Figure 1B). Loss of YFP signal could result either from fixative-induced conformation changes of the YFP proteins, or from the loss of cytosolic YFP proteins due to the leakiness of the permeabilized cell membrane. To investigate whether over-fixation is the major causative factor, we shortened the Fixation/Permeabilization (Fix/Perm) buffer treatment from 2 hours to 5 minutes, but still failed to preserve detectable YFP signal (Figure 1C). Indeed, even in the absence of fixatives, we observed significant reduction of cellular YFP signal after only 15 seconds of permeabilization buffer treatment, suggesting that the rapid loss of the fluorescent signal is due to diffusion of YFP molecules out of the permeabilized cell.

To further demonstrate that the leakiness of the permeabilized cell membrane is the cause of the fluorescent signal loss, we took advantage of the eGFP-*Foxp3* transgenic mice, in which an eGFP molecule was inserted in-frame to the 5' end of the *Foxp3* gene to encode a functional eGFP-*Foxp3* fusion protein. One of the major functional components in the permeabilization buffer used is saponin, which can complex with cholesterol to form pores in the cholesterol-rich cytoplasmic membrane but leaves the cholesterol-poor nuclear membrane largely intact. The nuclear eGFP-*Foxp3* fusion protein should be retained in the nucleus under such treatment and any reduction of eGFP signal should be attributed to the fixative. Splenocytes harvested from the eGFP-*Foxp3* mice were treated with Fix/Perm solution overnight, followed by intracellular staining with anti-*Foxp3* antibodies. No drastic loss of eGFP signal was observed and the eGFP⁺*Foxp3*⁺ population can be easily identified by FACS analysis (Figure 1D).

It has been shown that pre-fixation of the YFP⁺ cells with 2% paraformaldehyde is sufficient to effectively preserve the YFP signal, while the efficiency of intracellular staining of *FoxP3* is significantly compromised after 30 minutes of paraformaldehyde treatment (data not shown). We speculated that the loss of *FoxP3*-staining could be caused by over fixation of the cytoplasmic membrane, which impeded the entry of anti-*FoxP3* antibody into the cells. To systematically evaluate the effects of fixative pretreatment in intra-nuclear staining, we treated splenocytes harvested from the eGFP-*Foxp3* transgenic mice with 2% paraformaldehyde from 15 seconds to 30 minutes, prior to staining of the cells with anti-*Foxp3* antibodies. As shown in Figure 2A, pre-fixation from 15 seconds to 2 minutes does not drastically affect the percentage of *FOXP3*⁺ cells, whereas more than 5 minutes of paraformaldehyde treatment significantly decreases the antibody staining of the eGFP-*Foxp3* fusion proteins in the nucleus.

Figure 1

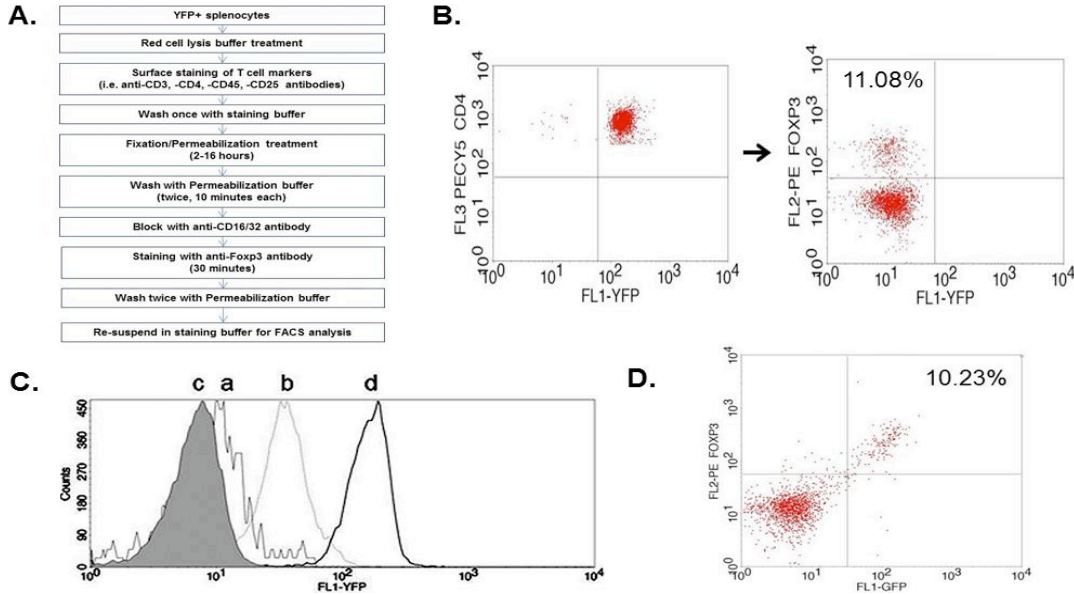


Figure 1. Loss of YFP signal in cells treated with Fixative/Permeabilization reagents used in intracellular staining procedures. **A.** A step-by-step protocol for intracellular staining of nuclear Foxp3 protein in T regulatory cells. **B.** YFP+, CD4+T cells harvested from Vav-Cre:Rosa-YFP mice were stained intracellularly with anti-Foxp3 antibodies following the procedure outlined in **A**. *Left panel*, before Foxp3 staining; *Right panel*, after Foxp3 staining. **C.** Splenocytes of Vav-Cre:Rosa-YFP mice were treated with Fix/Perm buffer for 5 minutes (**a**), or 15 seconds with permeabilization buffer (**b**). **c**, non-YFP control; **d**, untreated YFP+ T cells. Shown is a representative of FACS result from three independent experiments. **D.** Representative FACS result showing that nearly all the Foxp3 positive CD4+ T cells harvested from eGFP-Foxp3 mice are EGFP positive.

We thus speculated that the success of staining the nuclear FoxP3 molecules while preserving detectable YFP signals might hinge on the optimization of pre-fixation of cytoplasmic membrane to block the leakage of YFP molecules from cytosol, and to preserve the accessibility of the antibody to its nuclear target. To evaluate whether the short pre-fixation treatment is sufficient to block the leakage of the cytosolic YFP proteins, we treated splenocytes harvested from the Rosa-YFP mice with 2% of paraformaldehyde for less than 2 minutes before treating the cells with Fix/Perm buffer. With pre-treatment as short as 15 seconds we were able to detect weak YFP signals. Pre-fixing samples for more than 1 minute was more efficient to achieve a well-defined separation of YFP positive cells from YFP negative controls (Figure 2B). We next proceeded to add the short pre-fixation step to the intracellular staining protocol for Foxp3 detection in Rosa-YFP T regulatory cells. As shown in Figure 2C, a distinct population of CD4+ T cells can be readily identified as double positive for both YFP and Foxp3.

In summary, pre-fixing the cytoplasmic membrane for 1-2 minutes prior to the intracellular staining procedure is sufficient to retain cytosolic YFP proteins within the cells without compromising the antibody detection of cytosolic or nuclear proteins. Such a simple modification of the intracellular staining procedure (Figure 2D) not only allows the usage of FPs as cellular markers for co-localization

studies with FACS technology, but may also be applied to intracellular detection of cytosolic proteins to prevent loss of target molecules from membrane leakiness.

Figure 2

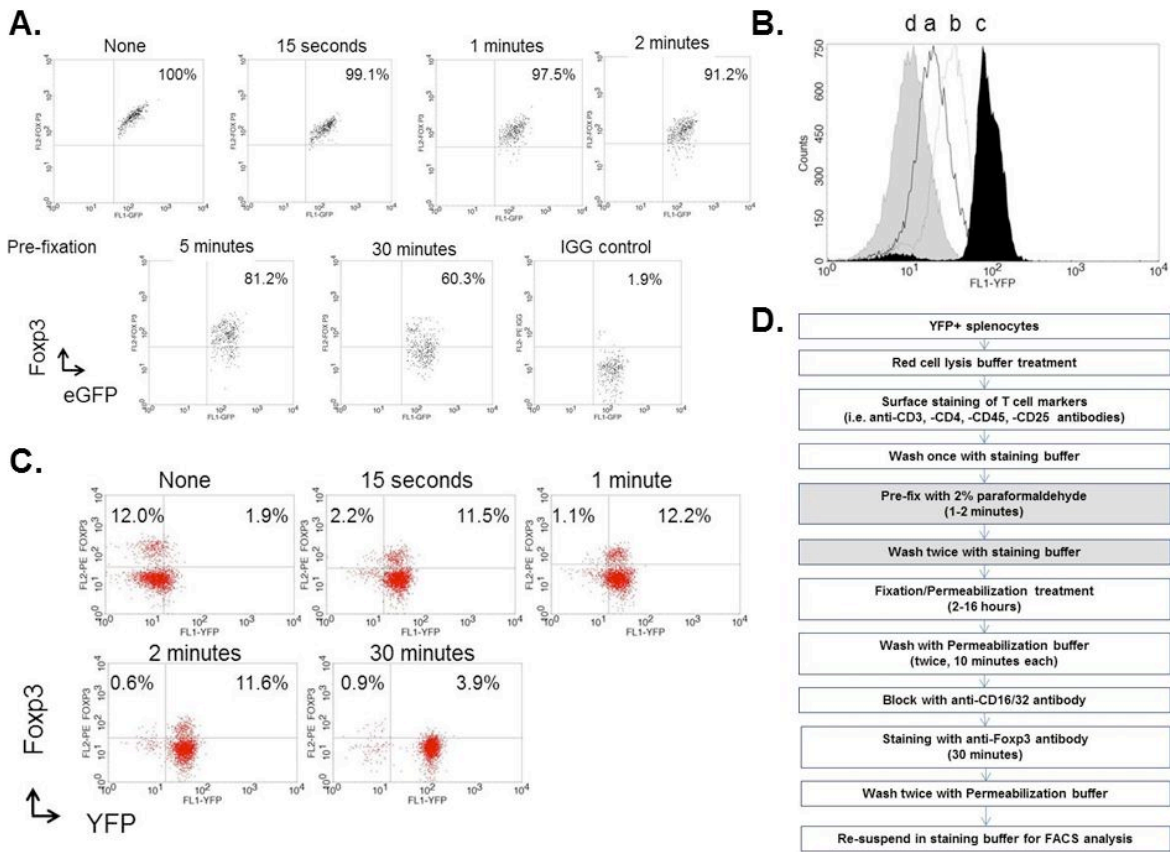


Figure 2. Short pre-fixation prior to intracellular staining is sufficient to retain detectable cytosolic YFP signals. **A.** Splenocytes harvested from eGFP-Foxp3 mice were stained intracellularly with anti-Foxp3 antibodies and analyzed by FACS (gated on CD4+eGFP+ T regulatory cell population). Percentages of cells with nuclear Foxp3 stained successfully under different pre-fixation conditions are shown. Shown are representative FACS results from five independent experiments. **B.** Splenocytes harvested from Vav-Cre:RosaYFP mice were pre-treated with 2% paraformaldehyde for 15 seconds (**a**), 1 minute (**b**), and 30 minutes (**c**), followed by Foxp3 intracellular staining and FACS analysis for YFP signals. **d**, sample without pre-fixation. **C.** Foxp3-staining of Vav-Cre:RosaYFP CD4+ T-cells pre-treated with 2% paraformaldehyde for various times (shown on top). Percentages of CD4+ T cells positive for both Foxp3 and YFP are shown on the upper right corners. Shown are representative results from five independent experiments. **D.** Modified intracellular staining protocol for nuclear Foxp3 detection. Steps in grey boxes were added to the original procedure in Figure 1A. For other cytosolic or nuclear proteins, optimization of the pre-fixation time will be required.

2. Establishing the Aire-Cre:iDTR animal model.

Taking advantage of the fact that the Aire-Cre transgene can truthfully recapitulate the endogenous Aire gene expression pattern, we crossed the Aire-Cre transgenic animal to Rosa26-iDTR mouse, in which the human diphtheria toxin receptor (DTR) gene, together with a floxed transcriptional blocking cassette at its 5' end, is site-specifically inserted at the Rosa26 locus. Action of the Cre-recombinase will delete the transcriptional blocking cassette from the genome and place the human DTR gene under the direct control of the ubiquitously active Rosa26 promoter. Only in cells transgenically expressing the Cre recombinase gene, human DTR are present at the cell surface; and they are susceptible to diphtheria toxin (DT)-mediated apoptotic cell death. Thus, in Aire-Cre:iDTR mice, DT-injection would, in theory, effectively eliminate mTECs in the thymus, as well as other Aire-expressing APCs in the periphery.

We successfully obtained a number of Aire-Cre:iDTR mice during this quarter. In our initial characterization of Aire-Cre:iDTR mice injected with DT, lymphocyte infiltration in multiple organs was observed, indicating loss of immune tolerance to self in response to mTEC deletion. Of note, these animals were treated at approximately 8-weeks of age, when T-regulatory cell and tolerogenic DC-mediated peripheral tolerogenic mechanisms were well-established, further indicating the dominant roles of central immune organ, the thymus, in mediating immune self-tolerance.

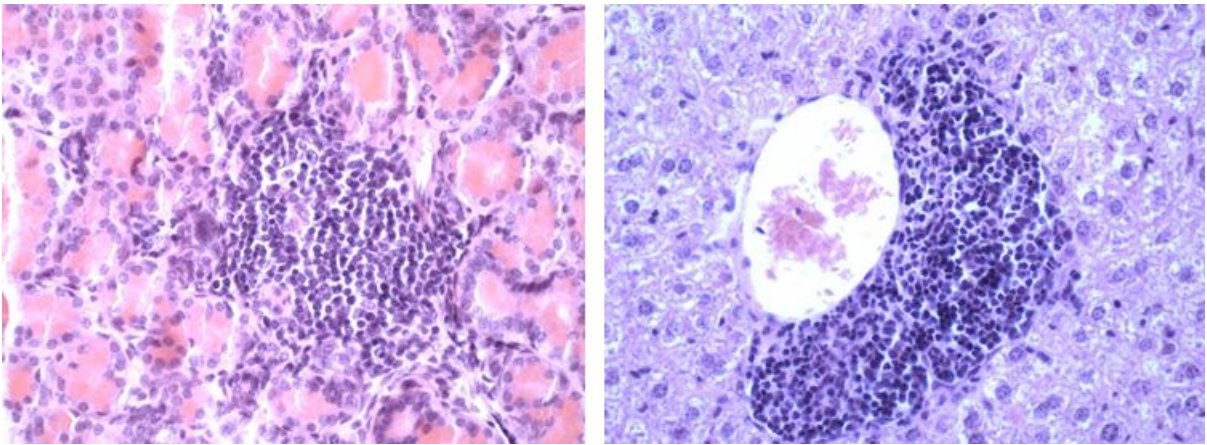


Figure 3. Multiple organ autoimmunity induced by DT-mediated mTEC-depletion. 8-week old Aire-Cre:iDTR mice injected with DT intramuscularly (i.m. at 5ng/kg body weight) for 2 consecutive days. Animals were sacrificed 4-weeks later and examined histologically (H&E). Left panel, salivary glands. Right panel, liver.

In summary, we have successfully developed an intracellular staining protocol which enables us to examine the presence of nucleic proteins, such as T-regulatory specific protein Foxp3 in a specific population of cells, while preserving their cytosolic YFP markers. In addition, we have established an inducible animal model, which will enable us to further unravel the essential roles of Aire-expressing cells in establishing and maintaining immune self-tolerance. Further characterization of these animal models will facilitate our understanding the etiopathogenesis of not only type 1 diabetes, but other autoimmune disorders as well.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

1) We have previously identified a population of insulin-expressing, Aire⁺ antigen-presenting cells (APCs) in the spleen which express a number of surface markers similar to pDCs (MHCII⁺, CD11c^{low}, B220⁺, Gr1⁺). In the next quarter, we will use both molecular and cell biology techniques to further characterize these cells. In addition, we will start to explore the therapeutic potential of these insulin-expressing, Aire⁺, APCs by transplanting these cells, either alone or together with FoxP3⁺eGFP⁺ T-regulatory cells into prediabetic ID-TEC pups. Diabetes incidence and progression will be monitored. As well, the ability of these APCs to home to the inflammatory pancreases, as well as secondary lymphoid organs of the ID-TEC pups will be examined. These results will be the first step for us to design more sophisticated gene therapy protocols to prolong the survival and efficiency of these tolerogenic insulin-expressing, Aire⁺ APCs in the future.

2) The establishment of the Aire-Cre:iDTR animal model enables us to investigate the essential roles of Aire-expressing cells in establishing self-tolerance at both central and peripheral levels. In the next quarter, we will examine the function of Aire-expressing, mTECs within the thymus for T-regulatory cell developments. In addition, the roles of Aire⁺ APCs in the peripheral lymphoid organs in maintaining T-regulatory cell homeostasis will be examined by a bone marrow transplantation model. These results will be instructive to develop therapeutic protocols to prolong the survival and efficiency of islet-specific T-regulatory cells to prevent autoimmune diabetes progression in our ID-TEC model.

Background and Significance

The essence of adaptive immunity is its capability to effectively distinguish self from non-self and take actions accordingly: eliciting protective immune response to invading pathogens while maintaining immune unresponsiveness to self-antigens under steady state. To establish a self-tolerant, functional T-cell repertoire, developing T-cells must survive both the positive and the negative selections mediated by self-antigen-presenting thymic stromal cells. T-cells fail to establish stable contacts with thymic epithelial cells of the thymic cortex will receive no survival signal and perish by negligence (positive selection), whereas T-cells bearing T-cell receptors (TCRs) with high affinity to self-antigens are considered to be autoreactive and undergo apoptosis within the thymic medulla (negative selection). While it is conceivable that blood-borne self-antigens can be captured and engulfed by the bone marrow (BM) -derived antigen presenting cells (APCs) and be trafficked back to the thymus, the mechanism of thymic presentation of autoantigens of tissue-specific nature remained elusive until about a decade ago when above-the-noise levels of transcripts of tissue-specific antigens (TSA) were found in medullary thymic epithelial cells (mTECs). One of the master regulators of TSA ectopic expression in mTECs is the autoimmune regulator (Aire) gene. Patients with mutations in the Aire gene develop autoimmune polyglandular syndrome I (APS-I), a rare autoimmune disorder affecting multiple organs and tissues, which is also known as autoimmune polyendocrinopathy, candidiasis, and ectodermal dysplasia (APECED). Targeted mutagenesis of the mouse Aire gene partially recapitulated the human disease symptoms – autoimmune responses, manifested as the presence of autoantibodies targeting multiple organs and tissues as well as lymphocyte infiltration, were observed in these animals. Of note, expressions of more than 300 TSAs are significantly down-regulated in mTECs, providing, for the first time, direct experimental evidence linking defective thymic TSA expression with peripheral multiple organ autoimmunity.

However, in most human autoimmune disorders of organ/tissue-specific nature, such as autoimmune diabetes type 1A (T1D) and autoimmune thyroid disease (ATD), one specific organ or tissue is primarily targeted. In addition, autoimmune responses targeting to one (or more) specific TSA(s), such as insulin and thyroglobulin, were often found to be the pivotal driving forces for disease progression. Thus, the broad effects of Aire inactivation on thymic TSA expression and its suggestive roles in mTEC differentiation and maturation render it difficult to study the impact of individual TSA in establishing immunologic tolerance of a specific organ with the Aire-KO animal model.

T1D is an autoimmune disease, in which the insulin-secreting pancreatic beta cells are targeted and destroyed by the body's own immune system. Although intensive insulin therapy and tight glycemia control can effectively prevent the devastating secondary complications, as demonstrated by DCCT and other large scale clinical studies, hypoglycemia incidence remains as the major adverse event leading to increase of morbidity and mortality in T1D patients. Of note, numerous researches have shown that even residual islets in T1D patients can significantly lower the risk of severe hypoglycemic incidence and can help to achieve better glycemic control. Thus, developing islet antigen-based immune therapies to restore islet immune tolerance is clinically important to prevent high-risk individuals from developing T1D and to halt islet destruction in recent onset T1D patients.

Our research has been focusing on the immunomodulatory roles of islet autoantigen expression in immune cells in establishing/maintaining islet tolerance. Using the insulin-deleted thymic epithelial cells (ID-TEC) mouse model (Fan et al. *The EMBO Journal* 28:2812, 2009), we have demonstrated conclusively the essential roles of mTEC-insulin expression in mediating central negative selection of insulin-reactive T-cells in establishing a T-cell repertoire tolerant to islet beta cells of the pancreas. In addition, we were able to identify a population of hematopoietic lineage-derived Aire-expressing antigen presenting cells (APCs) in the spleen which express surface markers shared by tolerogenic plasmacytoid dendritic cells (pDCs, CD11c^{low}, MHC Class II⁺, Gr-1⁺, B220⁺). Notably, insulin transcripts were predominantly found in this population of Aire⁺ cells.

Furthermore, deletion of insulin-expression in CD11c-expressing cells with the CD11c-Cre transgene resulted in elevation of insulinitis in animals with defective central negative selection (carrying diabetes-prone H2^{g7} MHC), suggesting a role of insulin-expression in CD11c cells in maintaining peripheral tolerance of islet beta cells.

In an effort to unravel the mechanism of insulin-expressing pDCs in exerting their protective roles of islet autoimmunity, we further characterized the Aire⁺ pDC-like cells in the spleen in this quarter. In addition, we further refined the intracellular staining protocol for effective detection of fluorescent molecule labeled T-regulatory cells.

3. Further characterization of Aire⁺ APCs in the spleen.

To further characterize the Aire⁺, pDC-like cells described previously, we FACS sorted the YFP⁺ cells (representing Aire⁺ cells) from Aire-Cre:RosaYFP mice and subjected them to surface marker analysis. To our surprise, only about 5-6% of all YFP⁺ cells express mPDCA-1, one of the pDC-specific surface markers routinely used to define the pDC population. Thus, the Aire⁺ cells identified in our model might not represent APCs of one specific lineage, rather, Aire-positivity, in conjunction with other surface markers, such as B220, MHCI, Gr-1 and CD11c intermediate/low, might actually reflect the common molecular property of various tolerogenic APCs. As reported previously, the level of MHC Class II and costimulatory molecules CD80 and CD86 expression are indicators of a maturation status of APCs: lowly expressed in immature DCs and tolerogenic pDCs at resting stage, but significantly up-regulated upon activation and antigen exposure. To further characterize the properties of Aire⁺ APCs isolated from the spleen, we used magnetic bead technology to separate YFP⁺ cells into two groups based on their levels of MHC Class II and costimulatory molecule expression. YFP⁺ cells predominantly belong to the MHC Class II^{high} group, whereas less than 0.5% expresses low levels of MHC Class II and co-stimulatory molecules. As shown in Figure 1A, about 40-50% of YFP⁺MHC Class II^{low} cells express high levels of pDC-specific marker, mPDCA-1, suggesting that they are resting pDCs. In contrast, only about 5% of YFP⁺MHC Class II^{high} cells are positive for mPDCA-1. The levels of mPDCA-1 expression in mPDCA1⁺YFP⁺MHC Class II^{high} cells were significantly lower than those of mPDCA1⁺YFP⁺MHC Class II^{low} and mPDCA1⁺YFP⁻ cells (Figure 1B). Thus, down-regulation of mPDCA-1 expression might be the result of YFP⁺ APC activation. As YFP⁺ APCs ectopically express and present self-antigens, such as insulin, it is conceivable that they might interact predominantly with T-cells bearing autoantigen-reactive TCRs, to induce anergy of self-reactive effector T-cells or to promote the survival/proliferation of T-regulatory cells. Indeed, we have observed a positive correlation between the numbers of T-regulatory cells and the numbers of YFP⁺ APCs in aged animals (data not shown), suggesting a role of YFP⁺ APCs in supporting T-regulatory survival.

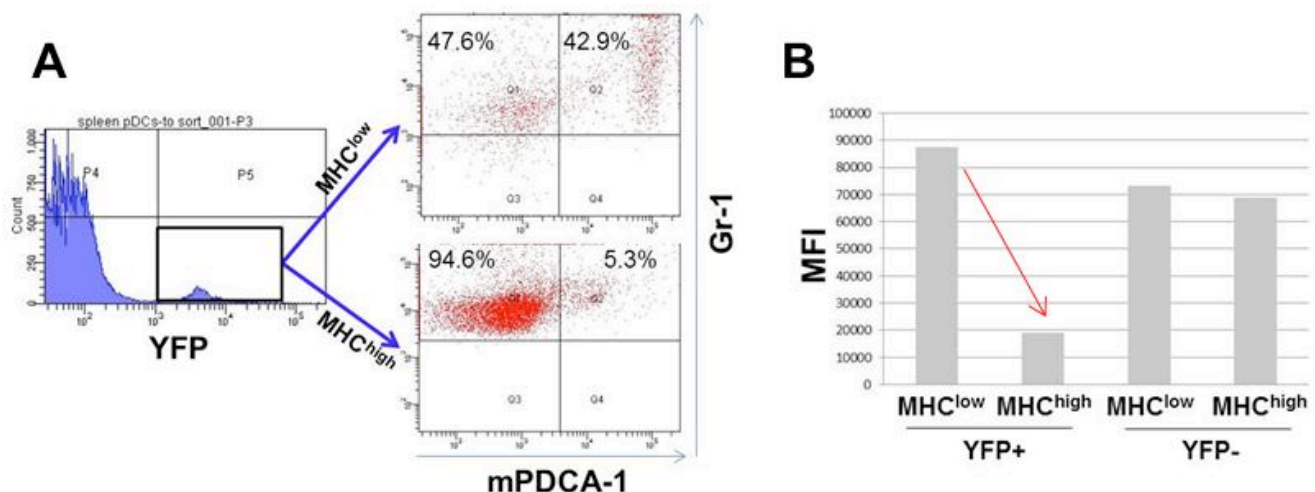


Figure 1. Characterization of YFP⁺ cells harvested from Aire-Cre:RosaYFP spleens. **A.** Representative FACS data showing that the YFP⁺ cells are predominantly mPDCA-1 negative. **B.** In mPDCA-1 positive populations, activated YFP⁺MHC^{high} cells express significant lower levels of mPDCA-1, in comparison to MHC^{low}YFP⁺ or YFP⁻ cells. MFI = mean fluorescent intensity.

4. Optimization of intracellular staining protocol to examine YFP⁺ T-regulatory cells.

In the previous quarter, we have developed a novel FACS method which enables us to intracellularly stain the nucleic FoxP3 proteins while preserving detectable levels of cytosolic YFP proteins for lineage identification. The success of procedure hinged on the optimization of pre-fixation of cytoplasmic membrane to block the leakage of YFP molecules from cytosol, and to preserve the accessibility of the antibody to its nuclear target. We have shown that a short prefixation with 2% of paraformaldehyde for 2 minutes fits the required fine balance. However, such a short time window of pretreatment might not be suitable for staining a large number of samples simultaneously; and the high concentration of paraformaldehyde fixative used rendered it hard to achieve precise control over the prefixation step. To overcome these problems and to optimize the protocol for broader use, we developed a formula to quantify the effect of prefixation on YFP⁺ cells undergoing intracellular staining procedure: Prefixative Factor (PF) = % of paraformaldehyde x prefixative time (minute). As shown in the Table below, four different concentrations of paraformaldehyde were systematically evaluated.

Time (min)/PF	A	B	C	D
Final conc. of paraformaldehyde	2%	1%	0.5%	0.01%
1	30 min/60	30 min/30	30 min/15	30 min/0.3
2	15 min/30	15 min/15	15 min/7.5	15 min/0.15
3	5 min/10	5 min/5	5 min/2.5	5 min/0.05
4	2 min/4	2 min/2	2 min/1	2 min/0.02
5	1min/2	1min/1	1min/0.5	1min/0.01

As shown in Figure 2A and highlighted in the Table above, PF $\geq$ 4 ensures sufficient fixation of the plasma membrane to preserve enough cytosolic YFP proteins for detection. Since over fixation of the plasma membrane prevents the entrance of anti-FoxP3 antibodies into the cells (shown previously), we projected that 4 $\leq$ PF $\leq$ 20 would represent the optimal conditions for intracellular staining of YFP⁺ cells (Gray area in Figure 2A). Indeed, we were able to stain FoxP3 efficiently in YFP⁺ cells under these conditions (Figure 2B and 2C and data not shown).

Summary

In this quarter, we characterized further Aire⁺ tolerogenic APCs in our Aire-Cre:RosaYFP animal models. Our results suggest that it is rather unlikely that these cells belong to one specific APC lineage, but rather, Aire-positivity represents the tolerogenic properties of self-antigen presenting APCs. As most of these cells express high levels of MHCII and costimulatory molecules, they might actively present autoantigens to T-regulatory cells to promote the homeostasis.

In addition, we have systematically examined and optimized the conditions for intracellularly staining of FoxP3 in YFP⁺ cells. The protocol enables us to trace the origins of FoxP3⁺ cells in adoptive T-cell transfer animal models, which will facilitate our efforts to understand the underlying mechanisms to maintain homeostasis of T regulatory cells under physiological and pathologic situations.

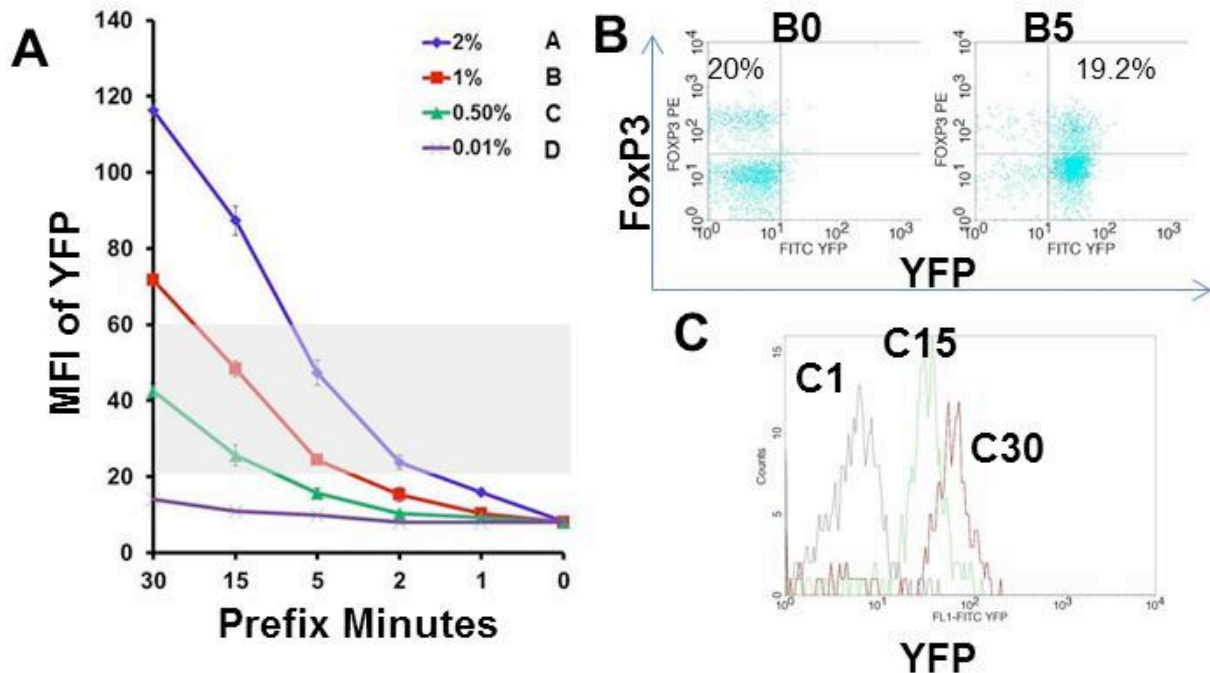


Figure 2. Intracellular staining of nucleic FoxP3 proteins in YFP+ cells. **A.** Preservation of YFP signals were evaluated in YFP+ cells prefixed with four different concentration of paraformaldehyde (**A**, 2%; **B**, 1%; **C**, 0.5% and **D**, 0.01%) for the designated periods of times. MFI above 20 usually provides sufficient separation of the YFP signals from the untreated controls. The experiments were repeated three times. **B.** Representative FACS results showing the effective detection of FoxP3 proteins without compromising of YFP signals. **B0** and **B5**, prefix with 1% paraformaldehyde for 0 and 5 minutes, respectively. The numbers in the FACS plots show the % of T regulatory cells present in the CD4+ T-cell population. As shown, prefixation under the B5 condition did not compromise FoxP3 staining. The experiments were repeated three times with similar results. **C.** Preservation of YFP signals in cells intracellularly stained for FoxP3. YFP+ samples were prefixed with 0.5% paraformaldehyde for 1 (**C1**), 15 (**C15**) and 30 (**C30**) minutes, followed by FoxP3 staining. Representative FACS results were gated on CD4+FoxP3+ cells. Shown are representative results of three independent experiments.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

3) We have previously identified a population of insulin-expressing, Aire+ antigen-presenting cells (APCs) in the spleen which express a number of surface markers similar to pDCs (MHCII⁺, CD11c^{low}, B220⁺, Gr1⁺). In the next quarter, we will use both molecular and cell biology techniques to further characterize these cells. In addition, we will start to explore the therapeutic potential of these insulin-expressing, Aire+, APCs by transplanting these cells, either alone or together with FoxP3⁺eGFP⁺ T-regulatory cells into prediabetic ID-TEC pups. Diabetes incidence and progression will be monitored. As well, the ability of these APCs to home to the inflammatory pancreases, as well as secondary lymphoid organs of the ID-TEC pups will be examined. These results will be the first step for us to

design more sophisticated gene therapy protocols to prolong the survival and efficiency of these tolerogenic insulin-expressing, Aire⁺ APCs in the future.

4) The establishment of the Aire-Cre:iDTR animal model enables us to investigate the essential roles of Aire-expressing cells in establishing self-tolerance at both central and peripheral levels. In the next quarter, we will examine the function of Aire-expressing, mTECs within the thymus for T-regulatory cell developments. In addition, we will examine the roles of Aire⁺ APCs played in the peripheral lymphoid organs, in maintaining T-regulatory cell homeostasis, using a bone marrow transplantation model. These results will be instructive to develop therapeutic protocols to prolong the survival and efficiency of islet-specific T-regulatory cells to prevent autoimmune diabetes progression in our ID-TEC model.

5) We will then continue to characterize the Aire-Cre:iDTR animal model developed in the lab, which have shown autoimmune responses targeting multiple organs upon DT-mediated depletion of Aire-expressing cells. Specifically, we will examine the affected organs systematically and investigate the underlying mechanisms of such loss of self-tolerance.

6) The golden standard to define tolerogenic APCs is to demonstrate their capabilities to: 1) inhibit autoreactive T-cell activation upon antigen stimulation, and/or 2) promote survival/proliferation of T-regulatory cells. In the next quarter, we will setup *in vitro* assays to examine whether Aire⁺ splenocytes possess these tolerance-induction properties. Furthermore, cytokine secretion profiles upon stimulation will also be examined, focusing on those that are essential for tolerance inductions, such as TGF-beta and IL-10. In addition, we will continue to investigate their potential therapeutic function in halting the progression of islet-autoimmunity in prediabetic ID-TEC pups.

In the fourth quarterly scientific progress report (06/28/11 - 09/27/11) of year 02, we now report on our new and cumulative results.

The establishment of the Aire-Cre:iDTR animal model enables us to investigate the essential roles of Aire-expressing cells in establishing self-tolerance at both central and peripheral levels. We then examined the function of Aire-expressing, mTECs within the thymus for T-regulatory cell developments. In addition, we examined the roles of Aire⁺ APCs played in the peripheral lymphoid organs, in maintaining T-regulatory cell homeostasis, using a bone marrow transplantation model. These results are instructive to develop therapeutic protocols to prolong the survival and efficiency of islet-specific T-regulatory cells to prevent autoimmune diabetes progression in our ID-TEC model.

Developing an intracellular staining protocol for efficient detection of nuclear proteins in YFP-expressing cells

In the previous quarter, we have systematically examined and optimized the conditions for intracellularly staining of T regulatory cell specific transcription factor FoxP3 in YFP⁺ cells. Our results indicate that the success of simultaneous detection of cytoplasmic YFP proteins and FoxP3 is hinged on the combined effect of effect of prefixation time and concentration of the fixative used. We defined the value of prefixation time (minutes) x % of fixative as prefixation factor (PF), and concluded that while PF $\geq$ 4 is required to retain enough YFP proteins for flow cytometry (FCM) analysis, PF $\geq$ 30 is detrimental to FoxP3 staining. Since various intracellular antigens may differ substantially in their retention and susceptibility to Fix/Perm treatment, we also examined the nuclear expression of *helios*, a member of nuclear expressed transcription factor of the Ikaros family, in YFP⁺ splenocytes to demonstrate the potential broad application of our intracellular staining protocol. As shown in Figure 1, effective detection of both Helios and YFP were achieved under conditions similar to FoxP3/YFP staining. Thus, PFs range from 4 to 20 represents the optimal conditions to obtain efficient nuclear protein staining, and well separation of the YFP signals from the background. It is conceivable that the optimal PF will vary in different models; for cells with weak fluorescent signals, a higher PF (10-20) could be preferred, and vice versa. Nevertheless, the simple modification of the intracellular staining procedure described above reliably enables the usage of cytoplasmic FPs as cellular markers for co-localization studies with FCM technology.

Direct contacts between FoxP3⁺ cells and Aire-expressing stromal cells in secondary lymphoid organs.

We have demonstrated previously that organ-specific self-antigens, such as insulin and islet autoantigen 69 (ICA69), are lowly expressed not only in the thymus, but also present in secondary lymphoid organs. While our ID-TEC animal model have demonstrated conclusively the essential roles of islet autoantigen expression within the thymus in establishing immune tolerance of pancreatic beta cells, the roles of islet antigen production in secondary lymphoid organs remain elusive. To answer these questions, we have developed Vav-Ins and CD11c-Ins animals, in which insulin expression are specifically abrogated in bone marrow-derived antigen presenting cells (APCs) and CD11c-expressing dendritic cells (DCs), respectively. As reported previously, our data suggest that islet autoantigen expression in secondary lymphoid organs play important maintenance roles in restricting the clonal expansion of insulin-reactive T cells in the periphery. As one potential mechanism for autoreactive T-cell inhibition is through the dominant suppressive activity of T regulatory (Treg) cells. Either derived from the thymus or converted from CD4⁺ effector T cells, these cells comprise of 10-20% of CD4⁺ T-cell population and express the

transcription factor FoxP3. Elimination of FoxP3+ Treg cells through genetic engineering or antibody based depletion will lead to unchecked proliferation of effector T-cells, resulting in lymphocyte infiltration into multiple organs and tissues. Interestingly, Treg cells are self-reactive (with cognate autoantigens) and undergo constant turnover and proliferation. However, the underlying mechanism for maintaining Treg cell homeostasis is still largely unknown at present. Based on these findings, **we hypothesized that the Aire- expressing APCs in the secondary lymphoid organs, play essential roles in Treg cell homeostasis in the periphery, by directly presenting organ-specific autoantigens to Treg cells to drive their proliferation.** As the first step to test this hypothesis, we took advantage of our previous findings that the Aire-Cre transgene can faithfully recapitulate the endogenous Aire gene expression pattern, and labeled all Aire-expressing cells with EYFP by generating the Aire-Cre:Rosa26R-EYFP (Aire-YFP) reporter mice. Both spleen and lymph nodes were harvested from the Aire-YFP reporter mice and were subjected to immunohistochemical analysis of Foxp3+ Treg cells and Aire-expressing tolerogenic DCs. As shown in Figure 2 (*top panels*), both Foxp3+ Treg cells and Aire-expressing DCs were localized in the marginal zone areas (MZ), adjacent to the follicular dendritic zone (FDC) of both the spleen and the lymph nodes. Furthermore, cell-cell contacts between Aire-expressing cells and Treg cells were observed (Figure 2, arrows in *lower panels*), suggesting the existence of immunologic cross-talks between the two cell types.

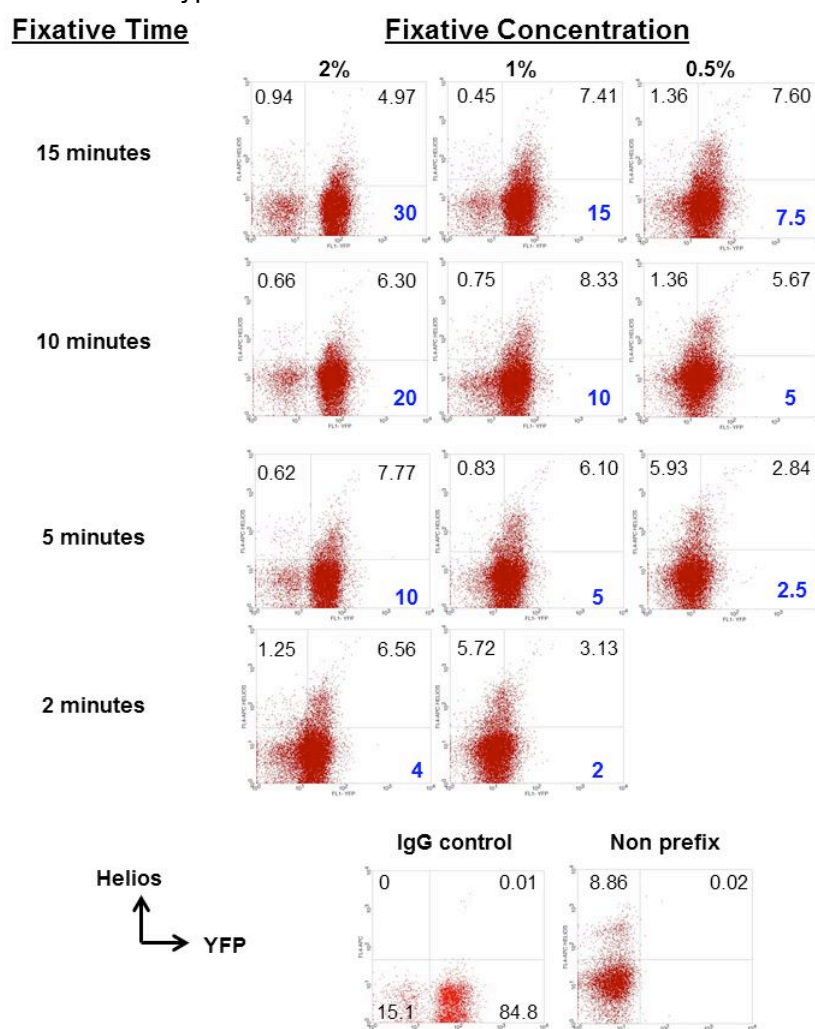


Figure 1. Flow cytometric detection of transcription factor Helios expression in YFP+ splenocytes. Splenocytes harvested from Vav-Cre:RosaYFP were pre-fixed with 0.5%, 1% and 2% paraformaldehyde for the times shown. Percentages of splenocytes positive for both Helios and YFP are shown on the upper right corners. Shown are representative results from two independent experiments. The blue numbers in each panel represents the PF factors under each condition. As shown, pre-fixation with PF range from 4-20 allows effectively staining of Helios, while preserving above background levels of YFP signals.

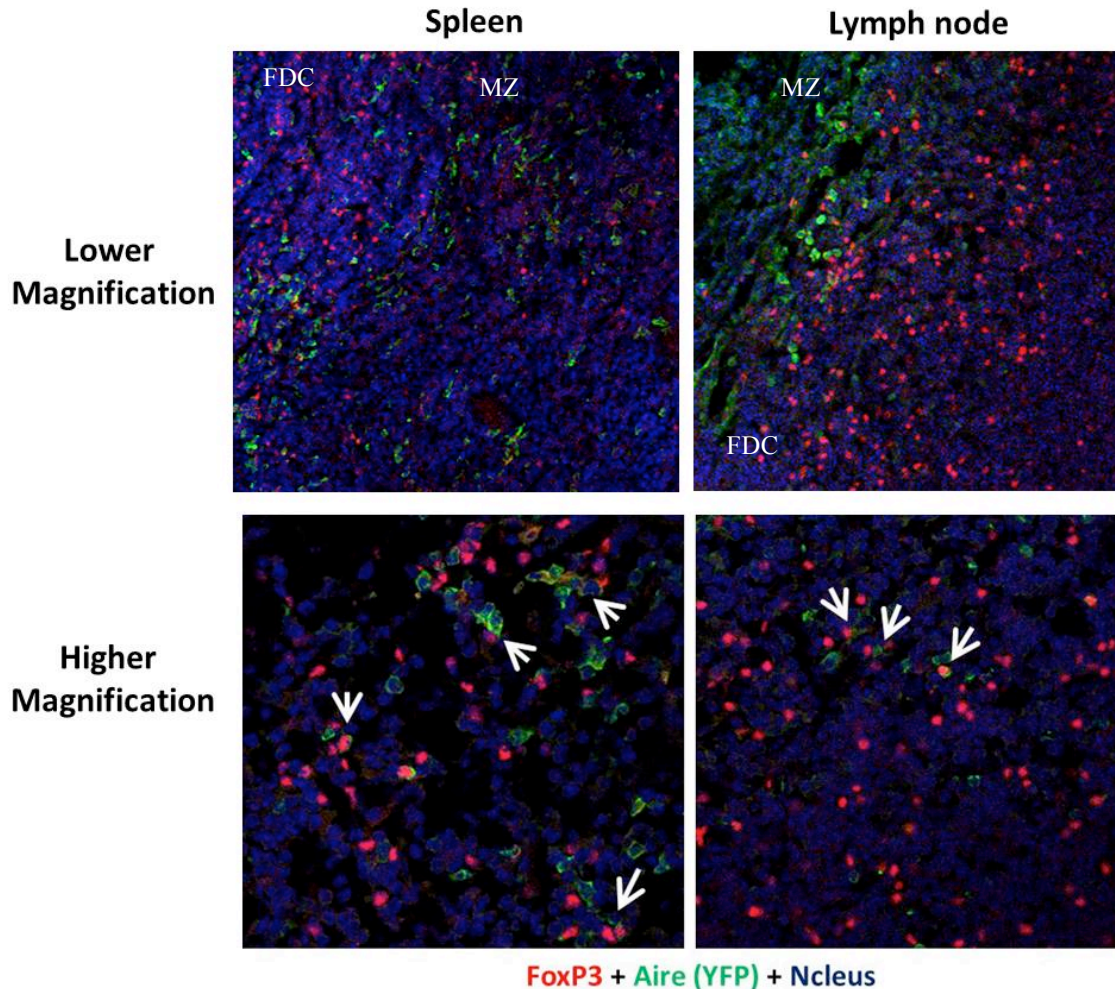


Figure 2. Immunohistochemical analysis of direct cellular interaction between FoxP3+ Treg cells and Aire-expressing tolerogenic DCs. Spleens and lymph nodes were harvested from Aire-Cre:Rosa26R-EYFP mice and fixed with 4% paraformaldehyde for 3 hours at 8°C. Cryosections of 7µm were co-stained with anti-FoxP3 (red) and anti-YFP (green) antibodies. Top Panel: low magnification images showing that Aire-expressing cells (green) are predominantly present in the marginal zone (MZ) area adjacent to the follicular dendritic zone (FDC) in both the spleen (Left panel) and the lymph nodes (Right panel). Top Panel: higher magnification images showing the existence of direct interactions between the Foxp3+ Treg cells and Aire-expressing DCs. Arrows indicate the close contacts between Tregs and Aire+DCs in both the spleen and the lymph nodes.

Use additional pages to present a brief statement of plans or milestones for the next quarter.

We will continue to characterize the Aire-expressing cells in secondary lymphoid organs. Specifically, we will systematically examine the expression of islet-specific genes in both the CD45+Aire+ splenocytes and the CD45-Aire+ stromal cells of the lymph nodes. In addition, their antigen-presenting capabilities and their response under immune stimulating conditions will be investigated in vitro. Furthermore, we are in the process of generating the Aire-Cre: IAb-fl/fl mice, in which the antigen presenting MHC II molecules will be specifically knocked out in Aire-expressing cells. This animal line will help us to demonstrate the essential roles of self-antigen presentation by Aire-expressing tolerogenic DCs in maintaining Treg homeostasis.

KEY RESEARCH ACCOMPLISHMENTS:

1. We have successfully generated and characterized the ID-BMC animal model in which insulin expression is abrogated only in bone marrow derived cells. We further demonstrated that the absence of insulin in bone marrow cells is not sufficient to induce autoimmune diabetes, thus, emphasize the dominant roles of central mechanism in T1D etiology.
2. We have identified two insulin-expressing stromal cell types in secondary lymphoid organs, and we will explore their therapeutic potency to strengthen peripheral tolerogenic mechanism to counteract preexisting faulty thymic negative selection conditions.
3. We have successfully generated ID-DC and ID-DC-H2g7 animals, in which insulin expression is specifically knocked out in both classic and tolerogenic dendritic cells. These animals will allow us to dissect further the interactions between central and peripheral immune mechanisms in establishing/regaining insulin tolerance.
4. Intracellular staining is a widely used flow cytometry (FCM)-based technique to detect the expression of cytosolic/nucleic antigens. However, intracellular staining of cells expressing cytosolic fluorescent protein (FP) markers was proven to be problematic as significant loss of the FP-signal was routinely observed. Using splenocytes harvested from mice constitutively expressing the enhanced yellow fluorescent proteins (YFP) as a model, we modified the widely used intracellular staining protocol and successfully achieved simultaneous detection of both the nuclear proteins and YFP in T-regulatory cells. The improved protocol can be used to perform antibody-based intracellular characterization of FP-labeled target cells, while maintaining their fluorescent reporter signals for easy tracing and identification.

REPORTABLE OUTCOMES:

Manuscripts (5 publications)

1. Lu C, Kumar PA, Fan Y, Sperling MA, Menon R: A novel effect of GH on macrophage modulates macrophage-dependent adipocyte differentiation. **Endocrinology** 151(5): 2189, 2010.
2. Perdomo G, Kim DH, Zhang T, Qu S, Thomas EA, Toledo FG, Slusher S, Fan Y, Kelley DE, Dong H: A role of apolipoprotein D in triglyceride metabolism. **Journal of Lipid Research** 51: 1298, 2010.
3. Mavalli M, DiGirolamo D, Fan Y, Riddle R, Campbell K, Sperling M, Frank S, Bamman M, Clemens T: Distinct growth hormone receptor signaling modes control skeletal muscle development and insulin sensitivity. Manuscript accepted for publication in the *Journal of Clinical Investigation*. **J Clin Invest**. doi:10.1172/JCI42447, 2010.
4. Trucco M: Beta-cell regeneration: from science fiction to challenging reality. **Pediatric Diabetes** 11(5):292, 2010.
5. Grupillo M, Lakomy R, Geng X, Styche A, Rudert WA, Trucco M, Fan Y: An improved intracellular staining protocol for efficient detection of nuclear proteins in YFP-expressing cells. **BioTechniques**, In press, 2011.

CONCLUSION:

The conclusions from the current year of funding are that we demonstrate conclusively the dominant roles of thymic insulin expression in establishing adaptive immune tolerance towards islet beta cells of the pancreas. Ablation of insulin expression in bone marrow derived antigen-presenting cells will not induce autoimmune diabetes. We also identified two types of insulin-expressing stromal cells with antigen-presenting capability in the stroma of secondary lymphoid organs, which might play roles in maintaining peripheral islet beta cell tolerance under central defective conditions.

The So What Section:

What are the implication of this research?

Diabetes affects 16 million Americans and roughly 5-15% of all cases of diabetes are type 1 DM. It is the most common metabolic disease of childhood, and physicians diagnose approximately 10,000 new cases every year. Type 1 diabetes is associated with a high morbidity and premature mortality due to complications. The annual cost from diabetes overall exceeds \$100 billion, almost \$1 of every \$7 dollars of US health expenditures in terms of medical care and loss of productivity.

What are the military significance and public purpose of this research?

As the military is a reflection of the U.S. population, improved understanding of the underlying etiopathology of T1D will facilitate the development of potential therapeutics to prevent the onset of the disease or the development of diabetic complications among active duty members of the military, their families, and retired military personnel. Finding a cure to T1D will provide significant healthcare savings and improved patient well-being.

Perspective

Beta-cell regeneration: from *science fiction* to challenging reality

Type 1 diabetes is due to an autoimmune reaction directed against the pancreatic beta cells. Since some regenerative capabilities of the endocrine pancreas have now been quite well documented, and recent research has shown that human stem cells can be derived from embryos or from genetically engineered somatic cells, is it practical or even possible to combine these lines of research to more effectively treat young diabetic patients? The recently published paper of Pedro Herrera and his group (1) inspires a number of considerations that might help to properly answer this question, while bringing to mind unforgettable scenes from a popular 90's film. Herrera's results show that after near-total ablation of beta cells in the endocrine pancreas, some new beta cells are actually generated. However, these new beta cells originate from already existing alpha cells, rather than from preexisting beta cells or from other precursor cells.

Metallic drops, warmed by the sparks of melting iron, quiver and flow on the ground like quicksilver. They coalesce, and together they expand, change conformation, and eventually assume their final shape: it's T-1000, the liquid metal-based robot in the famous movie "Terminator 2" directed by James Cameron. The T-1000 is the nemesis of the T-800, the other, more conventional robot, sent back in time as mankind's savior. The task for the T-800 is quite difficult, since the T-1000 seems to be indestructible: Even after enduring tremendous damage, it can easily reassemble, repair, and finally regenerate itself, quickly resuming its original form.

Fortunately for us, we do not have to be overly envious of the T-1000. Once damaged, parts of the human body also have the ability to repair, reassemble, and regenerate themselves, ultimately resuming their original conformations. With the exception of only a few tissues, our cells can be replaced fairly quickly and regenerate into functional tissues. Perhaps we

should not be surprised, then, that the endocrine pancreas is capable of functional regeneration, given adequate time and the appropriate physiological conditions. However, the nature of the requisite beta cell precursors, how they function, where they are physically located, and what influences them to regenerate the correct missing tissue, are all questions still awaiting definitive answers.

The 'stem cell' is, by definition, the one cell capable of duplicating itself and while maintaining its undifferentiated status, could also originate a progeny that differentiate into one or more different final products that are physiologically defined by their specific functions. Proceeding through the differentiation pathway, stem cells can be categorized as totipotent, pluripotent, multipotent, oligopotent, and unipotent, depending upon all their possibly irreversible, progressively acquired characteristics. Twins can actually develop from the same zygote, if its derived cells are properly separated very early in the embryological process. Although then, the vertebrate zygote might be considered the preeminent totipotent stem cell, it continues to divide to form the final individual: a mixture of similar, but not identical, daughter cells. Since we do not know yet which specific markers characterize the totipotent cell, we also do not know whether totipotent cells are still preserved, once the various tissues begin to differentiate into organs. If they are, how long could they continue to be functional? Intuitively, we can argue that precursors of some kind should still be present and active within the body indefinitely, because even elderly people are able to repair their damaged tissues. However, we still do not know the number of these regenerative precursors, where they may be hiding, and which level of differentiation they have already achieved.

Paraphrasing what I wrote some years ago (2), I would argue that a system based on a single regenerative center, serving all the peripheral needs, would be quite inefficient. If this were the case, even if an 'S.O.S.' transmitted from the periphery traveled quickly, the center, once alerted, would need too much information and too much time to generate

the specific precursors. In other words, the center would not be able to deliver an adequate number of the appropriate cells to the 'scene of the crime' quickly enough to avoid disaster. Therefore, to be most effective, these hypothetical regenerative centers would have to be scattered throughout the body. This is the same rationale for stationing firehouses throughout an entire city, to allow each unit to be able to more rapidly reach any fire location and efficiently intervene.

Our sophisticated regenerative system does not need to deploy totipotent cells into each organ. It would be enough to maintain in each organ sufficiently undifferentiated precursors with self-maintenance capabilities, as well as those necessary to replace the worn-out cells of the organ. Also desirable would be a physiological process that progresses relatively slowly to maintain tissue homeostasis but which could be converted, in the case of crisis, into a rapidly operating system. Consequently, this system would be highly effective at responding promptly to abruptly received, alarming feedback signals.

In the endocrine pancreas as well, long-lasting insulin-producing beta cells should be continuously, albeit quite slowly, replaced by newly generated cells. At the time of need, besides the possible replicative ability of the beta cells themselves (3), other regenerated cells should come from precursors, possibly located among pancreatic ductal cells (4). Thus, these regenerative units would be in close physical proximity to the endocrine tissue, the islets of Langerhans. When the pancreas is physically damaged, the pace of the physiologic reparative process accelerates and a more evident regeneration product can be observed.

This actually seems to be the message we receive from the recently published paper of Herrera's group (1) in which the inherent regenerative capacity of the adult pancreas to produce new beta cells was systematically studied. To generate a strong enough 'danger signal', an extreme situation was created in which near-total beta cell loss can be obtained at will to mimic type 1 diabetes pathology, but in the absence of autoimmunity. For this purpose, the authors used two *in vivo* genetic approaches: cell ablation combined with cell lineage tracing. Inducible, rapid cell removal (>99%) was obtained by administration of diphtheria toxin (DT) in transgenic mice in which the diphtheria toxin receptor (DTR) was expressed by the beta cells only. The systemic administration of DT permits an exquisite, specific ablation of almost all existing beta cells by apoptosis. Newly formed beta cells were easily monitored using a reliable cell lineage tracing. The results obtained surprisingly showed that the adult pancreas can actually generate new beta cells after their near-total loss, but mainly by reprogramming its glucagon-secreting alpha cells.

Taking all this information together, a physiologic scenario can be then envisaged in which tissue-specific precursors, present among pancreatic ductal cells (4), are generating alpha cells and beta cells (2). Beta cells, in turn, even if extremely slowly, replicate themselves to maintain homeostasis (3). However, once stimulated by a powerful danger signal, glucagon-positive alpha cells can then transdifferentiate into insulin-secreting beta cells to repair the damage (1).

In humans, without the help of lineage tracing, specific markers become necessary to recognize and eventually physically isolate tissue-specific precursors. However, even if these markers were already utilizable, intuitively one sees that isolating precursor cells from a patient's own pancreas would not be an easy task. Increasing their numbers *ex vivo* while avoiding the activation of differentiation pathways would also be problematic, as would facilitating their differentiation toward the wanted final product. Here again James Cameron anticipated a possible solution of the problem.

The T-1000, like an exceptional chameleon, blends into any scene, assuming the most appropriate appearance suggested by each environment it encounters. In the film, the T-1000 completely hides itself, becoming part of the black and white tiles of the floor of a hospital ward. It then regenerates from the linoleum into a human form. However, it does not resume its original human appearance; instead, it takes on the appearance of the security guard on duty where the patient, for whom it is looking, is unwillingly detained.

Faithfully recreating the environment necessary to guide and facilitate a desired type of differentiation *in vitro* appears to be quite difficult. An easier solution might be to somehow isolate and then physically introduce a precursor into the already existing, appropriate environment like, e.g., the embryonic pancreas (5). The precursor would then be allowed to get 'acquainted' with its new surroundings by assuming the most appropriate appearance (i.e., phenotype) to better fit the new context. By becoming one with the new environment, the precursor may quickly convert into the product best equipped to repair the damage. The signals sent through host-secreted factors or by cell-to-cell contacts seem to be powerful enough to guide the differentiation process toward the most needed product, even across different lineage barriers. This ability to 'transdifferentiate' (i.e., generate a progeny belonging to a tissue lineage different from the one of origin) certainly is an astonishing discovery. A few years ago, no one would even speculate that a mammalian stem cell,

present in an adult individual, could still possess such an impressive plasticity. The possibility that new beta cells could be generated from 'adult' stem cells even passing through an alpha cell phenotype (1) offers a particularly appealing alternative, because it avoids the potential ethical problems associated with the use of embryonic stem cells.

However, even assuming the existence of these beta cell-specific precursors, we still do not know whether they are immortal or are actually subject to senescence, leaving us perhaps with a narrow window for intervention. This aspect may be especially relevant in diabetic individuals in whom the reparative process has been kept under check by autoimmune patrolling for a long period of time. In a case in which prompt intervention (e.g., immediately after the clinical onset of the disease) is not possible, would we still be able to repair the already 'seasoned', deleterious damage of the endocrine pancreas? In the absence of precursors, once they all might be already dead, which resources can still be used?

The T-800 is abruptly catapulted into a foreign environment and another time period. Under the friendly guidance of a young boy, who patiently introduces human feelings, behaviors, and vernacular expressions into its newly accessible electronic memory, the T-800 assimilates this passively received and progressively accumulated information. Eventually it becomes able to interact in a more meaningful way with its surroundings. As a direct consequence, the T-800 starts experiencing human-like feelings and becomes so sensitive to human concerns that it decides on self-immolation as the only path that will halt or delay the destruction of the world it has begun to comprehend.

Many remarkable results have been obtained in the research laboratory by transfecting cells of a certain lineage (e.g., fibroblasts) with genes (i.e., *Oct4*, *Sox2*, *Klf4*) encoding different transcription factors able to convert these somatic cells into ones carrying the characteristics of pluripotent precursors. The human induced pluripotent stem (iPS) cells, in turn, could differentiate into cells of a different lineage, like insulin-producing cells, even if the quantity in which these precursors can be reliably generated seems to be quite limited (6). Furthermore, assuming that we could overcome these limiting aspects, the possibility of using human stem cell lines tailored *ad personam* is certainly revolutionary, even if it were quite inefficient, frequently unsuccessful, and consequently extremely expensive. This approach, while allowing us to bypass the big problem of allorejection, simultaneously opens

the door to the possibility that these dividing cells will not stop growing once a specific, predetermined mass has been reached. In the situation in which these cells do not spontaneously stop proliferating, we could have, unknowingly and tragically, transplanted cancer precursors into our patients.

Finally, assuming that we would be able to establish pluripotent stem cell lines for each patient, and eventually derive from them a specific progeny with the correct phenotype, and that we could generate a sufficient number of the missing beta cells to satisfy the needs of the diabetic recipient, we would still have to solve the problem of recurrent autoimmunity. In patients with type 1 diabetes, autoimmunity not only damages the original endocrine tissue, bringing them to the clinical onset of the disease, but also efficiently limits its reparative process. In fact, autoreactive, diabetogenic T-cell clones, escaped from thymic control (7), seem to be able to systematically kill newly generated beta cells with which the precursors try to replace the ones that are lost. Once transplanted into diabetic mice or humans, syngeneic, healthy beta cells are quickly killed by these same perpetrators, namely CD8⁺ effector T-cells (8).

This autoimmune process is successfully annihilated in the diabetes-prone [e.g., the non-obese diabetic (NOD)] mouse either by substituting all or a part of the immunocompetent cell pool of the recipient with bone marrow cells from a diabetic-resistant donor. Complete substitution is accomplished with conventional bone marrow transplant techniques, and partial substitution by establishing hematopoietic chimerism (9, 10). If these processes were to proceed in humans, they should be sufficient to ensure the recipient's well-being long enough to let him or her benefit from the positive consequences of the laborious, regenerative process. However, more important is the consideration that the successful engraftment of the transplanted bone marrow, or the establishment of a steady hematopoietic chimerism, would have to be obtained and eventually maintained without the use of immunosuppressive agents. This is because these potent drugs, by definition, would kill not only the immunocompetent cells of the recipient but also the beta cells themselves, which are particularly sensitive to the toxicity of the immunosuppressive agents (11). This would, of course, completely defeat the purpose of the transplant.

Perhaps more easy to implement is another approach successfully used in the mouse to stop autoimmunity. Dendritic cells (DCs) are the body's sentinels largely responsible for host surveillance against microenvironmental anomalies including pathogen invasion, infection, and damaged tissue architecture. In a functionally immature state (characterized by low to absent expression of costimulatory molecules such as

CD40, CD80, and CD86), DCs are powerful agents of immune hyporesponsiveness. Exogenous administration of functionally immature DCs achieves long-term and stable allograft survival in a variety of mouse and rat models and prevents a number of autoimmune diseases. Mechanistically, functionally immature DCs act by inducing anergy of the dangerous effector T-cells, either via direct cell contact and/or cytokines and by upregulating the number and function of regulatory T-cell subsets. It has also been shown that *in vitro* administration of nuclear factor-kappa B (NF-kappa B) decoys to DC as well as direct targeting of CD40, CD80, and CD86 with antisense oligodeoxyribonucleotides (AS-ODNs), reduce costimulatory molecule expression levels, producing functionally immature DCs capable of preventing or even reversing new-onset diabetes, once reintroduced into the diabetic recipient, the same NOD mouse from which they were originally collected (12). If we would be able to somehow stop autoimmunity, then it would be sufficient to characterize the factors able to trigger the regenerative process of the beta cell, allowing us to bypass quantitative and chronological limits of resident precursor cells and to reconstitute in this way an efficient glycemic control in our young patients (13).

Diabetic patients must check their blood glucose levels and be injected with insulin at least four times a day. Concurrently, they live with the constant threat of unpredictable incidents of hypoglycemia and the persistent worry of future damage associated with the disease state. Therefore, these patients are probably not enthusiastic about the prospect of waiting for the day in the distant future when the resolution of all the problems of the extremely interesting, yet extremely complex process of tissue regeneration are resolved, before they can have a cure. They would likely look forward to a cure sooner, rather than later. However, it is only in the movies where optimism always prevails against all odds. In science, optimism is generally tempered by the concerns and critiques of peers, which serve to raise questions and may actually rectify errors. Despite some scientific skepticism, the prospect of gene therapy-based treatments remains intriguing and the use of human stem cell research carries with it enormous scientific potential in the treatment and possible cure of many diseases. As we wait, then, for a successful and perhaps not too distant clinical application of the regenerative capabilities of our endocrine pancreas, we may have to be hopeful and just satisfied by the obtained evidence that supports the rights for the beta cell to proudly repeat the statement made famous by the T-800:

'I'll be back!'

Acknowledgements

I would like to thank my son, Matteo, for introducing me to Cameron's movies; and Sharon Rosenshine, who always helped me to improve my written English.

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An improved intracellular staining protocol for efficient detection of nuclear proteins in YFP-expressing cells

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Running Title: Simultaneous flow cytometric detection of nuclear protein and YFP

Key words: Intracellular staining, Flow Cytometry, Membrane Permeabilization, YFP, Foxp3 and T regulatory cells.

Word Counts: Abstract (108 words), Body of Manuscript (1084 words).

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Abstract

Intracellular staining is a widely used flow cytometry (FCM)-based technique to detect the expression of cytosolic/nucleic antigens. However, intracellular staining of cells expressing cytosolic fluorescent protein (FP) markers was proven to be problematic as significant loss of the FP-signal was routinely observed. Using splenocytes harvested from mice constitutively expressing the enhanced yellow fluorescent proteins (YFP) as a model, we modified the widely used intracellular staining protocol and successfully achieved simultaneous detection of both the nuclear proteins and YFP in T-regulatory cells. The improved protocol can be used to perform antibody-based intracellular characterization of FP-labeled target cells, while maintaining their fluorescent reporter signals for easy tracing and identification.

Transgenic animals expressing a fluorescent protein (FP) in specific cell types or lineages are essential tools for studying biological processes *in vivo*¹. Frequently, to target a specific cell population, the promoter element governing the transcription of a tissue-specific gene is used to control FP expression directly or indirectly through the action of the Cre recombinase in a Rosa26-FP reporter line². In the later approach, Cre-mediated removal of the loxp-tagged transcriptional stopper cassette situated between the ubiquitously active Rosa26 promoter and the FP gene will switch on the FP gene transcription, resulting in permanent labeling of cells of the specific lineage with FP³⁻⁴.

Nevertheless, it is essential to examine whether the promoter elements used to drive FP expression can truthfully recapitulate the tissue specificity of the endogenous gene *in vivo*⁴. Staining FP-positive cells with antibodies specific to the endogenous proteins for flow cytometry (FCM) analysis is an effective way to validate the nature of the FP-labeled cells⁵. In addition, antibody-based FCM can also facilitate the characterization of the molecular properties of the FP-labeled cells under various experimental conditions⁶. However, FCM detection of cytosolic or nuclear proteins in FP-expressing cells has been shown to be technically challenging as loss of FP signal is frequently observed when the intracellular staining procedure is used. The common approach to overcome this obstacle is to first isolate the FP-expressing cells with the fluorescence activated cell sorter (FACS) technique, followed by intracellular characterization⁷. Such an approach is not only time-consuming, but also impractical when studying rare cells.

In this study, we modified an intracellular staining protocol, which utilizes paraformaldehyde and saponin as fixative and membrane permeabilizing reagent, respectively, to achieve simultaneous detection of nuclear proteins and cytosolic FP molecules. The nuclear

Foxp3 proteins in T-regulatory cells of Vav-Cre:Rosa-YFP reporter mice³ were used as a model target.

After staining YFP+ splenocytes with antibodies specific to surface markers of T cells (i.e., CD3 and CD4), we subjected them to the intracellular staining procedure outlined in Figure 1A (detailed in *Supplementary Method*) for the detection of nuclear Foxp3 proteins. Although the Foxp3+ T-regulatory cell population could easily be identified, the YFP signals in these cells became essentially undetectable (Figure 1B). Loss of YFP signal could result either from fixative-induced conformation changes of the YFP proteins, or from the loss of cytosolic YFP proteins due to the leakiness of the permeabilized cell membrane. To investigate whether over-fixation is the major causative factor, we shortened the Fixation/Permeabilization (Fix/Perm) buffer treatment from 2 hours to 5 minutes, but still failed to preserve detectable YFP signal (data not shown). Indeed, significant reduction of cellular YFP signal was observed immediately following exposure of YFP+ cells to the Fix/Perm buffer, indicating that the rapid loss of the fluorescent signal is due to diffusion of YFP molecules out of the permeabilized cells (Figure 1C and data not shown).

As fixation with 1-2% paraformaldehyde is routinely used in FCM protocols to preserve fluorescent signals for analysis, we postulated that adding a fixation step prior to the Fix/Perm treatment might prevent YFP proteins from leaking out of the cytoplasmic membrane. Indeed, prefixing samples with 2% paraformaldehyde for 30 minutes can effectively retain the YFP signal; however, we then became unable to stain Foxp3 proteins (Figure 1D), presumably due to over fixation of the cytoplasmic membrane. We thus speculated that the success of staining the nuclear FoxP3 molecules, while preserving detectable YFP signals, might hinge on the

optimization of pre-fixation of cytoplasmic membrane: to block the leakage of YFP molecules from cytosol, while preserving the accessibility of the antibody to its nuclear target.

To find out the minimal fixative conditions which can be used to preserve the YFP signal, cells were prefixed with various concentrations of fixative for different lengths of time (*Supplementary Table 1*), followed by Fix/Perm treatment and FCM analysis. As summarized in Figure 2A, the success of cytoplasmic YFP protein retention was determined by the combined effect of prefixation time and concentration of the fixative. To give a relative quantification of the prefixation process, we defined the value of prefixation time (minutes) x % of fixative as prefixation factor (PF), and concluded that conditions with $PF \geq 4$ (grey area in Figure 2A) were sufficient to retain enough YFP proteins for FCM analysis.

To evaluate the negative effect of prefixation on Foxp3 staining, we took advantage of the eGFP-Foxp3 transgenic mice, in which an eGFP molecule was inserted in-frame to the 5' end of the *foxp3* gene to encode a functional eGFP-Foxp3 fusion protein⁸. One of the major functional components in the permeabilization buffer is saponin, which can complex with cholesterol to form pores in the cholesterol-rich cytoplasm membrane but leaves the cholesterol-poor nuclear membrane largely intact^{9,10}. Thus, nuclear eGFP-Foxp3 fusion protein should be retained in the nucleus during intracellular staining while the eGFP signal will truthfully reflect the presence of Foxp3 protein (Figure 2B). Splenocytes harvested from the eGFP-Foxp3 transgenic mice were prefixed with 2% paraformaldehyde from 15 seconds to 5 minutes, prior to staining of the cells with anti-Foxp3 antibodies. Prefixation from 15 seconds to 2 minutes did not drastically affect the percentage of Foxp3+ cells, whereas we began to observe a decrease of the antibody staining of the eGFP-Foxp3 fusion proteins after the cells were treated with fixative for more than 5 minutes (Figure 2B and data not shown).

Based on the above findings, we proceeded to add the prefixation step to the intracellular staining protocol for Foxp3 detection in Vav-Cre: RosaYFP T regulatory cells. As shown in Figure 2C, a distinct population of CD4⁺ T cells can be readily identified as double positive for both YFP and Foxp3 under a number of amenable conditions. Since various intracellular antigens may differ substantially in their retention and susceptibility to Fix/Perm treatment, we also examined the nuclear expression of *helios*, a member of nuclear expressed transcription factor of the Ikaros family, in YFP⁺ splenocytes¹¹. As shown in supplementary Figure S1, effective detection of both Helios and YFP were achieved under conditions similar to those described in Figure 2C. Thus, PFs range from 4 to 20 represents the optimal conditions to obtain efficient nuclear protein staining, and well separation of the YFP signals from the background. It is conceivable that the optimal PF will vary in different models; for cells with weak fluorescent signals, a higher PF (10-20) could be preferred, and vice versa. Nevertheless, the simple modification of the intracellular staining procedure described above (Figure 2D) reliably enables the usage of cytoplasmic FPs as cellular markers for co-localization studies with FCM technology.

Acknowledgements

We thank Dr. Joy William for helpful discussions and critical reading of the manuscript. We also thank Mr. Carl Engman, Dr. Brett Philips and Dr. Valentina DiCaro for technical assistance. The study was supported in part by Department of Defense grant W81XWH-09-1-0742.

Competing Interests

The authors declare no competing interests.

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Figure Legends

Figure 1. Loss of YFP signal in cells treated with Fixative/Permeabilization reagents used

in intracellular staining procedures. A. A step-by-step protocol for intracellular staining of

nuclear Foxp3 protein in T regulatory cells. **B.** YFP⁺, CD4⁺T cells harvested from Vav-

Cre;Rosa-YFP mice were stained intracellularly with anti-Foxp3 antibodies following the

procedure outlined in **A**. *Left panel*, before Foxp3 staining; *Right panel*, after Foxp3 staining.

C. Representative fluorescent microscopy images of YFP⁺ splenocytes, showing rapid loss of YFP signal in YFP⁺ cells upon Fix/Perm exposure. Top panels: *Left*, prior to Fix/Perm exposure;

Right, 5 seconds after Fix/Perm addition to the medium. Lower panel: Average fluorescent

intensities of YFP⁺ cells (n=10) before or after of Fix/Perm buffer addition. ***, $p < 0.0001$

(Student's *t* test). **D.** Intracellular staining of Foxp3 is significantly compromised in YFP⁺

splenocytes prefixed with 2% paraformaldehyde for 30 minutes, while YFP signal is well-preserved. Shown are representative FCM results from three independent experiments.

Figure 2. Short Prefixation prior to intracellular staining is sufficient to retain detectable

cytosolic YFP signals. A. Systematic evaluation of YFP signal preservation in YFP⁺

splenocytes treated with various prefixing conditions, followed by Fix/Perm exposure. Gray

area: conditions under which sufficient YFP signals were preserved for FCM detection.

Percentages (%) of paraformaldehyde used as fixative are shown in the insert. The results are

summarized from four independent experimental repeats. **B.** Splenocytes harvested from eGFP-

Foxp3 mice were pretreated with 2% paraformaldehyde for various times (shown on the top of

each panel), and subsequently stained intracellularly with anti-Foxp3 antibodies and analyzed by

FCM (gated on CD4⁺eGFP⁺ T regulatory cell population). Percentages of cells with nuclear

Foxp3 stained successfully under different pre-fixation conditions are shown. These results are

representative of FCM analyses from five independent experiments. **C.** Foxp3-staining of Vav-Cre:RosaYFP CD4⁺ T-cells pre-treated with either 0.5% or 1% of paraformaldehyde for the times shown below each panel. Percentages of CD4⁺ T cells positive for both Foxp3 and YFP are shown on the upper right corners. Shown are representative results from five independent experiments. **D.** Modified intracellular staining protocol for nuclear Foxp3 detection. Steps in grey boxes were added to the original procedure in Figure 1A. For other cytosolic or nuclear proteins, optimization of the pre-fixation time may be required.

Figure 1

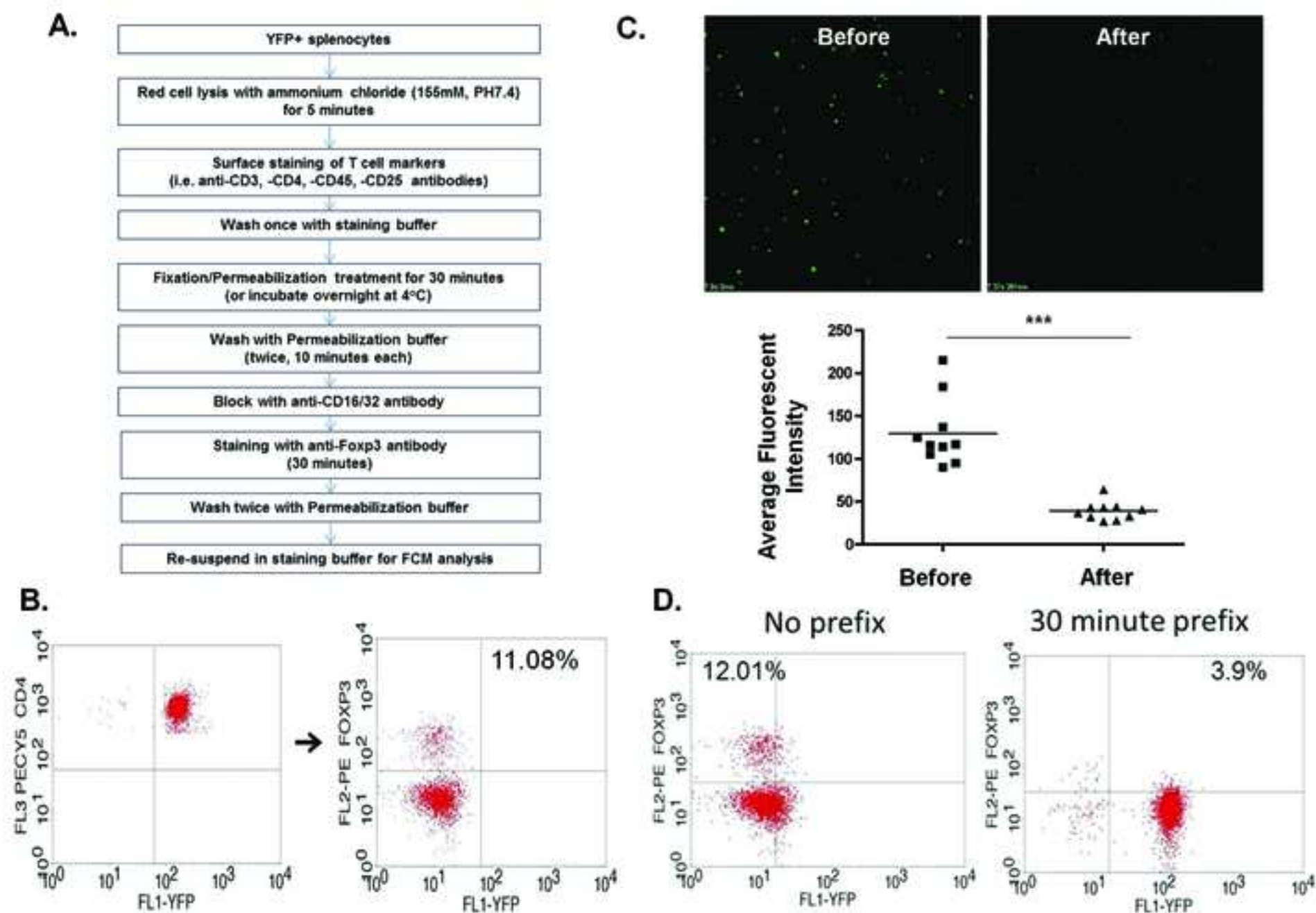
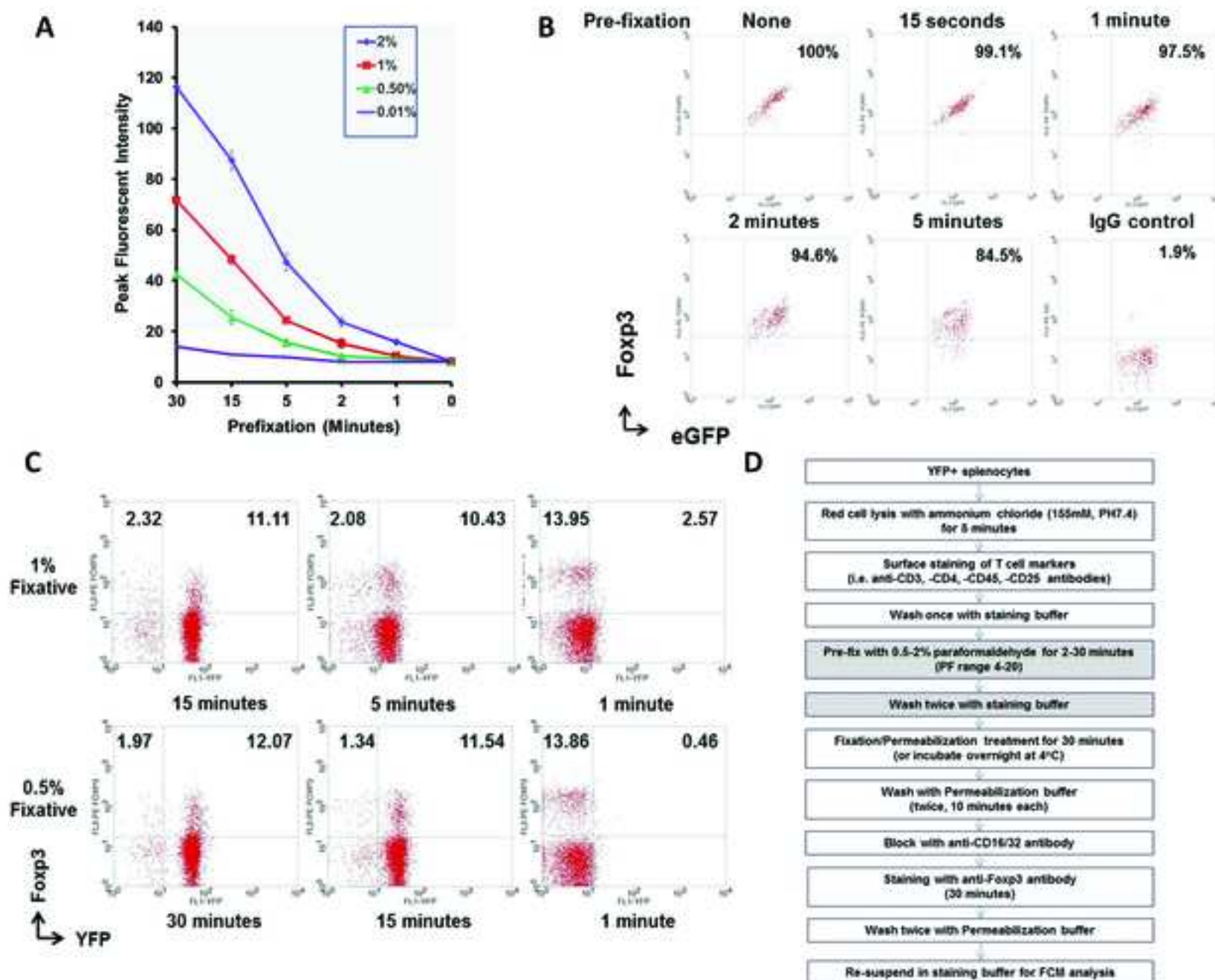


Figure 2

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Figure 2



Supplementary Material for Online Publication includes:

- 1. Supplementary Materials and Methods.**
- 2. Supplementary Table S1.**
- 3. Supplementary Figure S1.**

1. Supplementary Materials and Methods.

Mice

Rosa-YFP transgenic mice were purchased from Jackson Laboratory. Transgenic mice expressing the Vav-Cre transgene, in which the pan-hematopoietic promoter elements derived from the Vav1 oncogene were used to drive Cre recombinase expression, has been described^{3,4}. EGFP-Foxp3 N-terminal knock-in mice were also described previously⁸. All animal experiments were carried out under protocols approved by the Institutional Animal Care and Usage Committee of the University of Pittsburgh (protocol approval number 0811074).

Flow cytometry

Flow cytometric analysis was performed on the BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed with CellQuest Pro software (BD Biosciences). Single cell suspensions were prepared from spleen, subjected to erythrocyte depletion in red blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO), blocked with anti-CD16/32 antibody and stained with antibodies. The following antibodies were purchased from BD Biosciences: anti-CD16/32 (2.4G2), anti-CD4 PeCy5 (H129.9), anti-CD45-APC (30-F11), and anti-CD3-APC (145-2C11). Anti-CD25-APC (7D4) antibody was purchased from Miltenyi Biotec (Auburn, CA). Staining buffer: phosphate buffered saline (PBS, calcium and magnesium free, Invitrogen) supplemented with 1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich).

Intracellular staining of Foxp3 and Helios

Both the Foxp3 protein staining kit and the anti-helios antibody (Clone 22F6) were purchased from eBiosciences (San Diego, CA). Unless specified in the text, all procedures were performed following manufacturer's suggested protocol. Permeabilization buffer contains 0.1% saponin and

0.09% sodium azide in PBS. Fixative/Permeabilization (Fix/Perm) buffer is prepared by supplementing permeabilization buffer with 4% paraformaldehyde.

2. Supplementary Table S1. Prefixation factors (PFs) under various prefixation conditions

Prefixation Time (minutes)	Paraformaldehyde Concentration			
	2%	1%	0.5%	0.1%
30	60	30	15	3
15	30	15	7.5	1.5
5	10	5	2.5	0.5
2	4	2	1	0.2
1	2	1	0.5	0.1

Note: Prefixation factor (PF) = Prefixation time (minute) x Paraformaldehyde concentration (%). Cells treated with PFs in yellow could preserve sufficient YFP signals for FACS detection.

3. Supplementary Figure S1

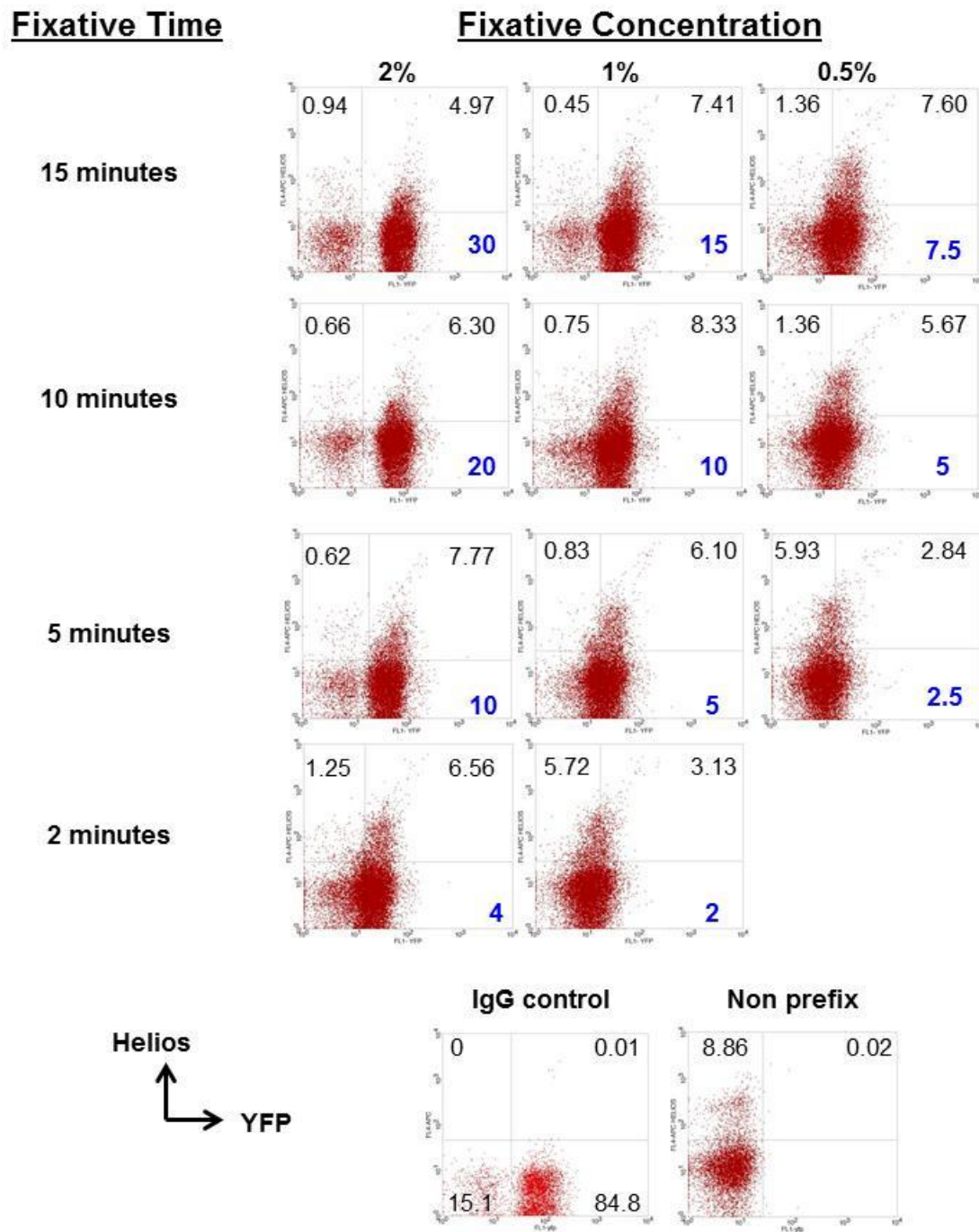


Figure S1. Flow cytometric detection of transcription factor Helios expression in YFP+ splenocytes. Splenocytes harvested from Vav-Cre:RosaYFP were pre-fixed with 0.5%, 1% and

2% paraformaldehyde for the times shown. Percentages of splenocytes positive for both Helios and YFP are shown on the upper right corners. Shown are representative results from two independent experiments. The blue numbers in each panel represents the PF factors under each condition. As shown, pre-fixation with PF range from 4-20 allows effectively staining of Helios, while preserving above background levels of YFP signals.