MHC Class II-Peptide Complexes Displayed on Activated T Cells Guide Treg Suppression

MHC Class II-Peptide Complexes Displayed on Activated T Cells Guide T_{reg} Suppression

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Background: Regulatory T cell (T_{reg}) therapy remains a promising strategy for minimizing immunosuppression and extending allograft survival. T_{reg} suppression requires initial activation through T_{reg}-target cell contacts and T cell receptor (TCR) engagement, however, the precise nature of these interactions in the context of allogeneic settings remains to be elucidated. Studies indicate that inflammation facilitates transfer of peptide-MHC-II complexes(pMHCII) from antigen presenting cells (APCs) to activated T cells. Thus, we hypothesize that during inflammation associated with organ transplantation, pMHC-II complexes relocate to alloreactive T effector cells and serve as T_{reg} activation signals.

Methods: Transfers of pMHC-II complexes onto activated T effector cell surface were studied in co-cultures of C57BL/6 (B6) $CD4^+$ T_{eff} cells and B6 B lymphocytes isolated from I-A^b-GFP transgenic mice. Suppression assays *in vitro* were done in co-cultures of APCs (CD90 depleted splenocytes), $CD4^+CD25^-$ effector T cells (T_{eff}), and $CD4^+CD25^+$ T_{regs} from various MHC backgrounds. Experimental read-out was T_{eff} cell proliferation following stimulation by allogeneic APCs. Similar combinations of T_{eff} and T_{reg} cells were tested *in vivo* in B6 Rag $1^{-/-}$ mice reconstituted with T_{reg}/T_{eff} (1:1 or 2:1 ratios) injected IV one day prior to grafting of allogeneic (C3H or BALB/c) tail skin grafts.

Results and Conclusions: Data from more than 150 T_{eff}-T_{reg}-APC co-culture experiments unequivocally show that *in vitro* T_{reg} suppression occurs when T_{reg} and T_{eff} cells have the same MHC-II background, but independently of T_{reg} matching with APC MHC-II. Suppression experiments to determine the origin of pMHC-II complexes were extended *in vivo* using T_{eff}, T_{reg}, and skin allografts with different MHC-II matching or mismatching. Results recapitulate the *in vitro* data confirming that T_{reg} activation/suppression proceeds through recognition of "suppress me" pMHC-II tags exposed on T_{eff} cells, leading to graft survival. Collectively, these data imply that T_{reg} regulation is the result of semidirect recognition of donor and/or recipient peptides exposed on activated effector cells. They also suggest that recognition of a limited set of T_{reg} activator signals on T_{eff} cells improves local T_{reg} function by directing suppression only toward activated cell targets.

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Figure Two: A model of pMHC-II mediated T_{reg} activation

Hypothetical model: Under inflammatory conditions, activated $CD4^+$ T_{eff} capture pMHC-II complexes from APCs. These complexes are recognized by T_{reg} TCRs, are necessary for T_{reg} activation, and guide T_{reg} function to local areas of inflammation.

Figure Three: T_{reg} suppression of $CD4^+$ T_{eff} activated in a TCR-dependent manner is MHC II restricted to T_{eff}

Mixed cell cultures were set in triplicates and incubated for 4 days. T cell proliferation was measured by ^3H thymidine incorporation in the presence or absence of T_{regs} from BALB/c or B6 origin. Bars indicate T_{eff} proliferation; percent inhibition of proliferation are indicated. Data are from 3 independent experiments for cultures activated with anti-CD3 and 5 independent experiments for cultures activated by B6 APCs. T_{eff} are BALB/c $CD4^+$ $CD25^-$ cells.

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A: On day -2, $Rag1^{-/-}$ recipients were injected IP with 600 ug of natural killer (NK) cell depleting antibody (NK1.1, clone PK136, BioXCell.) The next day, $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ cells

were sorted from B6, C3H, or BALB/c donors and adoptively transferred into recipients in multiple ratios. On the day of transplant, recipients received tail skin grafts from C3H or BALB/c donor mice. Grafts were covered by a protective bandage for one week, then monitored for graft survival/rejection.

B: Example accepted and rejected C3H skin grafts.

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Kaplan-Meier plots of allogeneic tail skin grafts transplanted onto B6 $Rag1^{-/-}$ recipients. On the day prior to transplantation, recipients were infused with wild type B6 T_{eff} and T_{regs} from B6, C3H, or BALB/c donors. All groups received 2 x 10^5 T_{regs} and 10^5 T_{eff}, except for the "T_{eff} only group" which received no T_{regs}. Corresponding survival data and statistical significance of individual curve comparisons can be found in Tables 2 and 4.

Tables

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Chapter 1: Background

1.1: Background

Definition

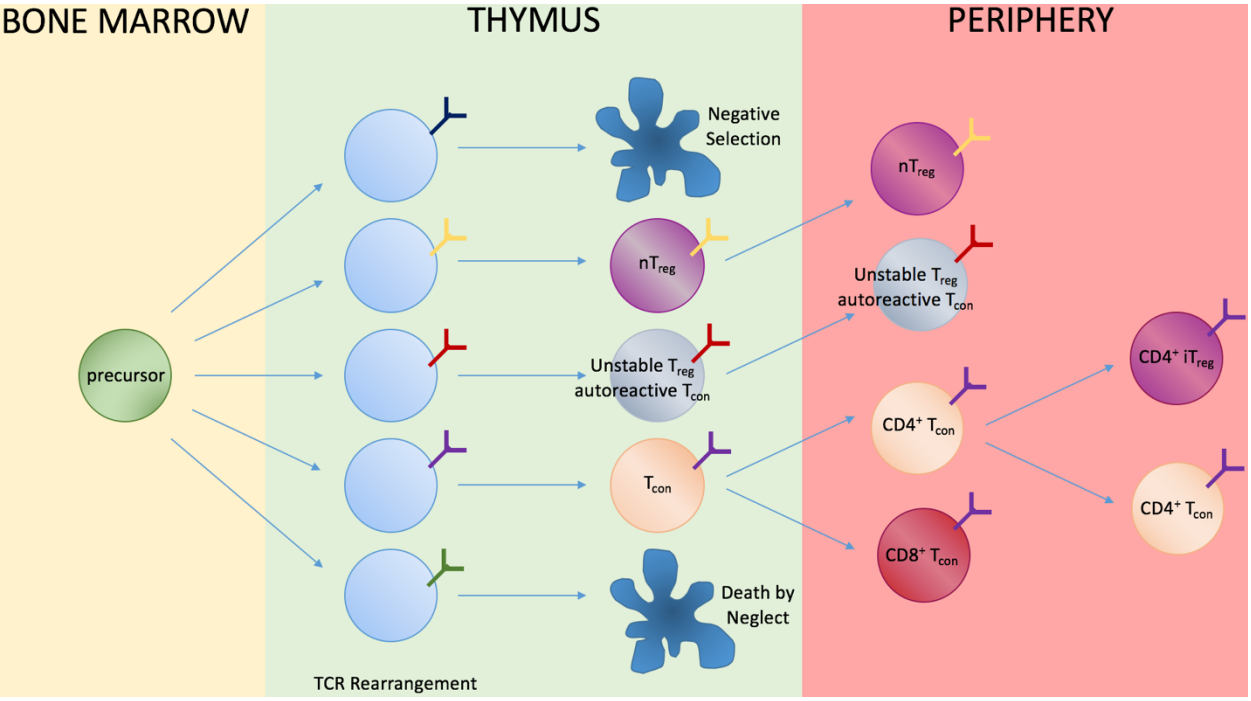
Regulatory T cells (T_{regs}) are an immunosuppressive subset of T cells, the major subset of which are $CD4^+$and express the lineage-specific transcription factor FoxP3, as well as high levels of the high-affinity IL-2 receptor alpha subunit, CD25.[1-11] T_{regs} play an important role in maintaining self-tolerance.[3-9, 12-14]

Thymic/Natural and Induced/Peripheral T_{regs}

T_{regs} can be categorized as either thymic or induced T_{regs} (tT_{regs} or iT_{regs}, respectively) based upon their developmental history. tT_{regs}, sometimes called natural (nT_{regs}), differentiate into a regulatory phenotype during T cell development and express FoxP3 when they exit the thymus. On the contrary, iT_{regs}, sometimes called peripheral (pT_{regs}), leave the thymus as conventional $CD4^+$ $FoxP3^-$ T cells (T_{con}) and convert to a regulatory phenotype upon encountering specific signals in the periphery.[1, 13, 15] In particular, low doses of target antigen, suboptimal dendritic cell activation, TGF-□, and low IL-2 production are conditions which favor the extrathymic generation of iT_{regs}.[15, 16] The signals which determine differentiation into tT_{reg} during thymic development are incompletely understood, however, TCR signaling plays a significant role[11, 17] and many strongly autoreactive cells which are not deleted during negative selection become tT_{regs}.[18, 19] Engagement of CD28 on T cells with B7 family ligands is critical for tT_{reg} development, as well as activation and survival of T_{regs} in the periphery.[11, 20, 21]

The different developmental histories of tT_{reg} and iT_{reg} result in populations with distinct T cell receptor (TCR) repertoires: while tT_{regs} are selected in the thymus for self-specificity, iT_{regs} have the identical TCR from the non-regulatory $CD4^+$ conventional T cell (T_{con}) from which they were induced.[22] Subsequently, tT_{regs} recognize peptides on self major histocompatibility complex (MHC) II and are not considered antigen specific, while iT_{regs} are believed to function in an antigen-specific manner.[13, 23, 24] Understanding these differences in specificity will inform future work on T_{reg} therapy and culture *ex vivo*.

Figure One: A Model of T Cell Differentiation



T cell progenitors migrate from the bone marrow to the thymus. After generating TCRs of random specificity, cells with a productive rearrangement are positively selected, while those that cannot signal die by neglect. Developing thymocytes differentiate into different T cell subsets depending upon their affinity for self MHC. $CD4^+$ T_{con} can later be induced to a regulatory phenotype in the periphery. Adapted from Okhura et al, 2013.[1]

Major Histocompatability Complexes (MHC)

Understanding MHC structure, function, and interactions with TCRs is crucial for understanding T_{regs}. MHC molecules are transmembrane proteins expressed on the cell surface which display peptides for T cells recognition.[25] Two subtypes of MHC, classes I and II, play complementary yet distinct roles in antigen presentation. We will focus predominantly on MHC II for the purpose of this discussion, but it is important to understand that MHC I and II are expressed on different cell types, present peptides derived from different sources, and engage different subsets of T cells.

MHC II proteins consist of two polymorphic, non-covalently associated peptide chains. Each has a cytoplasmic signaling domain, transmembrane region, and extracellular regions which come together to form a peptide-binding cleft.[25] MHC II present lysosomal and endosomal peptides typically derived from extracellular proteins.[25] The peptide-binding groove of MHC II molecules is open on both ends, allowing for increased flexibility and binding peptides approximately 10-30 residues long.[25]

MHC II molecules are mostly expressed on antigen presenting cells (APCs) including dendritic cells (DCs) and B cells; they are also displayed by other cell types, including endothelium, upon activation.[26] Specialized epithelial cells in the thymus which participate in T cell development also express MHC II.[27]

During T cell development, T cells of randomly generated specificity are selected based on their ability to recognize peptides presented on self-MHC molecules. The increased binding affinity of

a particular TCR with either class of MHC determines the T cell's subsequent differentiation into a $CD4^+$ or $CD8^+$ cell. After this stage of development, $CD8^+$ T cells are only able to engage MHC I, and $CD4^+$ T cells only bind MHC II. Mature T cells are also limited to recognizing self MHC molecules; this is called MHC restriction.[25, 28, 29]

MHC genes are the most polymorphic in mammalian genomes.[25] The reason for such extensive polymorphism is hypothesized to involve population-level protection against pathogens through generation of a diverse pool of peptide-binding clefts; although MHC are the primary targets of allogeneic immune responses, they did not evolve to make transplantation difficult.

There are several important differences between the MHC of mice and men which should be kept in mind. MHC II genes in humans are called Human Leukocyte Antigen (HLA) II and are distributed among three distinct loci: HLA-DP, -DQ, and –DR.[27, 30] The corresponding murine genes are I-A and I-E.[25, 30] While human T cells upregulate and produce MHC II molecules in response to IFN[26, 31], murine T cells are unable to synthesize their own MHC II due to a mutation in the pIII promoter of the CIITA transcription factor which coordinates MHC II expression.[26, 27, 30, 31]

T Cell Development

T cell development occurs in the thymus, where thymocytes receive signals from a variety of epithelial and antigen presenting cells that are necessary for their proliferation and maturation.[25] After migration into the thymic cortex from the fetal liver or bone marrow,

progenitors of T cells called pro-T cells are negative for CD3, chains, CD4, CD8, and TCRs.[25] These pro-T cells receive the survival signal IL-7 from cortical thymic epithelial cells (cTECs) and activate TdT and RAG genes to undergo V(D)J rearrangement of the TCR chain.[25, 32] If these precursors, now called pre-T cells, productively rearrange the TCR chain to associate with the invariant protein pre-T, CD3, and chains on the cell surface, signaling through the pre-TCR inhibits further rearrangement of the chain locus and induces cell proliferation, rearrangement of the TCR locus, and CD4 and CD8 expression.[25, 33] Only about half of pre-T cells complete a successful chain rearrangement, which means half of developing thymocytes die by neglect at this stage.[25] It is unclear if the pre-TCR signals in a ligand independent or dependent manner, and if so, what ligands are recognized by the pre-TCR.[25]

Cells which pass the pre-TCR checkpoint become $CD4^+$ $CD8^+$ double positive thymocytes and undergo VJ rearrangement in the chain locus.[25, 28] If this is a productive rearrangement, the new chain(s) will replace the invariant T to assemble a complete TCR complex on the cell surface.[25] At this point, the assembled TCRs may or may not recognize a peptide-MHC complex; they could be specific for self-peptides presented on self-MHC, foreign peptides on self-MHC, any peptide on foreign-MHC, or fail to recognize peptide-MHC complexes altogether.[25] Since the purpose of T cell development is to generate a pool of immunocompetent, non-self-destructive T cells, these double positive thymocytes must be further selected to ensure that mature T cells are capable of recognizing antigen presented by self and are not autoreactive.[25, 28] The first of these selection processes, positive selection, involves interaction between cTECs and dendritic cells (DCs) with double positive thymocytes.

In the thymic cortex, cTECs and resident DCs present self-peptides on self-MHC class I and II.[34] The strength of TCR signal resulting from these interactions determines cell fate. TCRs which fail to recognize self-peptide MHC complexes and cannot generate a signal undergo death by neglect and apoptosis; this process ensures that all T cells in the mature repertoire are capable of recognizing self-MHC, or are self-restricted.[25] TCRs that bind with low avidity to self-peptide/self-MHC complexes survive and are committed to become either $CD4^+$ $CD8^-$ or $CD8^+$ $CD4^-$ single positive T cells depending on the class of MHC they best recognize.[25]

The critical role of MHC II/TCR interactions in the initial steps of T cell development is highlighted by patients with Bare Lymphocyte Syndrome.[30] In this autosomal recessive immunodeficiency, mutations in the CIITA gene result in extremely low MHC II expression and a subsequent defect in $CD4^+$ T cell positive selection.[30, 35]

The next selection process, negative selection, primarily involves $CD4^+$ or $CD8^+$ single positive (SP) thymocytes recognizing self peptide-MHC complexes (pMHC) exposed on medullary DC and epithelial cells.[25, 36] During this process, TCRs which bind self pMHC with high avidity are programmed to die.[25, 28, 34] The purpose of negative selection is to eliminate T cells which may initiate autoimmunity.[37] As part of this selection process, mTECs express the transcription factor Autoimmune Regulator (AIRE)[38, 39], which promotes the expression of tissue-restricted antigens. Mutations in AIRE result in incomplete negative selection, escape of self-reactive T cells to the periphery, and autoimmune disease called autoimmune polyendocrine syndrome type-1.[38, 40]

Not all self-reactive T cells are programmed to die, however: some of these cells differentiate into T_{regs}.[41-44] While it has been hypothesized that slightly weaker interactions lead to T_{reg} fate and slightly stronger ones lead to cell death[18], the signals determining these alternative fates remain poorly understood.[22, 25, 44, 45]

FoxP3 Deficiency

The important role of T_{regs} in maintaining self-tolerance is most clearly demonstrated by the condition known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), in which loss of function mutations in the FoxP3 gene lead to T_{reg} deficiency and multi-organ autoimmunity.[7, 46-48] Immunosuppressive drugs can be used to control the disease, but the only curative therapy is a hematopoietic stem cell transplant. If left untreated, IPEX is fatal within the first year of life.[7, 46-48] Scurfy is the analogous condition to IPEX in mice, and has a similar phenotype characterized by multi-organ immunity.[1, 48] Experiments using mice which delete FoxP3 upon diphtheria toxin treatment further demonstrated the necessity of this transcription factor for the development and maintenance of T_{reg}.[7]

T_{reg} Therapy in Transplantation

Solid organ and stem cell transplants are life-saving treatments for patients suffering from end-stage organ failure and hematological malignancy.[49, 50] According to the US Department of Health and Human Services and United Network for Organ Sharing (UNOS), the number of patients receiving a life-sustaining solid organ transplant increased over 20% from 2012 to 2016. In the United States alone, over 33,600 patients received a lung, heart, liver, kidney, or other organ transplant in 2016.[51] In addition to live-saving transplants, life-enhancing reconstructive

transplantation in the form of vascularized composite allografts (VCA) has become a clinically feasible option, with multiple centers around the world completing face[52], hand[53, 54], genital[55, 56], and abdominal wall transplants[57, 58].

The discovery of calcineurin inhibitors, such as cyclosporine and tacrolimus, have significantly improved allograft survival. Yet while these drugs are effective at preventing acute rejection, chronic rejection remains a significant barrier to clinical transplantation success.[49, 59-61] Furthermore, nephrotoxicity, malignancies, cardiovascular disease opportunistic infections, and other side effects associated with pharmacologic immunosuppression highlight the need for better therapeutic options to induce transplantation tolerance.[50, 59, 61-64] Known side effects of the best available immunosuppressive drugs present a particular ethical dilemma in the context of non-lifesaving reconstructive transplantation.[54, 62]

The significant role of T_{regs} in maintaining self-tolerance and preventing autoimmunity[12] has suggested their use as a clinical therapy for the induction of transplant tolerance[12, 22] as well as motivation for the development of T_{reg}-sparing immunosuppressive regimens.[65] Additional clinical applications include the treatment of rheumatoid arthritis[66], inflammatory bowel disease[67], type one diabetes[68], atherosclerosis[69, 70], and other inflammatory diseases.

Many clinical trials investigating the efficacy of immunosuppressive cellular therapies are already underway.[61, 71] As of March 15, there are 501 studies pertaining to T_{regs} listed on the National Institutes of Health website; they include treatments for a variety of autoimmune diseases, including type one diabetes mellitus, GVHD, autoimmune hepatitis, lupus, Crohn's

disease, chronic obstructive pulmonary disease, asthma, psoriasis, coronary artery disease, as well as lung, kidney, and stem cell transplants.[72] A particularly important ongoing clinical trial is the ONE Study, which is a large, multi-center trial comparing multiple regulatory cell therapies with standardized drug treatment protocols.[50, 73]

A number of animal studies and clinical trials have focused on understanding T_{reg} mediated immunosuppression and tolerance induction in the context of allogeneic transplantation.[13, 49, 50, 61] Despite excellent research contributing to the current understanding of T_{reg} for clinical use, however, questions still persist regarding optimal cell source, dose, and specificity.

Purpose

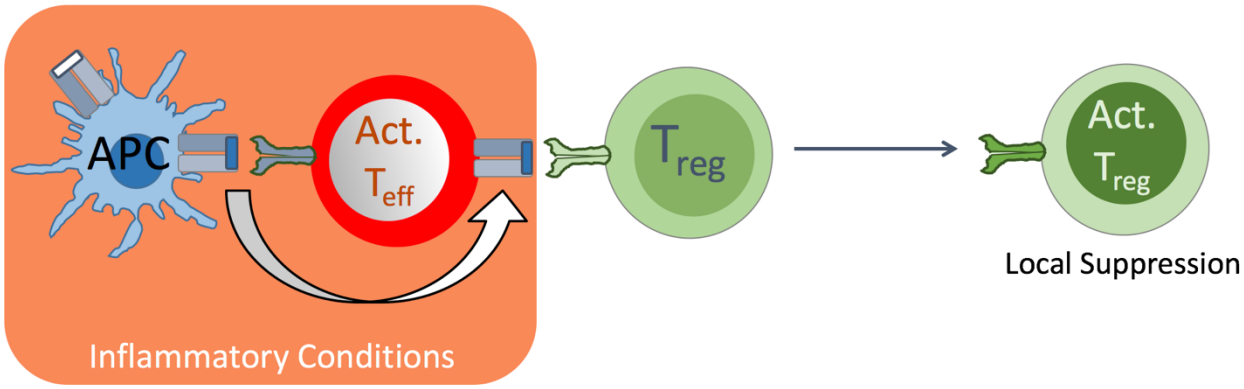
Through this project, we seek to better understand the signals that guide T_{reg} activation and suppression in an allogeneic transplant setting. We hope that mechanistic insights from these experiments will contribute to the design of future clinical T_{reg} therapies.

Hypotheses

Previous work by our group[74-76] and others[77-82] has been the impetus for our hypotheses surrounding T_{reg} activation. As shown in Figure Two below, we believe that T_{reg} activation requires initial cell contact with an activated T_{eff} cell, and furthermore, that these interactions involve engagement of T_{reg} TCR with MHC II displayed on the surface of effectors. Activated T_{eff} take up peptide-MHC II (pMHC-II) complexes from surrounding APCs via TCR-mediated endocytosis, trogocytosis, and/or exosome capture. Captured pMHC-II on activated T_{eff} serve as

docking and activation signals for T_{reg} and guide their suppressive activity to a local area of inflammation.

Figure Two: A model of pMHC-II mediated T_{reg} activation



Hypothetical model: Under inflammatory conditions, activated $CD4^+$ T_{eff} capture pMHC-II complexes from APCs. These complexes are recognized by T_{reg} TCRs, are necessary for T_{reg} activation, and guide T_{reg} function to local areas of inflammation.

Chapter 2: Data and Methods

2.1: Materials and Methods

Animals

Six- to eight-week-old male and female C3H (H-2^k), BALB/c (H-2^d), and C57BL/6 (B6; H-2^b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Rag 1 $^{-/-}$ mice on the B6 background were both purchased from Jackson and bred in our pathogen-free facility. I-A^b GFP mice on the B6 background were a gift from Marriane Bose. All mice were maintained at the Massachusetts General Hospital animal facility and treated according to institutional guidelines. All mice within individual experiments were gender matched.

Cell Enrichment

Donor spleens were washed in PBS, erythrocyte depleted with ACK lysis buffer, and enriched for either $CD4^+$ or $CD8^+$ T cells using the eBioscience/affymetrix Magnisort mouse CD4 and CD8 T cell enrichment kits, respectively. Enriched populations were then stained with αCD16/32, αCD4, αCD25, and other monoclonal antibodies (Table 1) and sorted on a BD FACSaria II Cell Sorter.

Table 1: Monoclonal Antibodies Used for FACS

Target	Clone	Conjugate	Company
Mouse CD3α	145-2C11	APC	Biolegend
Mouse CD3α	145-2C11	APC-Cy7	Biolegend
Mouse CD4	GK1.5	PerCP-Cy5.5	Biolegend
Mouse CD4	RM4-5	PerCP-Cy5.5	Biolegend
Mouse CD8α	5H10	PO	Life Technologies
Mouse CD16/32	93	None	Biolegend
Mouse CD19	6D5	BV421	Biolegend
Mouse CD25	PC61.5	PE-Cy7	eBioscience
Mouse/Human CD44	IM7	PE	Biolegend
Mouse CD62L	MEL-14	APC	Biolegend
Mouse/Rat FoxP3	FJK-16S	AF488	eBioscience
Rat IgG2aκ Isotype Control	eBR2a	AF488	eBioscience

Suppression Assays

Standard suppression assays[83] were performed using co-cultures of $CD4^+$ $CD25^-$ effector T cells (T_{eff}), $CD4^+$ $CD25^+$ regulatory T cells (T_{reg}), and CD90-depleted splenocytes as antigen presenting cells (APCs). Following four days of incubation, T cell proliferation was assessed by ^3H thymidine incorporation. Percent inhibition of proliferation was calculated by normalizing to cultures not containing T_{reg}. (Figure 5)

Immunocytochemistry

Naïve $CD4^+CD25^-$ T cells from B6 mice were co-cultured with or without I-Aβ-GFP APCs in the presence of PMA + ionomycin for 18 hours, fixed, and stained with an anti-TCR mAb. Microscopic images were recorded of sorted $TCR^+B220^-CD25^+$ activated T cells, 95% pure $B220^+$ B cells, and 93% pure $CD4^+$ T cells from the I-Aβ-GFP mouse following 18 hrs of PMA/ionomycin activation. Fixed cells were stained with an anti-TCR mAb.

Adoptive Transfer and Skin Transplantation

In a model adapted from Francis et al[3], B6 $Rag1^{-/-}$ mice were treated with an NK cell depleting antibody on day -2. The following day, T cell subsets were sorted from gender-matched B6, C3H, or BALB/c splenocytes and injected via the tail vein. On day zero, recipients were transplanted with a 1 centimeter square, gender-matched tail skin graft from either a C3H or BALB/c donor.[84, 85] Grafts were covered with a bandage for one week, then visually monitored for signs of rejection.[84] Animals with grafts surviving greater than 150 days were sacrificed, and skin grafts were harvested for histological analysis with H&E staining. (Figure 6C)

2.2: Results

In vitro *Studies*

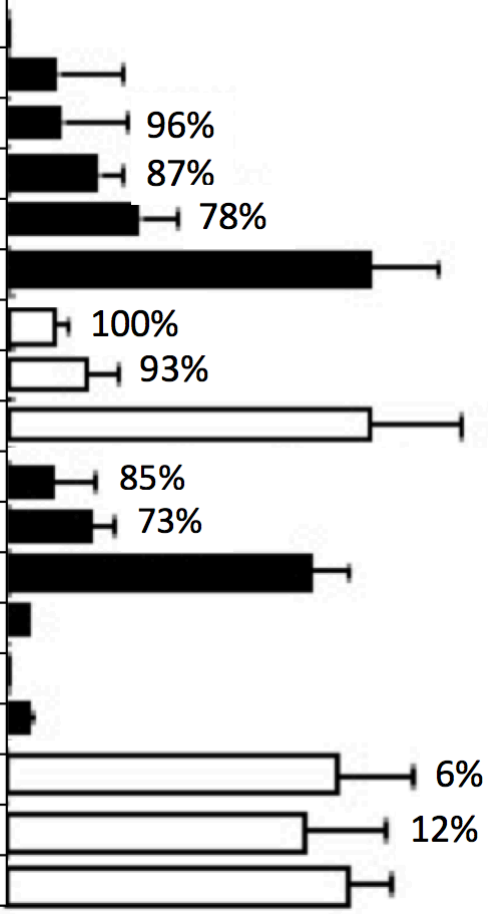
The *in vitro* experiments presented here as the basis for the *in vivo* work were conducted by Sharon Germana.

Standard *in vitro* suppression assays[83] were performed to evaluate T_{reg} activity (Figure 3). When syngeneic T_{eff} and T_{reg} from BALB/c mice (H-2^d) were stimulated with irradiated syngeneic APCs and cell-bound anti-CD3, T_{regs} suppressed T_{eff} proliferation. As expected, higher T_{reg}:T_{eff} ratios resulted in increased suppression. Under the same stimulation conditions, allogeneic B6 T_{regs} similarly suppressed T_{eff} proliferation in a dose-dependent manner (Figure 3).

Additional cultures were performed using allogeneic B6 APCs as stimulation (Figure 3). Under these conditions, only T_{reg} syngeneic to T_{eff} suppressed their proliferation. T_{reg} allogeneic to effector cells did not control the effector response, even when matched to the APCs. This suggests that T_{regs} are activated by a signal on activated self effector T cells, rather than on APCs.

Figure Three: T_{reg} suppression of $CD4^+$ T_{eff} activated in a TCR-dependent manner is MHC II restricted to T_{eff}

APC	Anti-CD3	T_{eff}	T_{reg}	T_{reg}:T_{eff} ratio	% inhibition
BALB/c	+				
	+	BALB/c			
BALB/c	+	BALB/c	BALB/c	1:3	96%
BALB/c	+	BALB/c	BALB/c	1:6	87%
BALB/c	+	BALB/c	BALB/c	1:12	78%
BALB/c	+	BALB/c			
BALB/c	+	BALB/c	B6	1:3	100%
BALB/c	+	BALB/c	B6	1:6	93%
BALB/c	+	BALB/c			
B6	-	BALB/c	BALB/c	1:1	85%
B6	-	BALB/c	BALB/c	1:2	73%
B6	-	BALB/c			
	-		BALB/c		
B6	-				
	-	BALB/c			
B6	-	BALB/c	B6	1:1	6%
B6	-	BALB/c	B6	1:2	12%
B6	-	BALB/c			



Mixed cell cultures were set in triplicates and incubated for 4 days. T cell proliferation was measured by ^3H thymidine incorporation in the presence or absence of T_{regs} from BALB/c or B6 origin. Bars indicate T_{eff} proliferation; percent inhibition of proliferation are indicated. Data are from 3 independent experiments for cultures activated with anti-CD3 and 5 independent experiments for cultures activated by B6 APCs. T_{eff} are BALB/c $CD4^+$ $CD25^-$ cells.

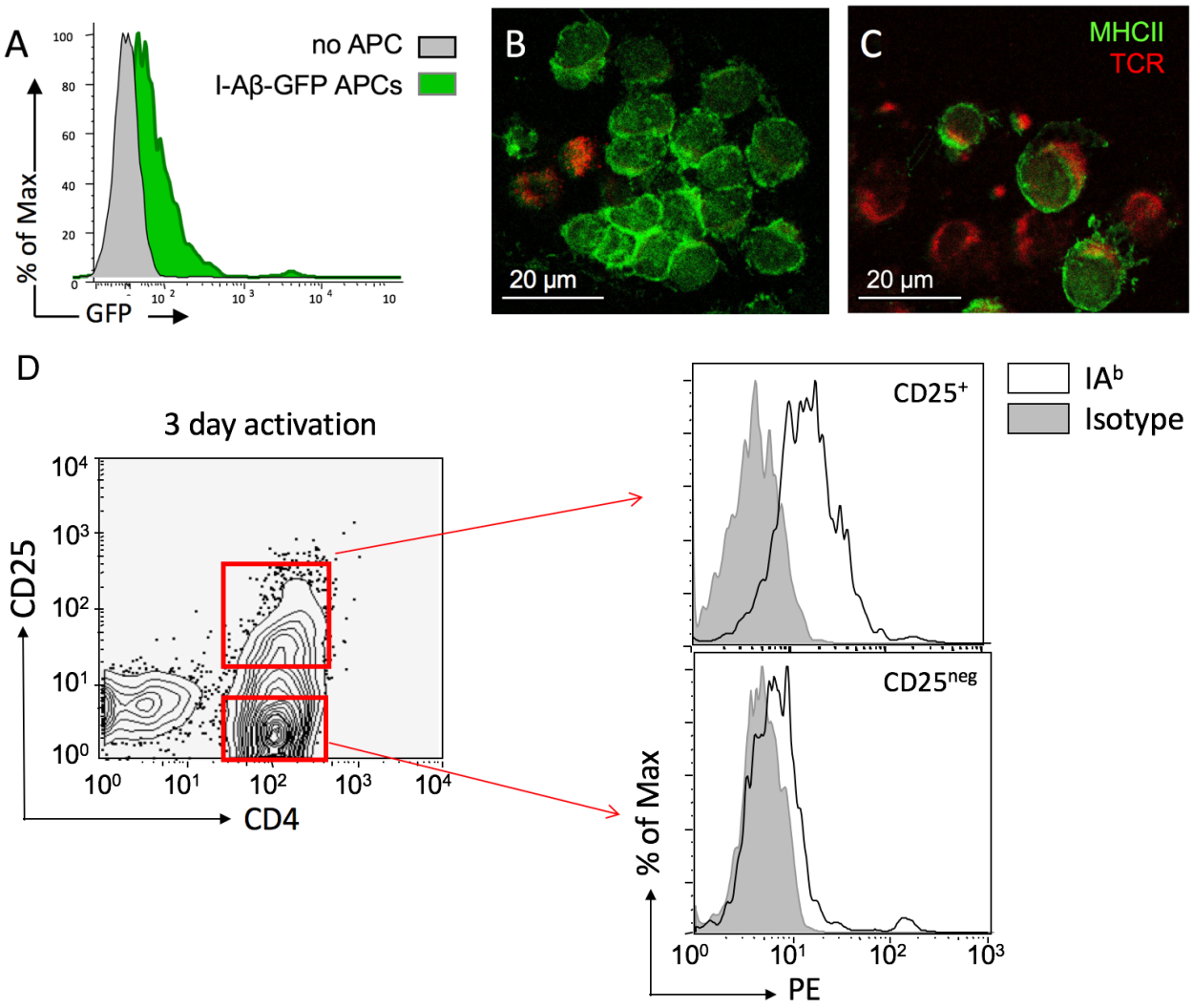
Combining the findings that T_{eff}-T_{reg} interactions are pMHC self restricted (Figure 3) with the fact that $CD4^+$ T cells are MHC II restricted, we next analyzed if activated T_{eff} cells express MHC II (Figure 4). In panel A, $CD4^+$ $CD25^-$ T_{eff} cells were isolated from wild type (WT) B6

mice and co-cultured with or without syngeneic I-A^b-GFP APCs; cells were activated in a TCR-independent manner using PMA and ionomycin. Following an 18-hour incubation, FACS analysis showed that TCR^+ $B220^-$ single cells cultured with the fluorescent APCs displayed increased GFP on their surface compared to those cultured with PMA and ionomycin but no APCs (Figure 4A). Immunocytochemistry analysis further showed that TCR^+ cells displayed GFP^+ I-A^b following TCR-independent stimulation and culture with I-A^b-GFP APCs. (Figure 4C)

In panel D, $CD4^+CD25^{neg}$ OT-II T_{eff} were stimulated with irradiated self APCs and cognate ovalbumin peptide for three days. Cells activated in a TCR-dependent manner were identified by CD25 expression, while cells negative for CD25 were considered non-activated. Activated T cells displayed increased MHC II compared to their non-activated counterparts.

Collectively, these data show that T_{eff} display additional MHC II on their surface following activation. Taken with data from previous experiments, they suggest that MHC II molecules on the surface of T_{eff} may contribute to the activation of T_{regs}.

Figure Four: Activated T lymphocytes express surface MHC-II



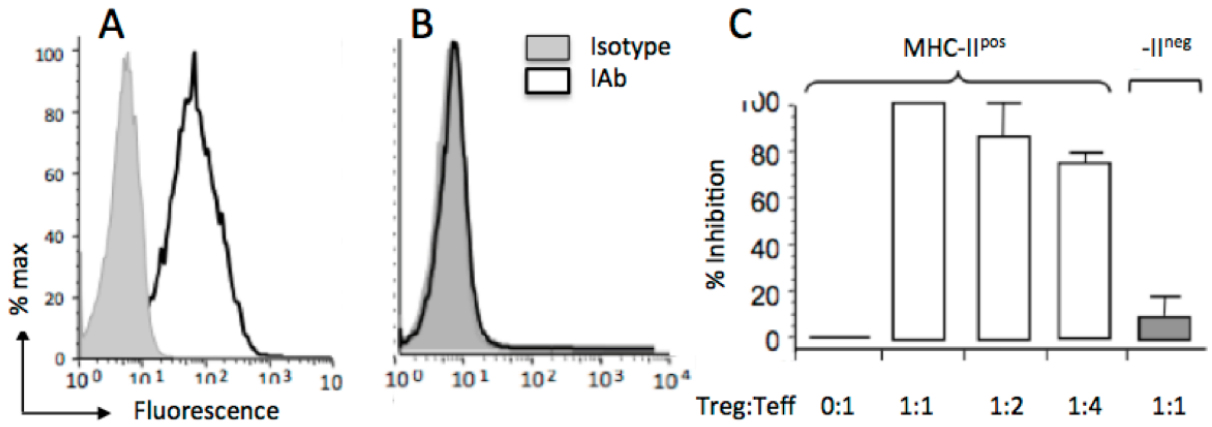
A: Naïve $CD4^{+}CD25^{neg}$ T cells from B6 mice were co-cultured with or without I-Aβ-GFP APCs in the presence of PMA + ionomycin for 18 hours. GFP expression on singlets of TCR^{+} $B220^{neg}$ $CD25^{+}$ activated T cells. Immunocytochemistry analysis of 95% pure $B220^{+}$ B cells **(B)** and 93% pure $CD4^{+}$ T cells **(C)** from the I-Aβ-GFP mouse following 18 hrs of PMA/ionomycin activation. Fixed cells were stained with an anti-TCR mAb (red).

D: Naïve $CD4^{+}CD25^{neg}$ T cells from OT-II mice were stimulated with irradiated self-APCs and chicken ovalbumin. After three days, cells were assessed for activation by CD25 expression and MHC II expression by FACS analysis.

Next, we investigated whether display of MHC II on the surface of T_{eff} was necessary for their suppression by T_{regs}. WT B6 T_{eff} were activated with irradiated, allogeneic CD90-depleted splenocytes either in the presence (Figure 5A) or absence (Figure 5B) of contaminating syngeneic I-A^b APCs. Consistent with results in Figure 4, T_{eff} activated in the presence of syngeneic APCs displayed self MHC II on their surface (Figure 5A). In contrast, sorted T_{eff} without contaminating APCs as a source of this molecule did not display MHC II on their surface. (Figure 5B)

MHC II expression on the surface of activated T_{eff} was related to their ability to be suppressed by T_{regs} in subsequent suppression assays (Figure 5C). MHC II^{pos} T_{eff} were suppressed by $CD4^+$ $CD25^+$ T_{regs}, with higher levels of suppression occurring at higher T_{reg}:T_{eff} ratios. The same T_{regs} were unable to control the proliferation of MHC II^{neg} $T_{eff,}$ however, at even the highest ratio tested in the MHC II^{pos} group. (Figure 5C)

Figure Five: Self pMHC-II complexes displayed on T_{eff} cells control T_{reg} suppression



Naïve WT B6 T cells were either enriched in the $CD4^{+}CD25^{neg}$ subset (8% of $I\text{-}A^{b+}$ cell contamination, **Panel A**), or further purified by FACS-sorting of TCR^{+} cells (99% pure, **Panel B**). The two subsets were then co-cultured with CD90-depleted, irradiated BALB/c APCs for 4 days. Panels **A and B** show respective $I\text{-}A^{b}$ expression on enriched and purified T_{eff} cells. **C.** Identical co-cultures of APC and T_{eff} cells were set up in the presence of various amount of sorted $CD4^{+}CD25^{high}$ from B6 mice; suppression was measured after 4 days. Inhibition of proliferation of T_{eff} cells expressing ($MHC\text{-}II^{pos}$) or not ($MHC\text{-}II^{neg}$) surface $I\text{-}A^{b}$ during the assay is indicated by bar height. T_{reg}:T_{eff} ratios are indicated below the corresponding bars.

Collectively, the results of these *in vitro* experiments suggest that T_{regs} recognize activated T_{eff} as targets for suppression by self-MHC II molecules displayed on their surface. *In vitro* co-culture experiments are susceptible to artifact, however, and next we sought to investigate this hypothesis in an *in vivo* model of skin transplantation adapted from Francis *et al*.[3]

In vivo *studies*

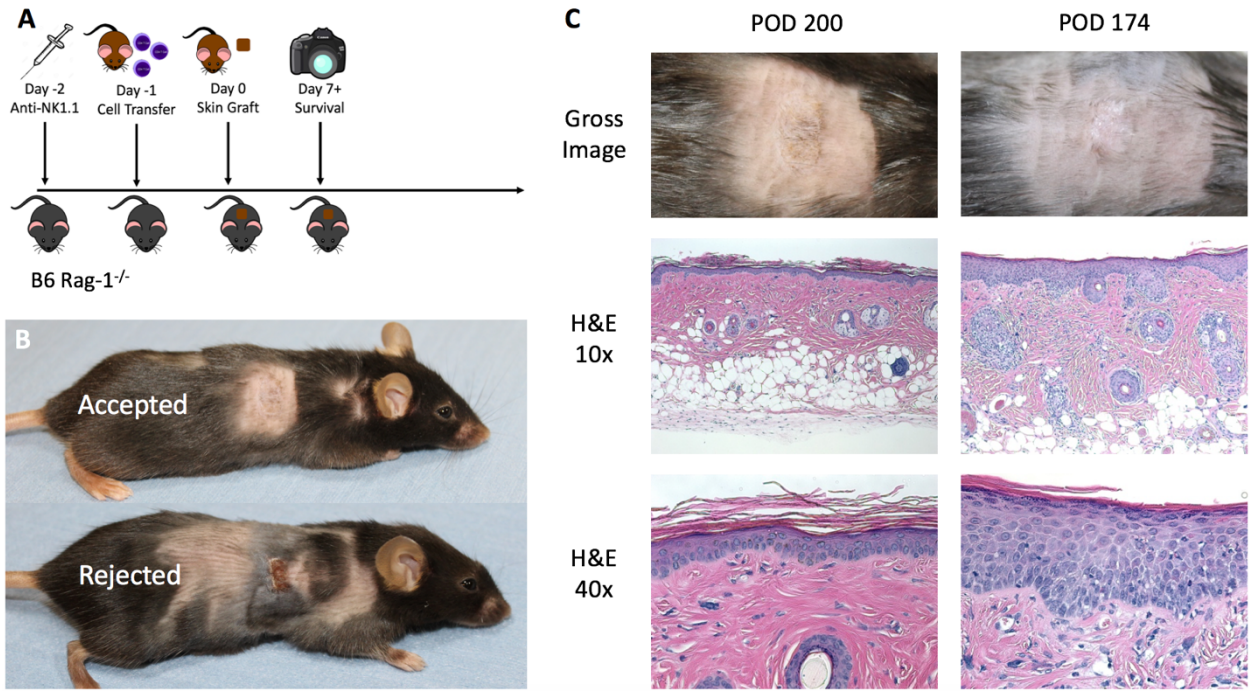
All *in vivo* experiments were performed by Madeline Fryer. The goal of these experiments was to develop and use an *in vivo* transplant model to assess the efficacy of T_{reg} at suppressing allograft rejection.

Briefly, C57BL6 (B6) RAG1 deficient mice were treated with a NK cell depleting antibody (clone PK136) one day prior to cell transfer to prevent cell rejection. FACS sorted T_{eff} and T_{regs} from different genetic backgrounds (B6, C3H, and BALB/c) were adoptively transferred into recipients via tail vein injection, and allogeneic skin grafts were placed the following day. Mice were monitored for graft rejection following bandage removal one week after transplant. (Figure 6A) We verified via FACS that anti-NK treatment allowed allogeneic T_{regs} to persist at least two weeks post-rejection (result not shown).

Skin transplants were visually monitored for signs of rejection (Figure 6B). Accepted skin grafts maintained their original texture, shape, and size; they also grew hair. Rejecting allografts developed a smooth, shiny texture, failed to grow hair, shrank, and eventually scabbed off the recipient. Grafts were considered rejected upon shrinking to less than 10% of their original size.

Several recipients of long-term (greater than 150 days) surviving allografts were sacrificed and grafts histologically examined to confirm that visual graft assessment was accurate (Figure 6C). In the left column, a graft surviving 200 days appeared grossly normal; the original shape, size, and texture were maintained, and the graft was growing hair. This corresponded with histological findings: the epidermis was a normal thickness and hair follicles and sebaceous glands in the dermis were not enflamed.

Figure Six: Adoptive transfer model for evaluating MHC II restriction of T_{regs} *in vivo*



A: On day -2, Rag1$^{-/-}$ recipients were injected IP with 600 ug of natural killer (NK) cell depleting antibody (NK1.1, clone PK136, BioXCell.) The next day, $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ cells were sorted from B6, C3H, or BALB/c donors and adoptively transferred into recipients in multiple ratios. On the day of transplant, recipients received tail skin grafts from C3H or BALB/c donor mice. Grafts were covered by a protective bandage for one week, then monitored for graft survival/rejection.

B: Example accepted and rejected C3H skin grafts.

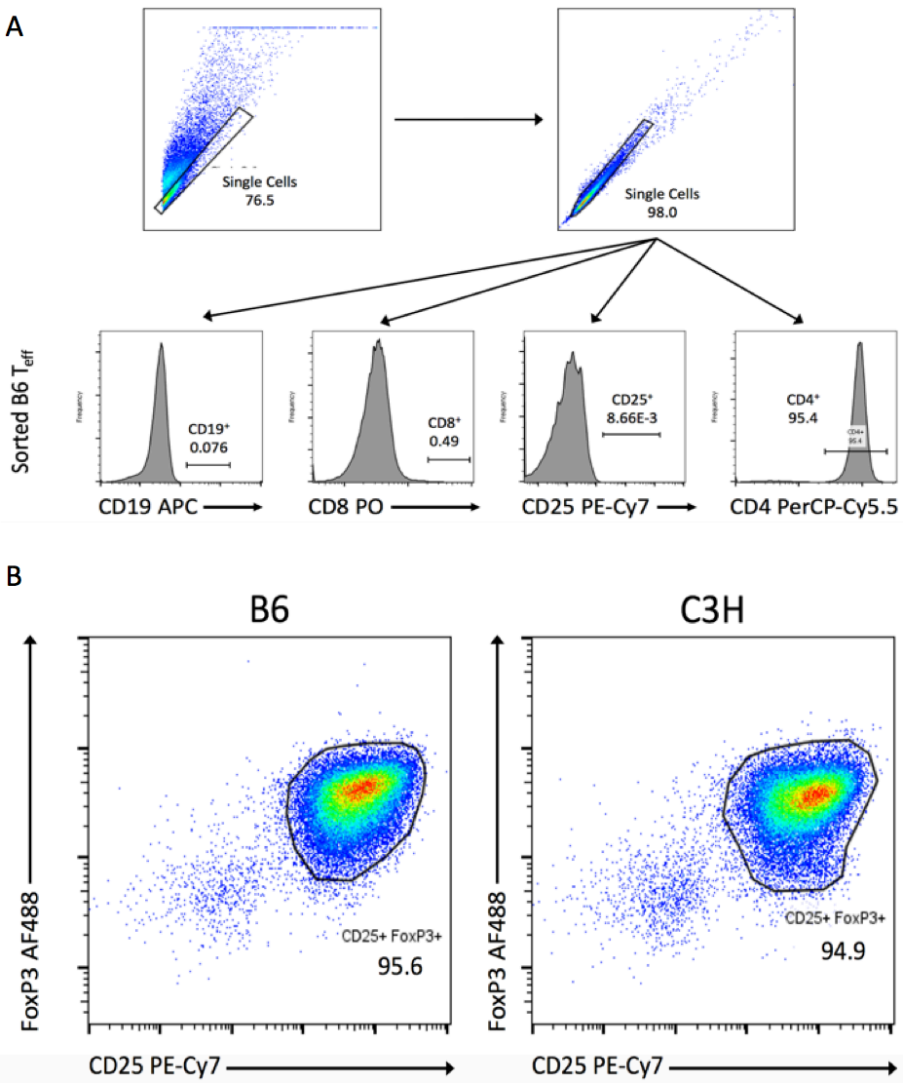
C: Gross evaluation of skin allografts correlated with histological findings. Skin grafts were excised with bordering native skin, preserved in 10% neutral buffered formalin, and stained with hematoxylin and eosin. (H&E, hematoxylin and eosin; POD, Post-Operative Day).

In comparison, the graft in the right column harvested on POD 174 was slightly shrunken, grew less hair, and had a smoother, shinier appearance. Upon histological examination, this graft had a markedly increased epidermal thickness, as well as inflammation of sebaceous glands and hair follicles within the dermis (Figure 6C).

Samples of sorted cell populations transferred into recipient mice were saved for additional FACS analysis to verify their purity. Figure 7A shows a sample analysis of sorted B6 $CD4^+$ $CD25^-$ T_{eff}. When gated on single cells, this population was contaminated with less than 0.5% each of B cells, $CD8^+$ T cells, and $CD25^+$ T_{regs}. The sorted population was greater than 95% positive for CD4 expression.

Figure 7B shows representative plots of adoptively transferred T_{regs}. Once again gated on single cells, sorted T_{regs} were roughly 95% double positive for CD25 and the transcription factor FoxP3. The high purity of transferred populations reduces the probability that observed survival outcomes are due to effects of contaminating cells.

Figure Seven: Transferred cell populations were highly purified

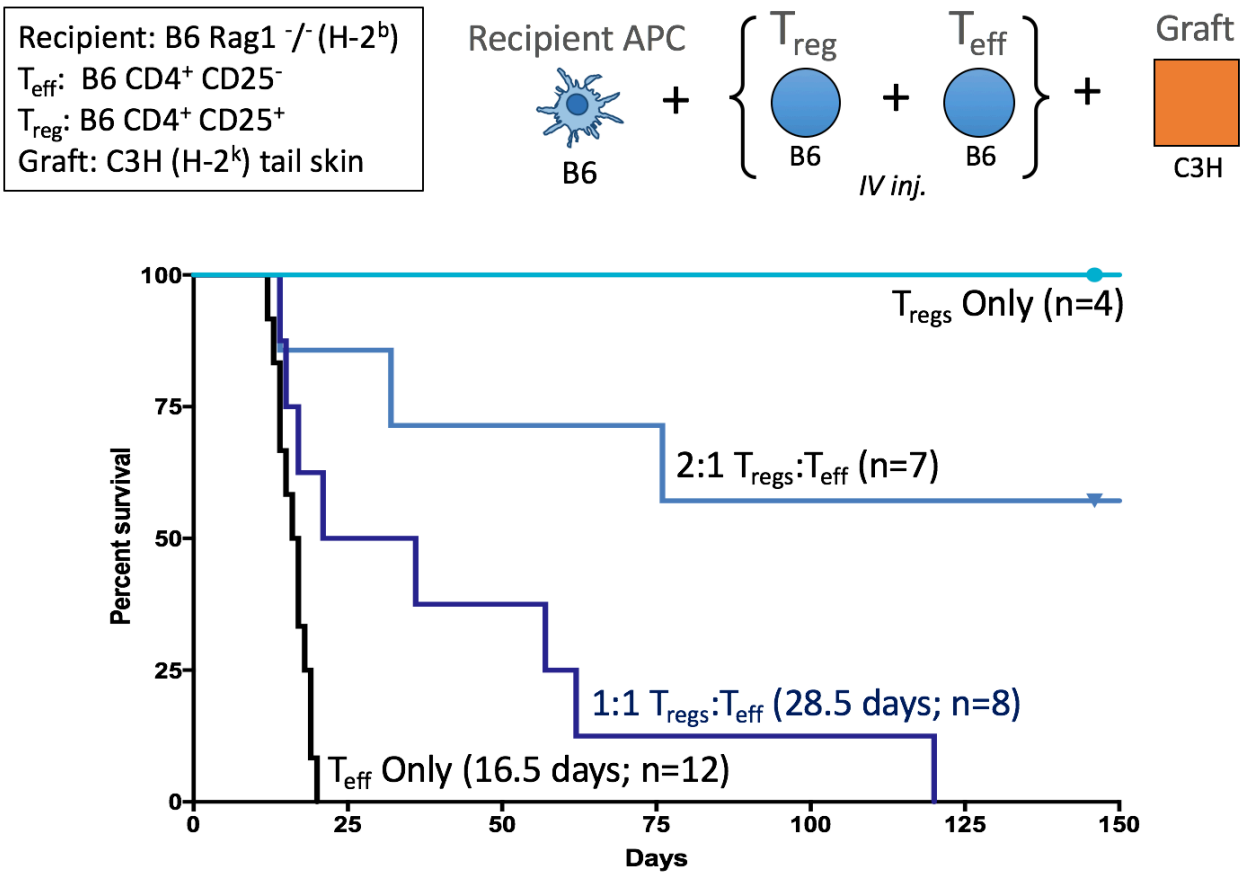


A: Representative samples of sorted $CD4^+$ $CD25^-$ T_{eff} were gated on singlets and analyzed by FACS for contaminating cells. Populations were contaminated with fewer than 0.1% B cells and 0.5% $CD8^+$ T cells.

B: Representative samples of sorted $CD4^+CD25^+$ T_{reg} from both B6 and C3H donors are 95% double positive for CD25 and FoxP3.

The first series of transplants performed all involved the transfer of $CD4^+$ T cells from a WT B6 ($H\text{-}2^b$) mouse into a B6 Rag1 deficient recipient; all skin allografts were from C3H ($H\text{-}2^k$) donors (Figure 8). Control mice receiving 10^5 B6 T_{eff} and a C3H skin transplant rejected their grafts in a median of 16.5 days, while mice receiving an equal number of sorted B6 T_{regs} failed to reject a C3H skin transplant for over 150 days ($p = 0.0013$; Figure 8; Tables 2 & 3). B6 T_{regs} transferred together with T_{eff} in a 1:1 ratio prolonged graft survival to 28.5 days, while transferring twice as many T_{regs} as T_{eff} resulted in greater than 50% graft survival for more than 150 days. These statistically significant (Table 3) differences in graft survival demonstrate that T_{regs} are able to suppress T_{eff} from the same genetic background. Furthermore, they validate the use of this model for evaluating the suppressive ability of T_{regs} to control T_{eff} of different concentrations and genetic backgrounds.

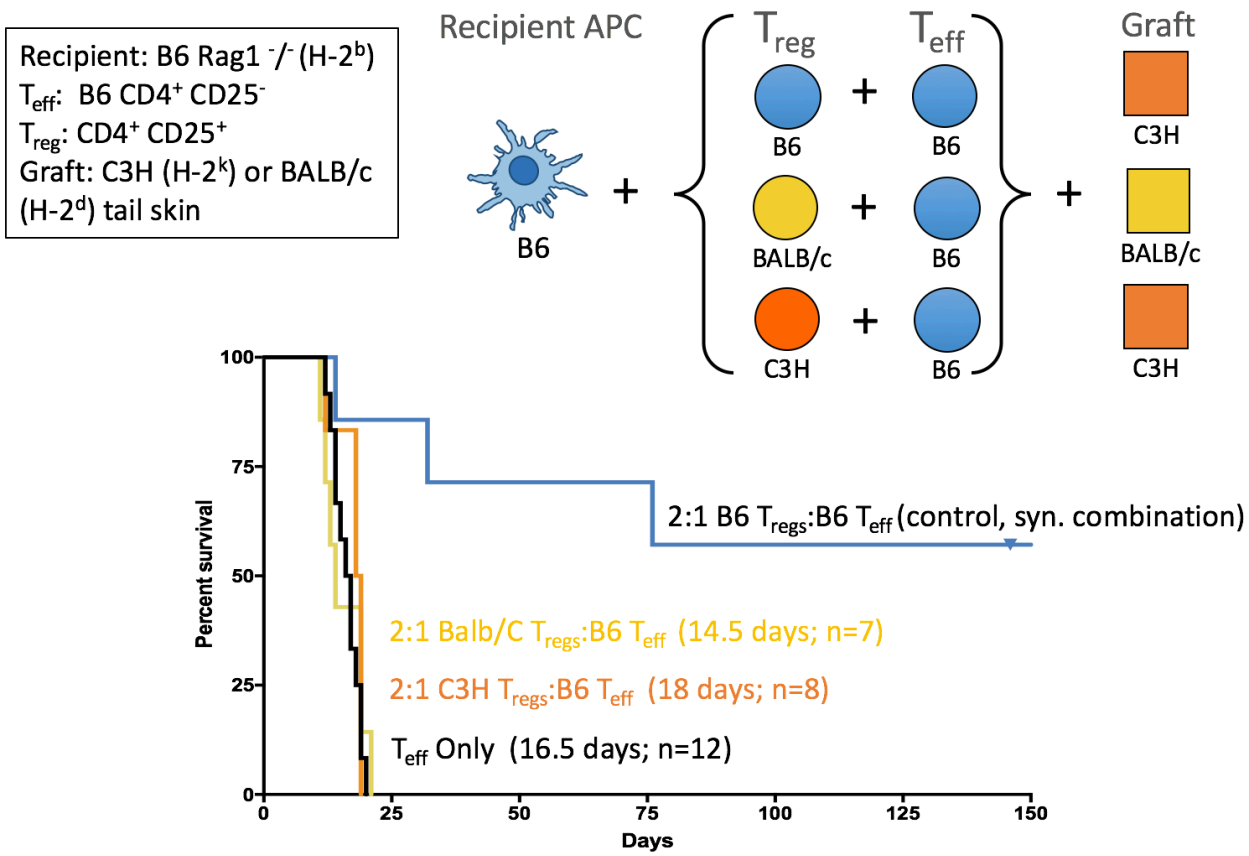
Figure Eight: Syngeneic T_{reg} prolong skin allograft survival in a dose-dependent manner



Kaplan-Meier plots of C3H tail skin grafts transplanted onto B6 $Rag1^{-/-}$ recipients. On the day prior to transplantation, recipients were infused with WT B6 $CD4^+$ cells. All groups received 10^5 of each cell type, except for the "2:1 T_{reg}:T_{eff}" group, which received 2 x 10^5 T_{reg}. T_{reg} = $CD4^+CD25^+$; T_{eff} = $CD4^+CD25^-$. Corresponding survival data and statistical significance of individual curve comparisons can be found in Tables 2 and 3.

Previous data imply that T_{eff}-T_{reg} interactions are self-MHC restricted and validate the *in vitro* results. If correct, this would also suggest that T_{reg} allogeneic to T_{eff} would be non-suppressive. We therefore tested the suppressive effect of C3H and BALB/c (H-2^d) T_{regs} facing graft-reactive B6 T_{eff} cells (Figure 9).

Figure Nine: Allograft-matched T_{reg} are unable to prevent graft rejection by allogeneic $CD4^+$ T_{eff}



Kaplan-Meier plots of allogeneic tail skin grafts transplanted onto B6 $Rag1^{-/-}$ recipients. On the day prior to transplantation, recipients were infused with wild type B6 T_{eff} and T_{regs} from B6, C3H, or BALB/c donors. All groups received 2 x 10^5 T_{regs} and 10^5 T_{eff}, except for the "T_{eff} only group" which received no T_{regs}. Corresponding survival data and statistical significance of individual curve comparisons can be found in Tables 2 and 4.

When C3H T_{regs} were transferred with B6 T_{eff} in a 2:1 ratio, they failed to extend C3H graft survival beyond that of B6 T_{eff} alone. (MST = 18.5, p = 0.3608; Figure 9; Tables 2 & 4) This suggests that the presence of "donor-specific" T_{regs} is not as important for preventing allograft rejection as T_{regs} which are genetically matched to the effector cells. This point was further emphasized by results from mice that received BALB/c T_{regs} and B6 T_{eff} in a 2:1 ratio, followed

by a BALB/c skin graft. Once again, matching between the T_{regs} and skin graft did not extend graft survival in the same manner that matching between T_{reg} and T_{eff} did. (MST = 14, p = 0.7044)

Table 2: Median Allograft Survival Time of C3H Skin Grafts (Figures 8 & 9)

Group	**MST (days)**	**Survival (days)**	**N**
B6 T_{reg} Only	undefined	150+, 150+, 150+, 150+	4
B6 T_{eff} Only	16.5	12, 13, 14, 14, 15, 16, 17, 17, 18, 19, 19, 20	12
1:1 B6 T_{reg}:T_{eff}	28.5	14, 15, 17, 21, 36, 57, 62, 120	8
2:1 B6 T_{reg}:T_{eff}	undefined	14, 32, 76, 150+, 150+, 150+, 150+	7
1:1 C3H T_{reg}:B6 T_{eff}	16	15, 15, 17, 18	4
2:1 C3H T_{reg}:B6 T_{eff}	18.5	12, 18, 18, 19, 19, 19	6
2:1 BALB/c T_{reg}:B6 T_{eff}*	14	11, 12, 13, 14, 19, 19, 21	7

Table 3: Log-Rank Significance of Allograft Survival (Figure 8)

C3H Skin Grafts	B6 $CD4^+$ T_{eff} Only	1:1 B6 T_{reg}:T_{eff}	2:1 B6 T_{reg}:T_{eff}
B6 T_{reg} Only	0.0013**	0.0025**	0.1540 (ns)
B6 $CD4^+$ T_{eff} Only	X	0.0093**	0.0007***
1:1 B6 T_{reg}: T_{eff}	0.0093**	X	0.0221**

Cells contain the p-value comparing survival of groups in intersecting row and column.

Table 4: Log-Rank Significance of Allograft Survival (Figure 9)

Skin Grafts*	2:1 C3H T_{regs}:B6 T_{eff}	2:1 BALB/c T_{reg}:B6 T_{eff}
2:1 B6 T_{reg}: B6 T_{eff}	0.0074**	0.0016**
B6 $CD4^{+}$ T_{eff} Only	0.3608 (ns)	0.7044 (ns)
2:1 C3H T_{regs}:B6 T_{eff}	X	0.8444 (ns)

Cells contain the p-value comparing survival of groups in intersecting row and column.
* Mice receiving B6 or C3H T_{regs} received a C3H graft.
Mice receiving BALB/c T_{regs} received a BALB/c graft.

Taken together, these *in vivo* data support our *in vitro* findings that T_{reg} activation is restricted to the MHC II expressed by T_{eff}, not by APCs. The transplants performed in Figure 9 also demonstrate that matching between T_{reg} and allografts is not sufficient for prolonging allograft survival.

2.3: Brief Discussion

Mature T_{con} are not constitutively active, but must receive signals from APCs through their TCR and costimulatory molecules before exerting their effector functions.[25] As another subset of $CD4^+$ T cells, it follows logically that T_{regs} in the periphery must also be locally activated by other cells.[5, 12, 86] Local, not constitutive, activation of T_{regs} is also beneficial from an evolutionary perspective. Constitutively active T_{regs} would expend more energy; constant immunosuppressive activity could also render individuals more susceptible to infections and malignancy. Notwithstanding, the specific signals responsible for activating and guiding T_{reg} suppression *in vivo* remain unclear.[18]

A series of suppression assays comparing the ability of T_{reg} syngeneic to T_{eff} and allogeneic to APCs with T_{regs} allogeneic to T_{eff} and syngeneic to APCs showed that T_{regs} more effectively inhibit the proliferation of syngeneic T_{eff}. (Figure 3) These results suggest that T_{regs} recognize and are activated by signals on T_{eff}. The stark differences in inhibition between $CD4^+$ T cells of different genetic backgrounds suggest the responsible signal is an MHC II molecule, not a generic T_{eff} marker.

These results were recapitulated in an *in vivo* model using T_{eff} and T_{regs} of different genetic backgrounds adoptively transferred into a B6 $Rag1^{-/-}$, followed by an allogeneic skin graft. (Figure 6) As in the *in vitro* experiments, T_{regs} genetically matched to T_{eff} inhibited their response in a dose-dependent manner. (Figure 8) Also recapitulating prior *in vitro* results, T_{regs} allogeneic to T_{eff} were unable to prolong allograft survival. (Figure 9) This inability to control effector responses was not dose-dependent (Table 2).

Our data show that T_{reg} are responding to pMHC-II complexes displayed on T_{eff}, but not APCs (Figure 3). These complexes are captured by murine T cells from APCs, but the impetus and mechanism underlying this transfer are not understood. One possible mechanism is through exosome transfer. Such a mechanism would eliminate the need for direct cell contact between APCs and activated T_{eff}, as well as facilitate the transfer of multiple surface proteins at one time. Unpublished data from our group has shown that surface protein transfer between APCs and T cells is roughly five times more efficient between syngeneic cells than allogeneic, suggesting at least a partial role for TCR involvement. Another possible method of transfer is TCR-mediated trogocytosis; it is unlikely to be the predominant transfer method, however, as this would suggest that the T_{eff} capturing pMHC-II and the T_{regs} which recognize them have identical TCR specificity.

Even if the mechanisms of pMHC-II transfer between APCs and T_{eff} were well understood, the relative contributions of captured versus synthesized pMHC complexes in human T cell biology remains unknown. We suspect that synthesized pMHC-II complexes are more prevalent than captured ones in human T cells, but a better understanding of the transfer mechanism in mice may shed some light on this issue. For example, if it were found that pMHC-II complexes are transferred as bystanders through a process evolved to capture multiple different surface proteins, it would suggest that endogenously synthesized molecules play a more significant role. On the other hand, if transfer is specific to MHC II, it would suggest a more conserved process and immunologically significant role for MHC II transfer in human T_{reg} activation.

Another interesting point raised by these data is the different ability of pMHC-II displayed on APCs and T_{eff} to activate T_{reg}. T_{reg} activation by syngeneic T_{eff}, but not syngeneic APCs (Figure 3), suggests that there may be a secondary signal expressed on activated effector cells which plays a role in T_{reg} activation.

T_{regs} control T_{con} in a variety of immune responses; they did not specifically evolve for the setting of allogeneic transplantation. This suggests that T_{regs} recognize and are activated by intrinsic, self peptides, rather than having specificity for a particular donor peptide antigen. The fact that T_{regs} are selected during T cell development for their ability to recognize self would also be in agreement with this hypothesis.

A comparative analysis of the immune parameters of more than 200 pairs of twins found that among immune cell subsets, T_{reg} were one of the least variable with age and immunological experience.[87] In other words, the T_{reg} TCR repertoire appears to be internally focused and less influenced by foreign antigens as is the T_{eff} repertoire.

Collectively, these data suggest that T_{regs} recognize self pMHC-II complexes displayed on activated T_{eff}. Considering this idea from the perspective of evolution and efficiency, recognition of self, intrinsic signals on the surface of activated T_{eff} by T_{regs} would present an elegant system for directing suppression towards activated cells. Furthermore, this hypothesis fits well with the current understanding of T_{reg} development in the thymus: strongly self-reactive developing thymocytes differentiate into T_{regs}.[88, 89]

Chapter 3: Discussions and Perspectives

3.1: Limitations

All of the experiments presented here were performed in mice; as previously referenced above, murine T cells do not synthesize MHC II. Therefore, any pMHC-II complexes displayed on T_{eff} must be captured from neighboring APCs. This limits the strength of the data because the model system does not work in the same way as human T cells do. The relative contribution of synthesized versus captured pMHC II cannot be assessed here.

These *in vitro* experiments demonstrate that upon activation, murine T_{eff} capture MHC II from self APCs. We failed to show, however, whether similar transfer occurs between allogeneic APCs and T_{eff}. Prior, unpublished data from our lab has shown that the transfer of surface molecules from APCs to other cells is roughly five times more efficient between syngeneic cells than allogeneic cells, yet we can only speculate about the mechanism responsible for this discrepancy and its effect on T_{reg} activation. It is possible, for example, that T_{reg} restriction is limited to pMHC II complexes displayed on the surface of activated T_{eff}, not necessarily the background of the T_{eff}; differences in transfer efficiency between allogeneic and syngeneic cells could obscure the interpretation of our data.

In addition to not testing for the presence of donor MHC II on T_{eff} cells, we also did not investigate MHC II expression on T_{regs}. Differences in the expression pattern of MHC II on T_{reg} and T_{eff} may provide insight into the mechanism of capture.

All data showing pMHC II display by activated T_{eff} was generated *in vitro*. The only readout for the *in vivo* studies was allograft survival. Additional experiments using FACS to analyze MHC II, CD25, CD28, B7.1/.2, CD40L, FoxP3, and PD-1 expression on transferred T cells isolated from recipient mice would provide relevant data to this story.

More obvious limitations to the data presented here are small *in vivo* sample sizes and the artificial nature of the model itself. While it is an elegant system for assessing T_{reg} and T_{eff} interactions *in vivo*, $Rag1^{-/-}$ mice are a highly manipulated system that does not perfectly recapitulate a wild type animal. The number of transferred cells is quite low, and minor fluctuations in cell counting or injection efficiency may have an impact on the ratio of T_{reg} to T_{eff} and allograft survival outcomes. Furthermore, as has been observed in many previous transplantation studies, mice are not men; interventions and therapies which induce tolerance in murine models rarely translate effectively to the clinic.

There are no histological samples here of acutely rejecting allografts, however, skin transplantation and rejection criteria are well established.

3.2: Future Research

Many additional questions are raised by the results of these experiments. Future studies should investigate the source of peptide in pMHC-II complexes which activate T_{regs}. Our current hypothesis is that these peptides are fragments of self MHC II molecules.[74, 75] This hypothesis is informed by prior observations that MHC II molecules on the cell surface of human dendritic cells (DCs) are taken back into endosomes, ubiquinated, and re-enter the MHC II presentation pathway.[25, 79] Additionally, studies by Viret, He, and Janeway have demonstrated that mice present fragments of MHC II on their own MHC II molecules, and furthermore, that a fragment of mouse I-Eα presented on I-A^b plays a significant role in positive selection of $CD4^+$ T cells during thymic development.[82, 90-93] The relative contributions of donor and recipient derived peptides in the pMHC-II complexes displayed on T_{eff} in a transplant setting are also not established.

We also hypothesize that the pMHC-II complexes displayed on activated T_{eff} are captured from nearby APCs. This transfer may occur through contact-dependent and independent mechanisms [78, 81] including uptake of exosomes[80, 94-96] and TCR-mediated endocytosis/trogocytosis.[97-104] Additional studies should be performed to understand the signaling pathways involved in the transfer and display of these complexes. Yet another unanswered question is if and how pMHC-II complexes are transferred to $CD8^+$ effector T cells, whose TCR are specific for MHC I.

Adapting the *in vivo* model used in our studies, a three-way mismatch using C3H T_{eff} and B6 T_{regs} into a B6 $Rag1^{-/-}$ recipient, followed by transplantation of a BALB/c skin graft, could

address the question of peptide source displayed by T_{eff}. If the source of pMHC-II complexes is APCs from the skin graft, we would expect that B6 MHC-II restricted T_{regs} would be unable to recognize the activated C3H T_{eff}, and the BALB/c skin graft would be rejected. Alternatively, if C3H T_{eff} take up a sufficient amount of pMHC-II complexes from recipient B6 APCs, it is possible that the B6 T_{regs} would recognize these complexes on the surface and suppress T_{eff} proliferation, extending allograft survival. Potential limitations to this study design include possible graft-versus-host-disease (GVHD) resulting from the transfer of of C3H T_{eff} into a B6 $Rag1^{-/-}$ host, T cell exhaustion[105], and rejection of B6 T_{regs} by C3H T_{eff}.

The role of secondary signals in modulating T cell responses has recently been better appreciated, particularly in the field of cancer immunotherapy. Similarly, results of our *in vitro* experiments suggest that activated T_{eff} display additional signals besides captured pMHC-II complexes to T_{regs} that play a role in T_{reg} activation. If there were no second signal being presenting by T_{eff}, then T_{reg} should be equally well activated by pMHC-II displayed on APCs and T_{eff}. The restriction of T_{reg} activation to pMHC-II expressed on T_{eff}, however, suggests that there are other signals involved. Additional work should be done to identify these signaling pathways and their potential application to T_{reg} therapies.

More broadly, future research in this area should focus on autologous, polyclonal T_{reg} therapy for the treatment of autoimmune disease and transplantation. The results of our study clearly demonstrate that T_{eff} responses are most effectively suppressed by syngeneic T_{regs}. Assuming that the TCRs of T_{regs} are predominantly self-reactive, the failure of allograft-matched T_{regs} to prolong skin allograft survival suggests that donor-specific T_{regs} may not be the most promising

therapeutic option. Future work should focus on the use of polyclonal T_{regs} because they are simpler to generate; furthermore, T_{reg} suppression is not antigen specific. Better understanding of T_{reg} activation is likely to be more clinically fruitful than efforts to generate T_{regs} with a particular antigen specificity.

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