IMMUNE SUPPRESSIVE MOLECULES AND REGULATORY T CELLS CONTROL HOST DEFENSE AGAINST *LISTERIA MONOCYTOGENES* INFECTION

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Jared Hamilton Rowe

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisor: Sing Sing Way M.D. Ph.D.

June, 2012

Acknowledgements

I have been extraordinarily fortunate to receive my research and professional training at institutions such as the University of Minnesota and the National Institutes of Health. The faculty, postdoctoral research fellows, clinical fellows, residents, graduate students, and undergraduate students have all made significant impacts on my development as an independent scientist and I am forever grateful for their contributions. I would like to specifically acknowledge the following individuals who have the greatest impact on my work and development over the course of my education:

Dr. Sing Sing Way, my advisor, for all of the countless hours you have spent demonstrating to me the hard work, intuition, intelligence, excitement, and friendship required for making worth while scientific discoveries. Your mentorship has provided me with the strongest foundation possible for taking the next step in my development as a scientific investigator and provided me with the tools necessary to take on any future challenges that present themselves. Thank you for always keeping your door open for questions and discussion.

James Ertelt and the other members of the Way Lab (past and current): Dr. Tanner Johanns, Dr. Marijo Aguilera, Dr. Lijun Xin, Minelva Nanton, Esra Buyukbasaran, Margaret Mysz, Calvin Law, and Joseph Lai. Thank you for your continued and unparalleled support, scientific discussions, and critical evaluation of the data presented in this report.

Dr. Chris Pennell whose mentorship, support, friendship, and direction has been invaluable from my development from an undergraduate student with no laboratory experience, to an MSTP student with potential to become an independent investigator, to a PhD candidate at the University of Minnesota.

Dr. Bryce Binstadt for his continued mentorship and dedication to my development as a physician scientist being both my thesis committee chairperson and clinical mentor.

Dr. Mathew Mescher for his mentorship and support during the development of my research project and for serving on my thesis committee.

The members of my thesis committee: Drs. Kristin Hogquist, Stephen Jameson, and David Masopust, for their support and critical evaluation of my research.

Dr. Brian Fife for his assistance in the development of Chapter 4 and helpful discussions regarding my project.

The 2007 entering class for the University of Minnesota Medical Scientist Training Program (MD/PhD). Your friendship, support, and camaraderie have been fundamental throughout the entire course of my training at the University of Minnesota Medical and Graduate Schools.

Dr. Tucker Lebien and the MD/PhD Steering Committee for taking a chance on an under qualified applicant. I would also like to thank Susan Shurson and Nick Berg for their continued assistance with all MD/PhD program matters.

Dr. Marc Jenkins, Dr. Stephen McSorley, and the members of their respective laboratories for providing the MHC Class II tetramers (Chapter 7) and transgeneic OVA (Chapters 5 and 6) and 2W1S mice (Chapter 7), which have made this work possible.

Dr. Michael Farrar for his generous contribution of the STAT5b-CA transgeneic mice (Chapter 5) used in this work.

Dr. Claudia Palena at the National Institutes of Health for being my first research mentor and instilling in me the excitement of laboratory discoveries.

Lastly, I would like to extend sincere gratitude for my wonderful family because of their unparalleled support and enthusiasm for my education. Especially, my wonderful and beautiful wife JoAnna for her continued love, support, faith, humor, understanding, and patience without which I could not have completed the work presented here. My parents, Russell and Terri, for their unending love and support. My siblings, Josh and Jessie, for continuing to put up with my nonsense and supporting me in all my endeavors. My grandparents; Wendell and Anne Hodge, and Al and Anne Rowe for all their support.

Dedication

This dissertation is dedicated to the thirteen million infants born prematurely each year.

Abstract

The mammalian immune response is an intricately regulated, dynamic process providing the host with protection from the vast number of pathogenic microorganisms. Herein, these studies identified the impacts of regulating host defense by immune suppressive mechanisms, following infection with the bacterial pathogen *Listeria monocytogenes*. These investigations identified that changes in the inflammatory signals present during *Listeria* infection resulted in drastically different outcomes in the priming of protective T cells, when these cells were stimulated by either the immune suppressive molcules Cytotoxic T Lymphocyte Antigen (CTLA)-4 or Program Death Ligand (PDL)-1.

Due to these findings, whereby changes in the immunologic environment altered the function of suppressive molecules, we examined whether physiologic changes in the immune system would impact host defense to infection. The natural expansion of the immune suppressive regulatory T cell (Tregs), while essential for sustaining pregnancy, resulted in increased maternal susceptibility to *Listeria* infection. Morevoer, *Listeria* entry into the host cell cytoplasmic compartment was suffient to reduce Treg suppressive potency and fracture Treg-mediated maternal tolerance. Lastly, we identified that the expansion of Tregs during pregnancy was primed by fetal-specific antigens and resulted in a long-lived population of cells, which provide "regulatory" memory. This is the first demonstration of Tregs with the ability to re-expand following a secondary antigen challenge (either fetal or pathogen-derived) and should spark future investigation into the role of regulatory memory.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction[†]

[†] Reprinted in part from Immunology. Jared H. Rowe, James M. Ertelt, and Sing Sing Way. "Foxp3^{+} regulatory T cells, immune-stimulation and host defense against infection."

Immune Activation and Inhibition

The fluid balance between immune activation required for optimal host defense against infection and immune suppression that maintains tolerance by averting autoimmunity is stringently regulated. This allows immune effectors with the potential to cause catastrophic damage to host tissues to be actively silenced during homeostasis, but also rapidly unleashed in response to infection. Accordingly, the cell-associated and cytokine signals that stimulate the activation of immune effectors have been intensely investigated for developing new therapeutic strategies for boosting desired immune responses during infection or immunization. On the other hand, understanding how ubiquitous immune suppression signals are selectively silenced during immune activation, and the extent they limit optimal host defense against infection has lagged behind.

The mammalian immune system contains multiple intricately regulated networks of opposing activation and inhibition signals. The balance between these contradicting signals provides instructions for the immune system to both mobilize protective antigen-specific responses during infections, but also quickly silence these effector cells once the infection is cleared thus minimizing immune-mediated injury to the host. The critical effector cell required to mediate protection against intracellular pathogens is the T cell subset of immune cells. Initial control of T cell-activation is controlled in part by CD28 and CTLA-4 (CD152), homologous receptors, that provide opposing activating and inhibitory roles, respectively (Kearney et al., 1995). The CTLA-4-mediated suppressive

effects on pathogen-specific T cell-activation have been demonstrated primarily in models of chronic bacterial, viral, and parasitic infection using monoclonal antibodies that specifically block CTLA-4 (Anderson et al., 2006; Furze et al., 2006; Jacobs et al., 2002; Kirman et al., 1999; Lepenies et al., 2007; Martins et al., 2004; McCoy et al., 1997; Taylor et al., 2007; Walsh et al., 2007). However, the role of CTLA-4 in inhibiting the activation and subsequent memory formation of effector T cells during acute infections is less well defined.

In addition to the profound control of T cell activation mediated by CTLA-4, other molecules immune inhibitory molecules have been demonstrated to play critical roles limiting T cell responses in specific immunological contexts. For example, Programmed death ligand-1 (PDL-1, B7-H1) belongs to a growing list of co-stimulation molecules within the B7 family that regulate T cell activation (Brown et al., 2010; Keir et al., 2007; Odorizzi and Wherry, 2012; Sharpe et al., 2007). After infection with Lymphocytic choriomeningitis virus (LCMV) and other persistent viral infections, PDL-1 sustains functional exhaustion for viral-specific $CD8^+$ T cells limiting their protection (Barber et al., 2006). Moreover, interrupting PDL-1 mediated signals using monoclonal antibodies during persistent infection or with therapeutic vaccination reinvigorates the activation of LCMV-specific $CD8^+$ T cells and accelerates pathogen eradication (Ha et al., 2008). Importantly, PDL-1-mediated immune suppressive properties initially described in mouse infection models extend to functional T cell exhaustion for humans infected with viruses that predominantly cause persistent infection. For example, $CD8^+$ T cells with specificity

to hepatitis C or human immune-deficiency virus each up-regulate the PDL-1 binding partner, PD-1, with progressively worsening infection (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006; Urbani et al., 2006). Furthermore, for rabies virus that primarily cause acute instead of persistent infection, targeted defects in PDL-1 also protects against lethal infection (Lafon et al., 2008). Taken together, these findings indicate PDL-1 compromises host defense against viral pathogens. However, the effects of PDL-1 signaling on protective T cell responses during intracellular bacterial pathogens remain unknown.

Regulatory T Cells and Immune Suppression

In addition to T cell-intrinsic molecules of inhibition, the identification of a distinct $CD4^+$ T cell subset with immune suppressive properties called *Regulatory T cells* (Tregs) was demonstrated to dictate dominant function over T cell activation (Sakaguchi, 2004; Sakaguchi et al., 1995; Suri-Payer et al., 1998). Although Tregs were initially identified as the $CD4^+$ T cell subset that constitutively express the IL-2 receptor, CD25, subsequent landmark studies have since established the lineage-defining and master regulator for Tregs is dictated by expression of the forkhead box P3 transcription factor, Foxp3 (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Infants who develop a fatal rare constellation of clinical features that includes refractory eczema, diabetes, thyroditis, colitis, infection susceptibility, and generalized wasting called the IPEX (Immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome have mutations in either the *foxp3* promoter or coding sequence resulting in defective Tregs (Bennett et al., 2001; Ochs et al., 2007; Wildin et al., 2001). Similarly, mice with naturally occurring or targeted defects in *foxp3* develop similar clinical features (lympho proliferation, colitis, weight loss, diabetes, and ruffled hair) associated with systemic autoimmunity, and become moribund within 20-25 days of life (Brunkow et al., 2001; Khattri et al., 2003; Wildin et al., 2001). Accordingly, $Foxp3^+$ Tregs are essential for maintaining peripheral immune tolerance in humans and mice, and these parallels in clinical features with Treg deficiency illustrate the usefulness of mouse models to investigate how Tregs may control other facets of the immune response.

In this regard, shortly after their identification as a distinct $CD4^+$ T cell lineage, numerous studies using various representative mouse models of parasitic, viral, and bacterial infection have described an important role for $CD25^+$ CD4 T cells in compromising host defense by suppressing the activation of protective immune components (Belkaid et al., 2002; Fulton et al., ; Kaparakis et al., 2006; Kursar et al., 2007; Netea et al., 2004; Suvas et al., 2004; Suvas et al., 2003; Taylor et al., 2006; Taylor et al., 2007; Zelinskyy et al., 2006). Since the ablation of CD25 expressing cells almost uniformly augmented resistance with reduced recoverable *in vivo* pathogen burden, Tregs were appropriately described as "*a dangerous necessity*" based on their detrimental roles in host defense and essential roles in sustaining immune tolerance (Belkaid, 2007). However with the subsequent identification of Foxp3 as the lineage-defining marker for Tregs, and the up-regulation of CD25 expression on activated T cells that occurs after infection, the conclusions of initial studies using CD25 expression as a surrogate marker for Tregs deserve critical re-evaluation using experimental strategies that identify and manipulate these cells based on Foxp3 expression.

Regulatory T Cells and Acute Infections

Pathogens that cause acute infection stimulate the activation of protective immune components almost immediately after infection. When the pathogen dose or initial rate of pathogen replication are below a preset threshold (lethal dose), innate immune components keep the infection at bay until pathogen-specific adaptive immune effectors that more efficiently mediate pathogen eradication are expanded and mobilized. On the other hand with higher inocula, these normally protective responses are overwhelmed and the host succumbs to infection. It is in this later context that initial studies using $Foxp3^{DTR}$ transgenic mice that co-express the high-affinity human diphtheria toxin (DT) receptor with Foxp3, allowing $Foxp3^{+}$ Tregs to be selectively ablated with low dose DT, first uncovered somewhat paradoxical protective roles for these cells in host defense. For example, the ablation of $Foxp3^{+}$ cells prior to intra-vaginal infection with herpes simplex virus (HSV)-2 caused accelerated mortality that was associated with delayed recruitment of protective immune cells into the vaginal tract and draining lymph nodes, and more recoverable virus at the site of infection (Lund et al., 2008). These protective effects were not limited only to mucosal infection with this pathogen because mice ablated of $Foxp3^{+}$ cells also contained increased titers of lymphocytic choriomeningitis virus after systemic infection that was associated with reduced lymph node chemokine levels (Lund et al., 2008). Similarly, $Foxp3^{+}$ Treg ablation prior to West Nile virus infection in mice caused increased mortality, worse clinical disease scores, and accelerated weight loss that were each associated with higher viral loads in the brain and spinal cord (Lanteri et al., 2009).

These results also parallel the lower frequency of Tregs in humans with symptomatic West Nile virus infection, and increased ratio of Treg to effector T cells in patients with mild compared with severe Dengue virus infection (Lanteri et al., 2009; Luhn et al., 2007). Accordingly, these first studies investigating infection susceptibility using $Foxp3^{DTR}$ mice to ablate Tregs based on Foxp3-expression established protective roles for these cells in host defense against specific viral pathogens.

In this regard, although Treg ablation using anti-CD25 antibody had been described to exacerbate inflammatory lesions in HSV-1 induced stromal keratitis, manipulating Tregs in this manner also accelerated the eradication of this virus (Suvas et al., 2004; Suvas et al., 2003). Therefore, despite the potential for other inherent differences in these more recent studies where Tregs were ablated based on Foxp3 compared with CD25 expression, these findings suggest differences in how Tregs are manipulated can lead to discordant conclusions. In particular, since CD25 expression is up-regulated by effector T cells upon activation, experimental approaches that exclusively identify and manipulate Tregs based on this surrogate marker do not discriminate between activated effector T cells stimulated by infection and bona fide Tregs. Therefore, initial conclusions regarding the role of Tregs in host defense for each specific pathogen using strategies that manipulate these cells based on CD25 expression should be interpreted with caution, and re-investigated using Foxp3-specific reagents for experimentally manipulating Tregs.

Consistent with these newfound beneficial roles for Foxp3$^+$ Tregs in host defense after viral infection, similar protective roles for Foxp3$^+$ cells have also been described for other types of pathogens. For example after infection with *Plasmodium berghei* in a mouse model of cerebral malaria, the expansion of Tregs using IL-2 cytokine antibody complexes confers protection against severe disease that is associated with reduced parasite burden (Haque et al.). These protective effects were due to expanded Foxp3$^+$ cells because their ablation in infected mice where Tregs are ablated with DT eliminated the impacts of IL-2 cytokine antibody complex treatment (Haque et al.). On the other hand for malaria infection in humans, Treg cell frequency appears to correlate more directly with parasite biomass and disease severity that illustrates more complex roles for Tregs, immune stimulation, and immune-related disease sequelae for human malaria infection in humans that are not recapitulated in mice (Hansen and Schofield, 2010; Minigo et al., 2009; Torcia et al., 2008; Walther et al., 2009; Walther et al., 2005). Interestingly, despite the increasing established importance of Tregs in *Plasmodium* infection, the experimental ablation of Tregs from baseline levels using Foxp3-specific reagents did not significantly impact infection susceptibility (Haque et al., ; Steeg et al., 2009). These findings illustrate the potential importance of Tregs in host defense for some infections are better appreciated using gain-of-function experimental approaches. Similarly, Treg expansion with IL-2 cytokine antibody complexes also averts the natural collapse in Foxp3$^+$ cells after *Toxoplasma gondii* infection and rescues mice from fatal immune pathology triggered by this infection (Oldenhove et al., 2009). Furthermore, Foxp3$^+$ Tregs also synergize with Th17 effector CD4$^+$ T cells in eradicating *Candida*

albicans after oral infection (Pandiyan et al.). Taken together, these findings indicated Foxp3^{+} Tregs play more generalizable protective roles that extend to host defense against parasitic and fungal pathogens.

Regulatory T cells and Persistent Antigen Stimulation

By contrast to acute infection where the expansion of pathogen-specific adaptive immune effectors generally coincides with pathogen eradication, pathogens that cause persistent infection have developed strategies to evade and co-exist with pathogen-specific immune components. On the other hand, allowing pathogen persistence by dampening immune activation may also be beneficial when immune-mediated collateral damage to the host outweighs injury caused by pathogen persistence. In this regard, Tregs play important roles in counterbalancing immune effectors during persistent infection. This was first described 10 years ago for *Leishmania major* infection where immune suppression by $CD25^+$ $CD4^+$ Tregs was found to promote pathogen persistence in the skin after intra-dermal infection (Belkaid et al., 2002).

More recently, these findings have been recapitulated for other persistent infections using more refined strategies that allow Treg manipulation based on Foxp3-expression. For example, the ablation of $Foxp3^+$ cells based on selective expression of the Thy1.1 congenic marker in mixed bone marrow chimera mice prior to pulmonary infection with *Mycobacterium tuberculosis* stimulates more robust effector $CD4^+$ T cell IFN-γ production and reduced pathogen burden at the site of infection (Scott-Browne et al., 2007). Similarly, $Foxp3^+$ Tregs provide a similar protective role in a model typhoid fever caused by persistent *Salmonella* infection in *Nramp1*-resistant mice (Johanns et al., 2010b). At early time points following infection when the activation of effector T cells is

blunted and progressively increasing *Salmonella* bacterial burden occurs, Treg ablation in $Foxp3^{DTR}$ mice accelerates the activation of effector T cells with significant reductions in recoverable bacteria (Johanns et al., 2010b). In turn at later time points during persistent *Salmonella* infection when effector T cells are already activated and progressive reductions in pathogen burden naturally occur, the impacts of $Foxp3^{+}$ cell ablation are marginalized with only modest incremental augmentation of effector T cell activation and no significant changes in pathogen burden (Johanns et al., 2010b). Thus, $Foxp3^{+}$ Tregs blunt effector T cell activation that impedes pathogen eradication, and the significance of Treg-mediated immune suppression can shift and dictate the tempo of some persistent infections. Although these results suggest Tregs play detrimental roles in host defense by preventing pathogen eradication, the reduced susceptibility against secondary infection related to low-level pathogen persistence for other pathogens (e.g. *Leishmania and Plasmodium*) illustrates Tregs may in fact provide protection against severe disseminated infection with potentially more fatal consequences (Hansen and Schofield, ; Mendez et al., 2004; Uzonna et al., 2001). It will be interesting to investigate if these Treg-mediated protective activities against secondary infection are more broadly applicable for other pathogens that cause persistent infection.

In addition to the persistence of antigen determinants during chronic infections, pregnancy represents a unique physiologic condition requiring immunologic tolerance to persistent paternal allo-antigens carried by the fetus. Consistent with this notion, circulating maternal $Foxp3^{+}$ Tregs expand during pregnancy peaking mid-gestation at

approximately 50% increased levels in human pregnancy (Santner-Nanan et al., 2009; Somerset et al., 2004). Reciprocally, defects in Treg expansion are associated with pregnancy complications (preeclampsia or spontaneous miscarriage), which could represent maternal immunological intolerance to the developing fetus (Prins et al., 2009; Santner-Nanan et al., 2009; Sasaki et al., 2004). These associations between maternal Tregs and pregnancy outcomes have also been explored experimentally in mouse models where $CD25^{+}CD4^{+}$ cells are essential in maintaining viable pregnancies (Aluvihare et al., 2004; Kahn and Baltimore, 2010). However, these previous approaches caused the complete ablation of these cells preventing the importance of the sustained expansion of Tregs, from pre-pregnancy levels, to be addressed. Similarly, since CD25 expression does not discriminate between bona fide Tregs and activated effector T cells, the actual requirement for expanded maternal $Foxp3^{+}$ Tregs during pregnancy remains undefined. More importantly, given the balance between immune suppression and activation controlled by Tregs, $Foxp3^{+}$ cell expansion during pregnancy may create holes in host defense mechanisms that confer susceptibility to prenatal pathogens. Therefore, while the effects of Treg ablation on susceptibility to parasitic, bacterial, fungal, and viral infection (Belkaid, 2007; Suvas and Rouse, 2006) have been described, the impacts from the physiological expansion of these cells during pregnancy remain undefined.

In summary, the immune system is intricately regulated allowing potent effectors to expand and become rapidly mobilized after infection, while simultaneously silencing potentially detrimental responses that averts immune-mediated damage to host tissues.

This relies in large part on the delicate interplay between immune suppressive regulatory $CD4^+$ T cells (Tregs) and immune effectors that without active suppression by Tregs cause systemic and organ-specific autoimmunity. Although these beneficial roles have classically been described to be counter-balanced by impaired host defense against infection, newfound protective roles for Tregs against specific viral pathogens (e.g. Herpes simplex-2, Lymphocytic choriomeningitis, West Nile virus) have been uncovered using transgenic mice that allow *in vivo* Treg-ablation based on Foxp3-expression. In turn, $Foxp3^+$ Tregs also provide protection against some parasitic (*Plasmodium* sp., *Toxoplasma gondii*) and fungal (*Candida albicans*) pathogens. By contrast for bacterial and mycobacterial infections (e.g. *Listeria monocytogenes*, *Salmonella enterica*, *Mycobacterium tuberculosis*), experimental manipulation of $Foxp3^+$ cells continue to indicate detrimental roles for Tregs in host defense. Furthermore, during pregnancy when the expansion of immunological tolerance against paternal allo-antigens is critical for maintaining maternal-fetal tolerance, Tregs appear essential in mediating these beneficial effects. These discordances are likely related to functional plasticity in Treg suppression that shifts in response to different form of antigenic stimulation.

Chapter 2

CTLA-4 blockade augments the T cell response primed by attenuated *Listeria monocytogenes* resulting in more rapid clearance of virulent bacterial challenge[‡]

[‡] Reprinted from *Immunology*. Jared H. Rowe, Tanner M. Johanns, James M. Ertelt, Joseph C. Lai, and Sing Sing Way. "CTLA-4 blockade augments the T cell response primed by attenuated *Listeria monocytogenes* resulting in more rapid bacterial clearance."

SUMMARY

CTLA-4 uniformly suppresses antigen-specific T cells during chronic infection with bacterial, parasitic, or viral pathogens. However, the importance of CTLA-4 in controlling the T cell response during acute infection or after priming with live attenuated vaccine vectors has not been well characterized. Since strategies aimed at blocking CTLA-4 are being actively developed to therapeutically augment T cell-mediated immunity, the effects of CTLA-4 blockade on T cell-activation during these conditions need to be more clearly defined. Herein, we examined the role of CTLA-4 in a prime-challenge model of acute bacterial infection using both attenuated and virulent strains of the intracellular bacterium *Listeria monocytogenes* (Lm). Although Foxp3+CD4+ T cells are the predominant CTLA-4 expressing cell type in naïve mice, antigen-specific Foxp3-CD4+ cells up-regulate CTLA-4 expression after primary Lm infection. CTLA-4 blockade results in increased numbers of Lm-specific CD4 and CD8 T cells after primary infection with attenuated Lm, and confers more rapid bacterial clearance after secondary challenge with virulent Lm. Accordingly, CTLA-4 plays an important suppressive role in T cell priming and protective immunity in a prime-challenge model of acute bacterial infection.

INTRODUCTION

The mammalian immune system contains multiple intricately regulated networks of opposing stimulatory and inhibitory signals that allow protective antigen-specific responses to be rapidly generated and mobilized during infection, and quickly silenced once infection resolves to minimize immune-mediated injury to the host. For example T cell-activation is controlled in part by a pair of homologous receptors, CD28 and CTLA-4 (CD152), that play stimulatory and suppressive roles, respectively (Kearney et al., 1995). The CTLA-4-mediated suppressive effects on pathogen-specific T cell-activation have been experimentally demonstrated primarily with in vivo models of chronic bacterial, viral, and parasitic infection using monoclonal antibodies that specifically block CTLA-4 (Anderson et al., 2006; Furze et al., 2006; Jacobs et al., 2002; Kirman et al., 1999; Lepenies et al., 2007; Martins et al., 2004; McCoy et al., 1997; Taylor et al., 2007; Walsh et al., 2007). CTLA-4 blockade in each of these experimental models of chronic infection uniformly augments the pathogen-specific T cell response magnitude, and in most cases is associated with reductions in pathogen burden. Accordingly, in vivo CTLA-4 blockade is actively being evaluated as therapies for chronic infection and in other settings where increased antigen-specific T cell immunity is desired (Hodi et al., 2008; Shrikant et al., 1999; Weber, 2007). Especially intriguing is the direct correlation between increased CTLA-4 expression on virus specific T cells and disease progression during ongoing HIV infection (Kaufmann et al., 2007). These results suggest CTLA-4 blockade may therapeutically bolster T cell immunity against HIV. Paradoxically however after simian

immunodeficiency virus (SIV) infection in non-human primates, CTLA-4 blockade caused increases in both T cell activation and viral replication (Cecchinato et al., 2008). Similarly in murine models of Plasmodium infection, the augmented T cell response attributable to CTLA-4 blockade lead to increased immune-mediated pathology (Jacobs et al., 2002; Lepenies et al., 2007). Therefore the effects of CTLA-4 blockade on infection outcome are context dependent and varies with each specific infection condition. Interestingly and in striking contrast to the uniformly described CTLA-4-mediated suppressive effects on T cell activation, a recent study revealed that CTLA-4 blockade during acute infection with Lymphocytic choriomeningitis virus (LCMV) in some settings can dampen the virus-specific T cell response resulting in increased circulating levels of virus within the first 8 days after infection (Raue and Slifka, 2007). Whether these apparent T cell stimulation roles for CTLA-4 are specific to the experimental conditions of acute LCMV infection or more broadly reflect a T cell stimulatory role for CTLA-4 during infection with pathogens that primarily cause acute infection is unclear.

Listeria monocytogenes (Lm) is an intracellular Gram-positive bacterium that primarily causes acute localized infections in the gastrointestinal tract in immune-competent individuals, and more severe systemic infections in immune-compromised individuals. Lm infection primes a robust antigen-specific CD8+ and CD4+ T cell response, and represents a widely used infection model whereby antigen-specific T cell priming, and the protective effects of these T cells can be evaluated (Pamer, 2004). Moreover the

existence of highly attenuated yet immunogenic mutant Lm make recombinant strains expressing heterologous antigens a promising class of live attenuated vaccine vectors (Frankel, 2005; Jiang et al., 2007; Orr et al., 2007; Paterson and Johnson, 2004). Accordingly in this study, the role of CTLA-4 in T cell priming and immunity was investigated using a prime-challenge model of Lm infection. We first characterized the kinetics whereby CTLA-4 is expressed on T cells after primary infection with the attenuated Lm ΔactA mutant that triggers a robust Lm-specific T cell response, but does not cause a productive infection due to defects in both intracellular and intercellular spread (Brundage et al., 1993). This Lm mutant is rapidly cleared after infection even in neonatal mice or adult mice with targeted defects in critical components of innate host defense (Harty and Bevan, 1995; Way et al., 2007; Way et al., 2003). We then determined the effects of CTLA-4 blockade on antigen-specific T cell response magnitude after primary Lm ΔactA infection, and the kinetics whereby virulent Lm is cleared after secondary challenge.

MATERIALS AND METHODS

Mice. C57BL/6 (H-2^b) female mice were purchased from the National Cancer Institute and used at 6-8 weeks of age. OT-II TCR transgenic mice were intercrossed with CD90.1 mice and maintained on a RAG-1-deficient background (Foulds et al., 2002). All experiments were performed under University of Minnesota IACUC approved protocols.

Bacterial infections. Lm-OVA, and Lm-OVA ΔactA derived from Lm-OVA through targeted disruption of the *actA* gene were used allowing for the immune response to the surrogate Lm-specific antigens H-$2K^b$ $OVA_{257-264}$ and I-A^b $OVA_{323-339}$ to be more precisely characterized (Foulds et al., 2002; Way et al., 2007). For infections, Lm was grown to early log phase (OD_{600} 0.1) in brain heart infusion media at 37ºC, washed, and diluted with saline to 200 μl and injected IV. At the indicated time points after infection, the number of recoverable Lm CFUs in the spleen of infected mice were quantified by homogenization in saline containing Triton X (0.05%), and plating serial dilutions of the organ homogenate on brain heart infusion agar plates (Rowe et al., 2008; Way et al., 2007).

Reagents, in vitro cultures, cell staining, and adoptive transfer. For in vivo CTLA-4 blockade, purified hamster anti-mouse CD152 (CTLA-4) (clone UC10-4F10) IgG or hamster IgG control antibodies were purchased from BioXCell (West Lebanon, NH), and injected IP in the following manner: one day prior to primary infection (500 μg per

mouse), days 4 and 8 after primary infection and day 1 prior to rechallenge (250 μg per mouse). For in vivo T cell depletion, 500 μg anti-mouse CD8 (clone 2.43) was inoculated IP one day prior to virulent Lm challenge as described (Orgun et al., 2008). Antibodies and other reagents for cell surface and intracellular staining were purchased from BD Biosciences or eBioscience. For in vitro culture, splenocytes were plated in 96-well round bottom plates (5 x 10^6 cells/ml), and stimulated with the indicated peptides (10^{-6} M) with Brefeldin-A (BD GolgiPlug reagent) for 5 hours as described (Rowe et al., 2008; Way et al., 2007). For adoptive transfer, 10^5 CD4+ T cells from OT-II (CD90.1+) were transferred intravenously into recipient (CD90.2+) mice one day prior to Lm-OVA infection.

Statistics. The differences in number and percent T cells, and geometric mean CFUs between groups of mice were evaluated by using the Student's t test with $P < 0.05$ taken as statistically significant (Graph Pad, Prism software).

RESULTS

CTLA-4 expression by CD4+ T cells after Lm-ΔactA infection.

Our initial studies examined the level and kinetics whereby CTLA-4 is expressed after primary Lm ΔactA infection. For both CD4+ and CD8+ T cells, increased levels of CTLA-4 were detected by staining cells before and after permeabilization compared with cell surface staining alone, and are in agreement with other studies reporting maximal CTLA-4 detection after intracellular staining (Raue and Slifka, 2007; Takahashi et al., 2000). Using this technique, we found dramatically lower levels of CTLA-4 expression by CD8+ compared with CD4+ T cells prior to and at each time point after infection (Figure 2.1A,B). In naïve mice, CTLA-4 expression by ~7% of all CD4 T cells is consistent with the expected percentage (5-10%) of Foxp3+ regulatory T cells known to constitutively express CTLA-4 (Read et al., 2006; Read et al., 2000; Takahashi et al., 2000). After Lm ΔactA infection, an increased percentage of CD4+ T cells expressed CTLA-4 reaching maximal levels day 5 where an ~2-fold increased percentage of CTLA-4+ cells among all CD4+ T cells was observed (16% compared with 7%) (Figure 2.1B). This increased percentage of CTLA-4+ cells among CD4+ T cells also reflected an increase in absolute numbers of CTLA-4+ CD4+ cells among splenocytes reaching maximal levels day 5 after infection (Figure 2.1C). Comparatively, neither the percent nor total number of CTLA-4+ CD8+ T cells changed significantly from baseline levels during the same time period after infection. These results indicate that within the first

week after Lm ΔactA infection, CTLA-4 expression by CD4+ T cells is dynamically regulated.

To determine if increased CTLA-4 expression among CD4+ cells after Lm ΔactA infection reflects expansion of Foxp3+CTLA-4+ cells or induced CTLA-4 expression by Foxp3-CD4+ cells, we examined CTLA-4 expression among Foxp3+ and Foxp3- cells (Figure 2.2A). These studies revealed most CTLA-4+ CD4 cells were Foxp3+ prior to infection, and the percentage of this cell population did not change significantly after infection. In contrast, CTLA-4 expression among Foxp3- CD4+ T cells compared with naive mice was increased at each time point after infection, reaching maximal levels at day 5 (Figure 2.2A). Thus while Foxp3+CD25+CD4+ cells comprise the majority of CTLA-4+ cells before infection, Foxp3-CD25-CD4+ T cells upregulate CTLA-4 expression in response to Lm ΔactA infection.

To more precisely characterize the level and kinetics whereby CTLA-4 is expressed by CD4+ T cells after infection, we examined CTLA-4 expression among congenically marked (CD90.1+) antigen-specific CD4+ T cells from TCR transgenic (OT-II) mice after adoptive transfer and at various time points after Lm-OVA ΔactA infection. CD4 T cells from OT-II mice have specificity to the $OVA_{323-339}$ class II peptide contained within Lm-OVA ΔactA used in these experiments. 10^5 purified CD4+ T cells from OT-II mice (CD90.1+) were transferred intravenously into naïve recipient B6 (CD90.2+) mice one day prior to infection, and tracked ex vivo by staining for CD90.1+CD4+ cells. After Lm-

OVA ΔactA infection, CTLA-4 levels increased significantly on these adoptively transferred $OVA_{323-339}$-specific CD4+ T cells, and with similar kinetics whereby Lm infection induces CTLA-4 expression on all CD4 T cells reaching maximal levels day 5 after infection (Figure 2.2B). These results demonstrate that CTLA-4 expression is dynamically regulated by antigen-specific CD4+ T cells during the first week after primary Lm infection. Moreover by eliminating potentially self-reactive components of adaptive immunity through intercrossing these OT-II mice with RAG1-/- mice, greater than 99% of these $OVA_{323-339}$ -specific (CD90.1+) CD4+ T cells are Foxp3- both directly ex-vivo prior to adoptive transfer and at each time point within the first week after infection. Together these results demonstrate both constitutive CTLA-4 expression by Foxp3+ CD4 cells and induced CTLA-4 expression among antigen-specific Foxp3- CD4 cells after primary Lm infection.

CTLA-4 blockade increases the antigen-specific T cell response magnitude

CTLA-4 in experimental models of chronic bacterial, parasitic, and viral infection suppresses the antigen-specific T cell response because CTLA-4 blockade uniformly augments the antigen-specific T cell response magnitude (Anderson et al., 2006; Furze et al., 2006; Jacobs et al., 2002; Kirman et al., 1999; Lepenies et al., 2007; Martins et al., 2004; McCoy et al., 1997; Taylor et al., 2007; Walsh et al., 2007). In contrast, CTLA-4 blockade during defined conditions after acute LCMV infection inhibits virus-specific T cell expansion resulting in increased levels of circulating virus (Raue and Slifka, 2007). To evaluate if these discordant roles for CTLA-4 are caused by inherent differences in

co-stimulation signals required for optimal T cell activation after acute and chronic infection, we examine the effects of CTLA-4 blockade on the antigen-specific T cell response after primary Lm ΔactA infection. For these experiments, mice were treated with either anti-mouse CTLA-4 monoclonal antibody (UC10-4F10) used previously by other investigators for in vivo CTLA-4 blockade or hamster IgG isotype control antibody beginning one day prior to infection and throughout the experiment (Anderson et al., 2006; Furze et al., 2006; Jacobs et al., 2002; Kirman et al., 1999; Lepenies et al., 2007; Martins et al., 2004; McCoy et al., 1997; Taylor et al., 2007; Walsh et al., 2007). Eight days after primary Lm ΔactA infection, mice treated with CTLA-4 blocking compared with control antibody had ~2.5-fold increased ($P < 0.05$) percentage and total numbers of both antigen-specific CD8+ and CD4+ T cells enumerated by intracellular cytokine staining after stimulation with the Lm-OVA specific MHC class I $OVA_{257-264}$ or class II $LLO_{189-201}$ peptides, respectively (Figure 2.3A). Similarly by day 30 after infection, and despite a significantly reduced magnitude response compared with day 8, increased percentage and total numbers of antigen-specific T cells were maintained for CTLA-4 blocking compared with control antibody treated mice (Figure 2.3B). These results demonstrate that CTLA-4 blockade increases the antigen-specific T cell response magnitude at both early (day 8) and memory (day 30) time points after primary Lm ΔactA infection.

Since Lm antigen load quantified by the number of recoverable CFUs 24 hours after infection directly correlates with the Lm-specific T cell response magnitude (Mercado et

al., 2000), we evaluated the possibility that the increased magnitude antigen-specific T cell response attributable to CTLA-4 blockade could result from differences in Lm ΔactA antigen load at early time points after infection between CTLA-4 blocking and control antibody treated mice. Consistent with our previous results characterizing the kinetics of Lm ΔactA clearance in other mice (Rowe et al., 2008; Way et al., 2007), only ~10% of the initial Lm ΔactA inoculum is recovered after 24 hours, and the infection is completely cleared by 72 hours for most mice treated with either CTLA-4 blocking or control antibody (Figure 2.4). Importantly no significant differences in bacterial counts between CTLA-4 blocking and isotype control antibody treated mice were detected after Lm ΔactA infection (Figure 2.4). These results indicate that differences in Lm antigen load at early time points after infection do not account for the increased magnitude antigen-specific T cell response in mice treated with CTLA-4 blocking antibody.

CTLA-4 blockade increases the kinetics of bacterial clearance after virulent Lm challenge

Lastly we evaluated how increased magnitude antigen-specific T cell response conferred by CTLA-4 blockade after priming with Lm ΔactA would alter the kinetics of bacterial clearance after challenge with virulent Lm. In our recent studies, suppressing the antigen-specific T cell response magnitude with anti-PDL-1 blocking antibody delays the kinetics whereby virulent Lm is cleared (Rowe et al., 2008). Accordingly we hypothesized that CTLA-4 blockade resulting in increased antigen-specific response magnitude would increase the clearance kinetics of virulent Lm after secondary challenge. To test this

hypothesis, groups of naïve or mice initially primed with Lm ΔactA each treated with either CTLA-4 blocking or control antibody were challenged with an inoculum of virulent Lm normally lethal for naïve mice (1 LD_{50}). Within the first 72 hours after virulent Lm challenge, both CTLA-4 blocking or control antibody treated naïve mice had progressively increasing numbers of Lm CFUs, became moribund and were euthanized (Figure 2.5). Importantly there were no significant differences in numbers of recoverable Lm CFUs between naive CTLA-4 blocking and control antibody treated mice indicating CTLA-4 does not play an important role in innate host defense to virulent Lm. Consistent with the highly efficient nature whereby antigen-specific T cells primed with Lm ΔactA confer protection to virulent Lm challenge, greater than 100-fold decreased Lm CFUs were present for control antibody treated Lm ΔactA primed compared with naïve mice beginning 24 hours after challenge (Figure 2.5). Remarkably, the rate of bacterial clearance was further enhanced for CTLA-4 blocking antibody treated mice since an additional ~10-fold reduction ($P < 0.05$) in recoverable Lm CFUs was present for Lm ΔactA primed CTLA-4 blocking antibody treated compared with control antibody treated mice 24 hours after Lm challenge (Figure 2.5). By 72 hours post-challenge, virulent Lm was cleared for the majority of Lm-ΔactA primed mice regardless of CTLA-4 blockade while increased numbers of Lm CFUs were found for both groups of naïve control mice (Figure 2.5). Thus, CTLA-4 blockade increases the kinetics whereby virulent Lm challenge is eradicated in Lm ΔactA primed mice.

Since CD8 T cells are the adaptive immune effectors that mediate protective immunity to Lm infection as demonstrated in various mice with unique targeted immune defects (Harty and Bevan, 1995; Orgun et al., 2008; Way et al., 2003), we sought to examine if CD8 T cells were responsible for the increased kinetics of virulent Lm clearance attributable to CTLA-4 blockade in Lm ΔactA primed mice. Accordingly, we quantified the effects of CD8 T cell depletion prior to challenge with virulent Lm in groups of Lm ΔactA primed mice treated with either anti-CTLA-4 or control antibody. Beginning at day 1 post-challenge, dramatically more Lm CFUs were recovered from both anti-CTLA-4 and control antibody treated mice depleted of CD8 T cells (Figure 2.5). Importantly, CD8 T cell depletion eliminated the difference in kinetics of virulent Lm clearance between anti-CTLA-4 and control antibody treated mice (Figure 2.5). Thus after CTLA-4 blockade, CD8 T cells are the primary mediators of protective immunity to virulent Lm infection. Taken together, these results demonstrate CTLA-4 suppresses the Lm-specific CD8 T cell response during primary Lm ΔactA infection resulting in delayed protective immunity after secondary virulent Lm challenge.

DISCUSSION

In this report we demonstrate using a prime-challenge model of attenuated followed by virulent Lm infection that CTLA-4 plays a suppressive role in antigen-specific T cell activation. These results are consistent with other studies that have characterized the role of CTLA-4 in antigen-specific T cell activation after infection that together uniformly indicate a T cell suppressive role for CTLA-4 (Anderson et al., 2006; Furze et al., 2006; Jacobs et al., 2002; Kearney et al., 1995; Kirman et al., 1999; Lepenies et al., 2007; Martins et al., 2004; McCoy et al., 1997; Shrikant et al., 1999; Taylor et al., 2007; Walsh et al., 2007). In each of these studies and the results presented here, CTLA-4 blockade augments the T cell response magnitude. Although antigen-specific T cells play important roles in host protection during infection, increased magnitude T cell activation conferred by CTLA-4 blockade can either improve or worsen infection outcome. For example, CTLA-4 blockade in some models of chronic bacterial and parasitic infection results in more rapid pathogen clearance and protective immunity (Anderson et al., 2006; Furze et al., 2006; Martins et al., 2004; McCoy et al., 1997; Taylor et al., 2007; Walsh et al., 2007), while in other infections the benefits of more rapid pathogen clearance are outweighed by immune mediated host tissue injury caused by CTLA-4 blockade (Jacobs et al., 2002; Lepenies et al., 2007). Interestingly for viruses such as SIV capable to establishing latent infection within T cells, CTLA-4 blockade causing non-specific T cell activation increases viral replication levels (Cecchinato et al., 2008; Doherty et al., 1999; Scanga et al., 2007). These results may be directly related to increased transcription of

latent viral genes after non-specific immune activation triggered by *Mycobacterium tuberculosis* or *Mycobacterium avium* infection in transgenic mice containing intergrated copies of the HIV genome (Cecchinato et al., 2008; Doherty et al., 1999; Scanga et al., 2007). Nevertheless, these studies together with the data we present here clearly indicate that CTLA-4 confers important suppressive effects on T cell activation and expansion during infection.

It remains unclear why CTLA-4 does not play a suppressive role in T cell-activation following acute LCMV or vaccinia virus infection, and in the setting of CD28-deficiency after acute LCMV infection appears to play an important stimulatory role in priming virus-specific CD8+ T cells. Although protective immunity to both LCMV and Lm infection is mediated primarily by pathogen-specific CD8+ T cells, a striking difference between our results and the data reported for acute LCMV infection is the dramatically increased CTLA-4 expression by CD8+ T cells after LCMV infection that does not occur after primary Lm ΔactA infection. Whether differences in CTLA-4 expression by CD8+ T cells triggered by LCMV compared with Lm alone account for the discordant roles for CTLA-4 in T cell activation and expansion between these infection models is unclear. Although CTLA-4 was not upregulated to any appreciable extent on CD8 T cells after Lm infection, our results indicate that in addition to constitutive expression on Foxp3+ regulatory T cells, CTLA-4 is also transiently upregulated on newly activated antigen-specific CD4 T cells. However, using our current experimental conditions, we cannot distinguish if the augmented Lm-specific CD8 and CD4 T cell response after anti-CTLA-

4 antibody is attributable to CTLA-4 blockade on Foxp3+ and/or Foxp3- CD4 T cells. Since others have demonstrated CTLA-4 blockade also blocks the suppressive function of Tregs that results in autoimmunity, the augmented Lm-specific CD8 and CD4 T cell response after CTLA-4 blockade could be consistent with overall blockade of Tregs (Read et al., 2006; Read et al., 2000; Takahashi et al., 2000). However, since these studies did not specially prime conditions where CTLA-4 is upregulated on non-Tregs CD4 cells, the potential role for CTLA-4 expression on these cells cannot be excluded. Our ongoing studies are designed to more specifically characterize how CTLA-4 and CD4 T cells control protective immunity during Lm and other infections.

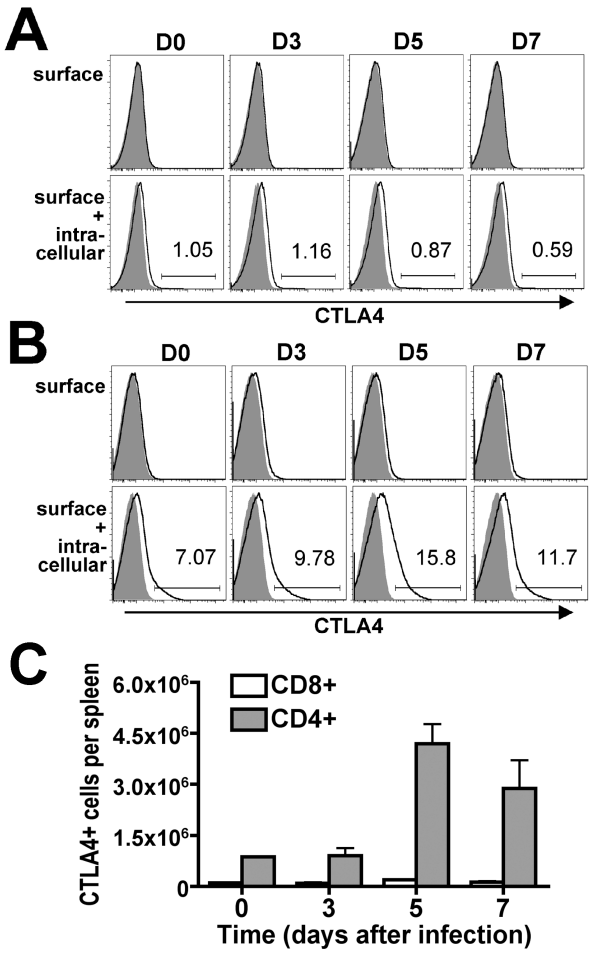

Figure 2.1 - CTLA-4 expression among CD8+ T cells (A) and CD4+ T cells (B) at the indicated time points after infection with 10^6 Lm-OVA ΔactA. CTLA-4 expression was measured by staining with anti-CTLA-4 (open histograms) or isotype control (shaded histograms) antibody either before cell permeabilization (surface), or staining before and after permeabilization (surface + intracellular). The numbers indicate percent CTLA-4+ cells compared with isotype control antibody. C. Absolute number of CTLA-4+ CD8 and CD4 T cells among splenocytes at the indicated time points after Lm-OVA ΔactA infection. These data are representative of 4 to 6 mice per time point from two independent experiments with similar results. Bar, standard error.

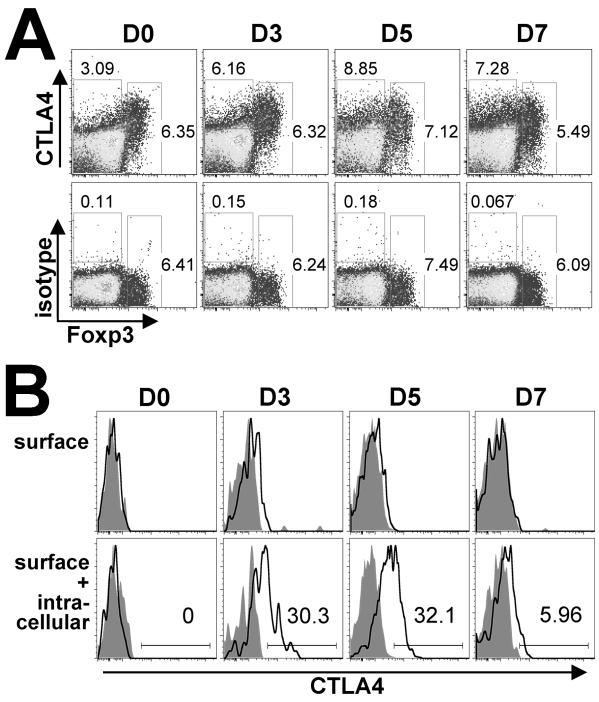

Figure 2.2 - A. CTLA-4 expression determined by both cell surface and intracellular staining compared with Foxp3 expression among CD4+ T cells at the indicated time points after infection with 10^6 Lm-OVA ΔactA. The numbers indicate the percentage CD4+ cells in each gate and are representative of 4 mice per time point from two independent experiments. B. Histogram plots indicating CTLA-4 expression among OT-II CD90.1+ $OVA_{323-339}$-specific CD4+ T cells at the indicated time points after infection. CTLA-4 expression was measured by staining with anti-CTLA-4 (open histograms) or isotype control (shaded histograms) antibody either before cell permeabilization (surface), or staining before and after permeabilization (surface + intracellular). The numbers indicate percent CTLA-4+ cells compared with isotype control antibody, and is representative of 4 to 6 mice per time point from two independent experiments with similar results.

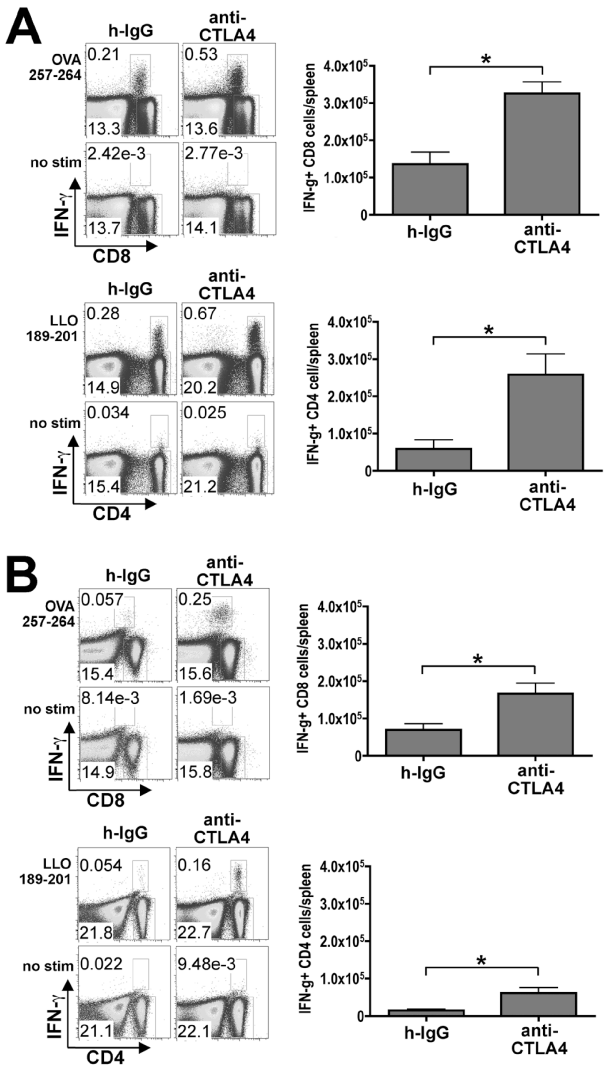

Figure 2.3 - A. Percent and total number IFN-γ producing CD8+ (top) and CD4+ (bottom) T cells among splenocytes from hamster isotype control (h-IgG) or CTLA-4 blocking antibody treated mice day 8 after infection with 10^6 Lm-OVA ΔactA and stimulation with $OVA_{257-264}$ peptide (MHC class I), $LLO_{189-201}$ peptide (MHC class II), or no stimulation. B. Percent and total number IFN-γ producing CD8+ (top) and CD4+ (bottom) T cells among splenocytes from hamster isotype control (h-IgG) or CTLA-4 blocking antibody treated mice day 30 after infection with 10^6 Lm-OVA ΔactA and stimulation with $OVA_{257-264}$ peptide (MHC class I), $LLO_{189-201}$ peptide (MHC class II), or no stimulation. These data are representative of 6 mice per experimental group from two independent experiments with similar results. Bar, standard error. * $P < 0.05$.

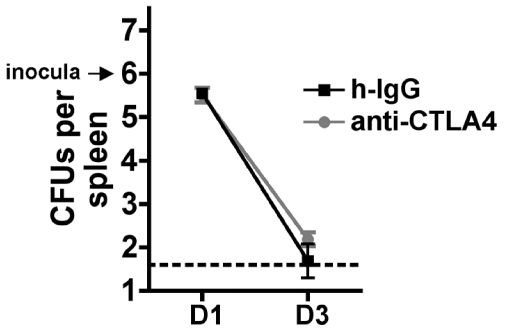

Figure 2.4 - Number of recoverable Lm CFUs (log 10) per spleen for CTLA-4 blocking (circle symbol, gray line) or hamster isotype control (h-IgG, square symbol, black line) antibody treated mice 24 hours (D1) or 72 hours (D3) after infection with 10^6 Lm-OVA ΔactA. These data are representative of 4 mice per experimental group. Bar, standard error.

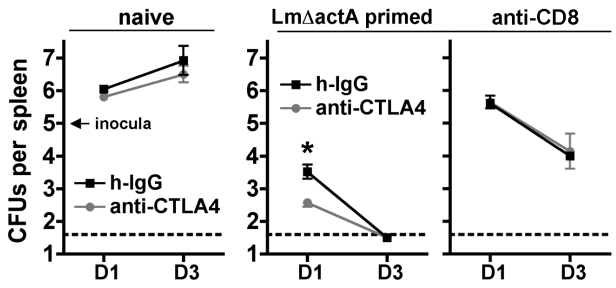

Figure 2.5 - Number of recoverable Lm CFUs (log 10) per spleen at the indicated time point after infection with 10^5 Lm-OVA for naïve mice (left-sided panel), mice primed 30 days previously with 10^6 Lm-OVA ΔactA (middle panel), and Lm-OVA ΔactA primed mice depleted of CD8 T cells one day prior to challenge (right-sided panel) each treated with CTLA-4 blocking (circle symbol, gray line) or hamster isotype control (h-IgG, square symbol, black line) antibody. These data are representative of 6 to 8 mice per experimental group from two independent experiments with similar results. Bar, standard error. * $P < 0.05$.

Chapter 3

PDL-1 blockade impedes T cell expansion and protective immunity primed by attenuated *Listeria monocytogenes*[§]

[§] Reprinted from *The Journal of Immunology*. Jared H. Rowe, Tanner M. Johanns, James M. Ertelt, and Sing Sing Way. "PDL-1 blockade impedes T cell expansion and protective immunity primed by attenuated *Listeria monocytogenes*."

SUMMARY

Infection with attenuated *Listeria monocytogenes* (Lm) is a robust in vivo model for examining how antigen-specific T cells are primed, and subsequent challenge with virulent Lm allows for the protective effects of T cell priming to be quantified. Herein, we investigated the role of PDL-1 in T cell priming and immunity conferred after primary infection with Lm ΔactA followed by virulent Lm challenge. In striking contrast to the inhibitory role of PDL-1 on T cell immunity in other infection models, marked reductions in the magnitude of T cell expansion and the kinetics of T cell proliferation were observed with PDL-1 blockade after primary Lm ΔactA infection. More importantly PDL-1 blockade beginning before primary infection and maintained throughout the experiment resulted in delayed bacterial clearance and T cell expansion after secondary challenge with virulent Lm. These results indicate that for immunity to intracellular bacterial infection, PDL-1 plays an important stimulatory role for priming and expansion of protective T cells.

INTRODUCTION

PDL-1 (B7-H1) belongs to the B7 family of co-stimulatory molecules that also includes B7-1 (CD80), B7-2 (CD86), and B7-DC (CD273 or PDL-2) (Keir et al., 2007; Sharpe et al., 2007). T cell engagement with each of these co-stimulatory receptors can confer both stimulatory or inhibitory signals, and the overall magnitude of the antigen-specific T cell response after immunization or infection is controlled by multiple T cell activation and suppression signals. Therefore, understanding how these various opposing signals together control antigen-specific T cell activation is required for the more rational design of vaccines that aim to target T cell-mediated immunity. Although PDL-1 can provide both T cell inhibitory and activation signals examined with both in vitro models of T cell activation or in vivo models of autoimmune disease, functional studies with in vivo infection models have uniformly demonstrated that T cell stimulation by PDL-1 suppresses T cell proliferation and effector function (Barber et al., 2006; Day et al., 2006; Dong et al., 1999; Jun et al., 2005; Kanai et al., 2003; Latchman et al., 2004; Maier et al., 2007; Petrovas et al., 2006; Subudhi et al., 2004; Trautmann et al., 2006; Urbani et al., 2006). For example during chronic LCMV infection, in vivo PDL-1 blockade restores proliferation and cytolytic function to virus-specific "exhausted" CD8 T cells and results in viral clearance (Barber et al., 2006). Similarly, PDL-1 blockade restores activation and proliferation to virus-specific T cells during both chronic hepatitis B and acute herpes simplex virus in mouse infection models (Jun et al., 2005; Maier et al., 2007), and proliferation and cytolytic function for HIV-specific or hepatitis C virus-specific CD8 T

cells from human patients with these chronic infections (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006). Accordingly, reinvigorating viral T cells through PDL-1 blockade has been proposed as a novel therapeutic intervention for treatment of chronic viral infection.

Listeria monocytogenes (Lm) is an intracellular Gram-positive bacterium that primarily causes localized infections in the gastrointestinal tract in immune-competent individuals, and more severe systemic infections in immune-compromised individuals. During infection, Lm primes a robust antigen-specific CD8 and CD4 T cell response, and accordingly Lm infection is a widely used experimental model whereby priming and activation of antigen-specific T cells, and the protective effects these T cells are examined (Pamer, 2004). Furthermore because of the relative ease by which recombinant Lm strains can be generated, and the existence of many highly attenuated and immunogenic Lm mutant strains, recombinant Lm expressing protective antigen from other pathogens are being explored as a new class of live attenuated vaccine vectors (Frankel et al., 1995; Orr et al., 2007; Zhao et al., 2006). In this study we examined the effects of PDL-1 blockade on T cell priming and expansion after primary infection with Lm ΔactA, and protective immunity following secondary challenge with virulent Lm. The highly attenuated nature of Lm ΔactA compared with WT Lm normalizes antigen load after primary infection thereby bypassing potential difference in innate susceptibility between groups of mice allowing for a more accurate comparison of the resulting T cell response (Harty and Bevan, 1995; Mercado et al., 2000; Way et al., 2007; Way et al.,

2003). In this report, we first demonstrate that PDL-1 expression is markedly upregulated during Lm ΔactA primary infection, and PDL-1 blockade does not alter antigen load or the kinetics of bacterial clearance following infection with this attenuated Lm strain. Additional studies using Lm ΔactA primary infection to normalize initial antigen load between anti-PDL-1 antibody and control antibody treated mice demonstrate that PDL-1 blockade delays the kinetics of T cell priming and reduces the magnitude of T cell expansion after primary Lm ΔactA infection. Importantly, PDL-1 blockade beginning prior to T cell priming and maintained throughout secondary challenge reveals an important role for PDL-1 in optimal bacterial clearance and secondary T cell expansion after challenge with virulent Lm.

MATERIALS AND METHODS

Mice. C57B6 (H-2K^b) female mice were purchased from the National Cancer Institute and used at 6-8 weeks of age. OT-1 TCR transgenic mice have been described and were intercrossed with CD90.1 mice and maintained on a RAG-1-deficient background (Foulds et al., 2002). All experiments were performed under University of Minnesota IACUC approved protocols.

Listeria monocytogenes. The recombinant Lm strain Lm-OVA and Lm-OVA ΔactA derived through targeted deletion in the *actA* gene were used allowing for an analysis of the immune response to the surrogate Lm-specific H-2K^b OVA$_{257-264}$ antigen as described (Foulds et al., 2002; Way et al., 2007). For infections, Lm were grown to early log phase (OD_{600} 0.1) in brain heart infusion media at 37ºC, washed, and diluted with saline to 200 µl final volume and injected IV. At the indicated time points after infection, the number of recoverable bacteria in the organs of infected mice were quantified by homogenization in saline containing Triton X (0.05%), and plating serial dilutions of the homogenate on brain heart infusion plates as described (Edelson and Unanue, 2002; Way et al., 2003).

Reagents, in vitro cultures, cell staining, and adoptive transfer. For *in vivo* PDL-1 blockade, anti-mouse PDL-1 (clone 10F.9G2) or rat IgG2b isotype control (clone LTF-2) antibodies (Barber et al., 2006; Maier et al., 2007) were purchased from Bio Express, and injected IP in the following manner: one day prior to primary infection (500 µg per

mouse), days 4 and 8 after primary infection and day 1 prior to rechallenge (250 μg per mouse). The CD8 T cell response to $OVA_{257-264}$ was examined with H-$2K^b$ dimer X loaded with $OVA_{257-264}$ peptide according to the manufacturer's instructions (BD Biosciences). Antibodies for cell surface staining, reagents for intracellular cytokine and Annexin V staining were purchased from BD Biosciences and used according to manufacturer's recommendations. For *in vitro* culture, splenocytes were plated into 96-well round bottom plates (5 x 10^6 cells/ml), and stimulated with the indicated peptides (10^{-6} M) with Brefeldin-A (BD GolgiPlug reagent) for 5 hours as described (20). For adoptive transfer, 10^5 CD8 T cells from OT-1 (CD90.1) mice were CFSE labeled (5 μM final concentration) and transferred intravenously into recipient mice one day prior to Lm infection.

Statistics. The differences in number and percentage of antigen-specific cells, and geometric mean CFUs between groups of mice were evaluated by using the Student's t test with $P < 0.05$ taken as statistically significant (Graph Pad, Prism software).

RESULTS

PDL-1 expression after primary Lm-ΔactA infection

Although expressed constitutively on most lymphoid cells, PDL-1 expression is upregulated on virtually all splenocytes during chronic viral infection (Barber et al., 2006). Functionally, PDL-1 expression during chronic viral infection actively suppresses virus-specific CD8 T cells since PDL-1 blockade restores the function of virus-specific "exhausted" T cells into "effector" cells capable of viral clearance (Barber et al., 2006). To gain insight into how and when PDL-1 may alter T cell priming during acute bacterial infection, we examined the kinetics of PDL-1 expression on splenocytes after primary Lm infection. Remarkably, beginning 24 hours after infection with 10^6 CFUs of Lm ΔactA, PDL-1 was upregulated on the majority of all splenocytes (Figure 3.1). At this time point, the level of PDL-1 expression was highest among the CD11c+ splenocyte population consistent with the important role this subset of antigen presenting cells plays in T cell priming after Lm infection (Jung et al., 2002a). PDL-1 was also dramatically upregulated on other splenocyte subpopulations including CD11b+, B220+, CD4+ and CD8+ cells. PDL-1 upregulation in response to Lm ΔactA infection was maintained through day 3, dramatically reduced by day 6, and returned to levels present in naive mice day 14 after infection. The kinetics for PDL-1 upregulation and return to baseline on CD11c+ cells was similar to the expression kinetics of other molecules important for T cell priming such as CD80, CD86, MHC class I (H-$2K^b$) and class II (I-A^b) (Figure 3.1 and data not shown).

PDL-1 blockade reduces antigen-specific T cell expansion after primary Lm-ΔactA infection

To evaluate the overall role of PDL-1 in T cell priming accommodating the brisk nature whereby PDL-1 is upregulated during Lm infection, we pretreated mice with either anti-PDL-1 blocking or isotype control antibody beginning one day prior to infection. Remarkably, in vivo PDL-1 blockade effectively blocked access to both constitutive levels of cell-surface PDL-1 and the increased expression levels present after infection since only background levels of PDL-1 staining could be detected in anti-PDL-1 treated mice (Figure 3.2A). These reductions were specific to PDL-1 since anti-PDL-1 blockade did not alter the cell-surface expression of other costimulation molecules in response to Lm infection (Figure 3.2A). Other experiments indicate that PDL-1 blockade does not alter the antigen load or kinetics of bacterial clearance after Lm-OVA ΔactA infection since the numbers of recoverable CFUs is virtually identical at early time points (24, 48, and 72 hours) after infection between anti-PDL-1 treated and control mice (Figure 3.2B). In this experimental model beginning day 5 after Lm ΔactA infection, a ~70% reduction in the antigen-specific CD8 T cell response enumerated by $OVA_{257-264}$ dimer staining was present for anti-PDL-1 treated compared with control mice ($P < 0.001$) (Figure 3.3A). This reduced magnitude antigen-specific CD8 T cell response was maintained through the peak (day 8) and contraction phase (days 15 to 30) of the T cell response. Similarly during the peak T cell response, there was a ~70% reduction in number of antigen-specific CD8 T cells in splenocytes from anti-PDL-1 treated compared with control mice

(Figure 3.3B). Moreover, 50 to 70% reductions in both the percentage and total number of cytokine producing CD8 and CD4 T cells among splenocytes were present after stimulation with the MHC class I peptide $OVA_{257-264}$ or class II peptide $LLO_{189-201}$, respectively (Figure 3.3C,D). These results indicate that PDL-1 blockade impedes antigen-specific T cell expansion after primary Lm ΔactA infection.

PDL-1 blockade impedes antigen-specific T cell priming

Early priming events after Lm infection play critical roles regulating both the kinetics and magnitude of the antigen-specific T cell response (Mercado et al., 2000). To determine if reductions in T cell expansion resulting from PDL-1 blockade are due to defects in early T cell priming, we examined the degree of proliferation and magnitude of expansion in adoptively transferred antigen-specific T cells in the first few days following Lm ΔactA infection. Beginning at 48 hours after infection adoptively transferred antigen-specific OT-1 CD8 (CD90.1) T cells could be readily detected, and at this early time point consistent reductions in both cell number and degree of CFSE dilution among antigen-specific T cells adoptively transferred into anti-PDL-1 compared with control mice were present (Figure 3.4A). Reduced levels of CFSE dilution and expansion among antigen-specific T cells in anti-PDL-1 antibody treated compared with control mice became more pronounced by day 3 after infection (Figure 3.4A). By day 4 after infection when CFSE was fully dilute among transferred cells in both groups of mice, significant reductions in both percentage and total numbers of antigen-specific OT-1 cells in mice treated with anti-PDL-1 compared with control antibody were still present (Figure 3.4B). In similar

experiments we evaluated the possibility that reduced numbers of antigen-specific T cells present in anti-PDL-1 treated compared with control mice were the result of increased apoptotic cell death since in other systems, direct stimulation of activated human T cells with immobilized anti-PDL-1 antibody in vitro can lead to increased rates of apoptotic cell death (25). These experiments revealed no difference in annexin V staining among antigen-specific OT-1 cells recovered from anti-PDL-1 treated and control mice (Figure 3.4C), and indicate that potential differences in the rate of apoptotic cell death attributable to anti-PDL-1 antibody in other systems does not contribute significantly to observed reductions in number of antigen specific T cells primed after Lm infection. Together, these results indicate that after primary Lm-ΔactA infection, PDL-1 blockade interferes with early events in T cell priming resulting in reduced proliferation and expansion of antigen-specific T cells.

PDL-1 blockade delays bacterial clearance after challenge with virulent Lm

To evaluate the overall impact PDL-1 plays in protective immunity primed by attenuated Lm infection we examined the susceptibility of anti-PDL-1 antibody treated and control mice to challenge with an inocula of virulent Lm lethal for naïve mice (1 LD_{50}). Consistent with other studies demonstrating the remarkable efficiency whereby prior infection with Lm-ΔactA primes protective T cell immunity allowing virulent Lm to be rapidly cleared after challenge (Harty and Bevan, 1995), there were ~ 4 log-fold reductions in recoverable Lm CFUs in the livers by day 3 after challenge for Lm ΔactA primed compared with naive control mice each treated with isotype control antibody

(Figure 3.5A). However the degree of protection primed with Lm ΔactA was significantly reduced by PDL-1 blockade since ~2 log-fold increased numbers of Lm were present for anti-PDL-1 antibody treated compared with control mice ($P = 0.001$). Moreover, this is likely an under-representation of the true difference between these groups since 4 of 7 control mice (rat IgG2b treated) had Lm CFUs below the limits of detection, while Lm CFUs were present for all anti-PDL-1 treated mice (7 total). To evaluate if the increased susceptibility to virulent Lm challenge for anti-PDL-1 compared with control mice reflects either a failure or delay in bacterial clearance, Lm CFUs were also examined at additional later time points. By day 5 after challenge, the number of recoverable Lm for both anti-PDL-1 antibody treated and control mice were below the limits of detection (Figure 3.5A). These results indicate that PDL-1 blockade delays bacterial clearance after secondary challenge with virulent Lm.

Lastly we examined the role of PDL-1 in antigen-specific T cell expansion after secondary infection by enumerating the antigen-specific T cell response to $OVA_{257-264}$ prior to challenge (day 30 after Lm ΔactA primary infection), and days 3 and 5 after secondary challenge with virulent Lm (Figure 3.5B). Consistent with the marked reductions in T cell expansion after primary infection with Lm ΔactA attributable to PDL-1 blockade, significantly delayed kinetics and reductions in overall magnitude of T cell expansion after secondary challenge with virulent Lm was observed for anti-PDL-1 antibody treated compared with control mice. Taken together, these results indicate that

PDL-1 blockade impedes antigen-specific T cell priming and protective immunity during Lm infection.

DISCUSSION

PDL-1 T stimulation uniformly results in suppression of pathogen-specific T cell proliferation and effector function after evaluation in various in vivo infection models (Barber et al., 2006; Day et al., 2006; Jun et al., 2005; Maier et al., 2007; Petrovas et al., 2006; Trautmann et al., 2006; Urbani et al., 2006). However, whether PDL-1 mediated suppression of antigen-specific T cell proliferation is beneficial or detrimental for the host appears to be pathogen and context dependent. For LCMV clone 13 and other viruses that cause chronic infection, active T cell inhibition through PDL-1 results in antigen-specific T cells that are phenotypcially "exhausted" and cannot eradicate virus resulting in chronic infection (Barber et al., 2006; Day et al., 2006; Maier et al., 2007; Petrovas et al., 2006; Trautmann et al., 2006; Urbani et al., 2006). On the other hand for acute HSV-1 infection causing stromal keratitis, PDL-1 mediated suppression of virus-specific T cell priming and apoptosis limits the severity of disease caused by activated T cells (Jun et al., 2005). Herein we report experimental data demonstrating a stimulatory role for PDL-1 in T cell proliferation and expansion, and a protective role for PDL-1 using an in vivo model of priming model of protective immunity to Lm infection.

First we demonstrate the marked upregulation of PDL-1 expression from baseline levels on virtually all immune cells after Lm infection, with CD11c+ having the highest levels of expression. The role of PDL-1 expression in priming T cell immunity after Lm infection was examined treating mice with either anti-PDL-1 blocking or control

antibody prior to and during infection. To bypass potential roles PDL-1 may play in innate resistance to virulent Lm infection leading to differences in Lm antigen load, infection with the ΔactA Lm mutant was used. This highly attenuated Lm lacks the bacterial virulence protein necessary for actin recruitment and intracellular and intercellular spread and therefore is rapidly cleared even in mice lacking key components of innate resistance (Harty and Bevan, 1995; Way et al., 2007; Way et al., 2003). Similarly we now demonstrate that infection with Lm ΔactA normalize antigen load in anti-PDL-1 treated and control mice at early time points when potential differences can critically impact the immune response magnitude (Mercado et al., 2000). Using this in vivo model to prime Lm specific T cells, we demonstrate consistent ~70% reductions in the antigen-specific T cell response for anti-PDL-1 antibody treated compared with control mice quantified using both MHC multimer and intracellular cytokine staining techniques. Additional experiments using adoptively transferred antigen-specific T cells from TCR transgenic mice indicate that PDL-1 plays an important role in early T cell priming since cells recovered from anti-PDL-1 compared with control mice had delayed CFSE dilution and decreased overall expansion. Importantly after challenge with virulent Lm in mice primed with Lm ΔactA, PDL-1 blockade throughout the experiment lead to delayed bacterial clearance and secondary expansion of antigen-specific T cells.

Therefore during Lm infection, unlike the other previously described in vivo infection models, PDL-1 is required for optimal T cell proliferation and expansion. These results are consistent with the stimulatory role for PDL-1 previously described in other non-

infectious in vivo and in vitro models of T cell activation (Dong et al., 1999; Kanai et al., 2003; Subudhi et al., 2004). Our results demonstrating PDL-1 stimulates T cell immunity after primary infection with Lm-ΔactA are also consistent with a recent study describing a defect in Lm-specific CD8 T cell expansion after infection with WT Lm using a different anti-PDL-1 blocking antibody and a different mouse strain (Seo et al., 2007). In this study, the increased susceptibility conferred by PDL-1 blockade to primary WT Lm infection was also demonstrated underscoring the importance of using attenuated Lm for primary infection to normalize antigen load at early time points when the resulting adaptive T cell response is being evaluated. This critical difference or potentially other intrinsic differences between the two experimental systems lead these authors to miss the important functional consequence of PDL-1 blockade resulting in delayed bacterial clearance after re-challenge with virulent Lm that we now report. These results collectively challenge the dogma that T cell stimulation through PDL-1 during infection results in decreased proliferation and suppression of effector function, and instead demonstrates that the role of PDL-1 in T cell immunity is context dependent and varies with the type of infection. Determining the molecular basis for how T cell stimulation through PDL-1 and other co-stimulation signals can result in such drastic differences in proliferation and functional capacity during specific infections is an important area for future investigation.

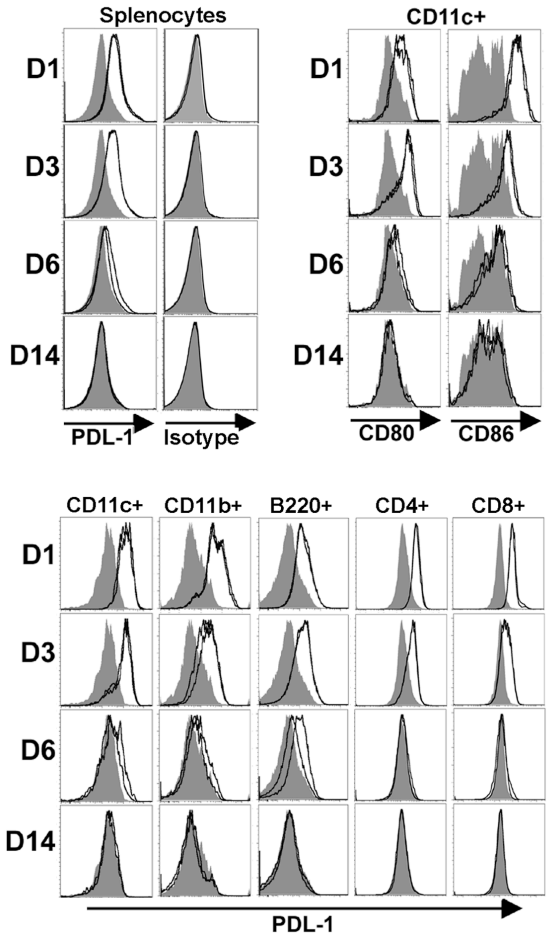

Figure 3.1 - PDL-1, CD80, and CD86 expression among all splenocytes or specific splenocyte cell subsets at the indicated time points after infection with 10^6 Lm ΔactA (open histograms) compared with no infection mice (shaded histograms). These data are from 2 mice per experimental group per time point, and representative of three independent experiments with similar results.

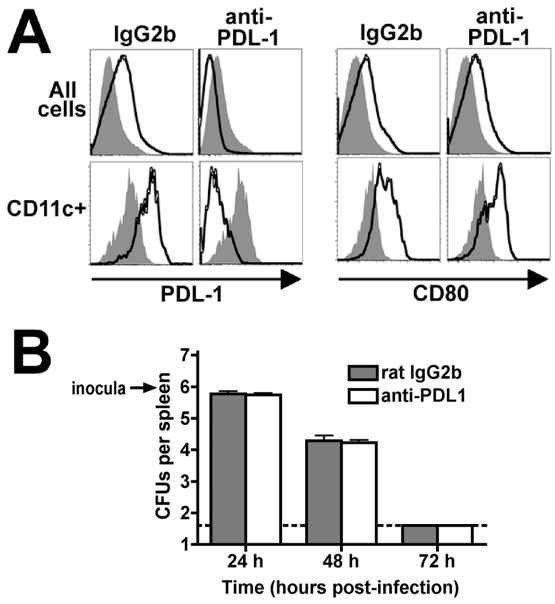

Figure 3.2 - A. PDL-1 and CD80 expression among all splenocytes (top) or CD11c+ splenocyte cells (bottom) from mice 3 days after infection with 10^6 Lm ΔactA (open histograms) or no infection (shaded histograms) treated with either rat IgG2b isotype control or anti-PDL-1 antibody prior to infection. These data are from 2 mice per experimental group per time point, and representative of three independent experiments. B. Number of recoverable Lm CFUs per spleen in rat IgG2b control (shaded bars) or anti-PDL-1 antibody (open bars) treated mice at the indicated time points after infection. These data are from 4 to 5 mice per group, reflective of two independent experiments. Bar, one standard error.

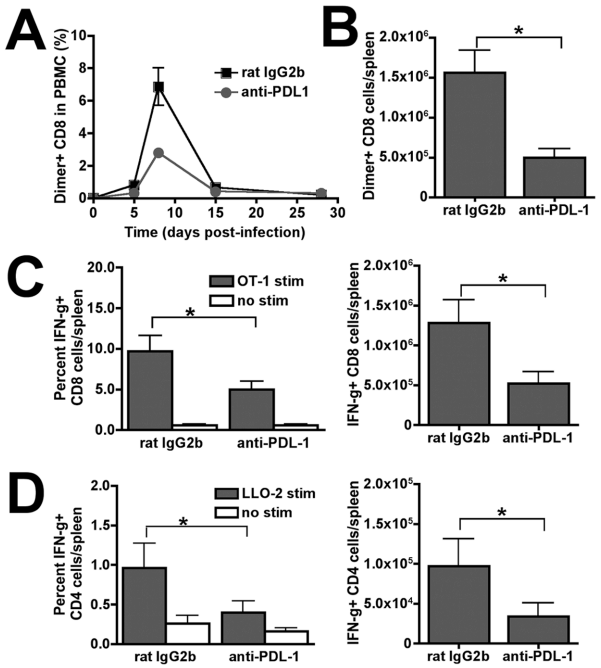

Figure 3.3 - A. Percent antigen-specific CD8 T cells among PBMCs in anti-PDL-1 antibody treated and control mice at the indicated time points after infection with 10^6 Lm ΔactA quantified by H-2K^b OVA$_{257-264}$ dimer staining. B. Number of H-2K^b OVA$_{257-264}$ dimer+ CD8 T cells among splenocytes day 8 post-infection. C. Percent and number of IFN-γ producing CD8 T cells among splenocytes after stimulation with OVA$_{257-264}$ peptide or no stimulation day 8 post-infection. D. Percent and number of IFN-γ producing CD4 T cells among splenocytes after LLO$_{189-201}$ peptide stimulation day 8 post-infection. These data represent 7 to 12 mice per experimental group and is representative of three independent determinations. Bar, standard error. * $P < 0.05$.

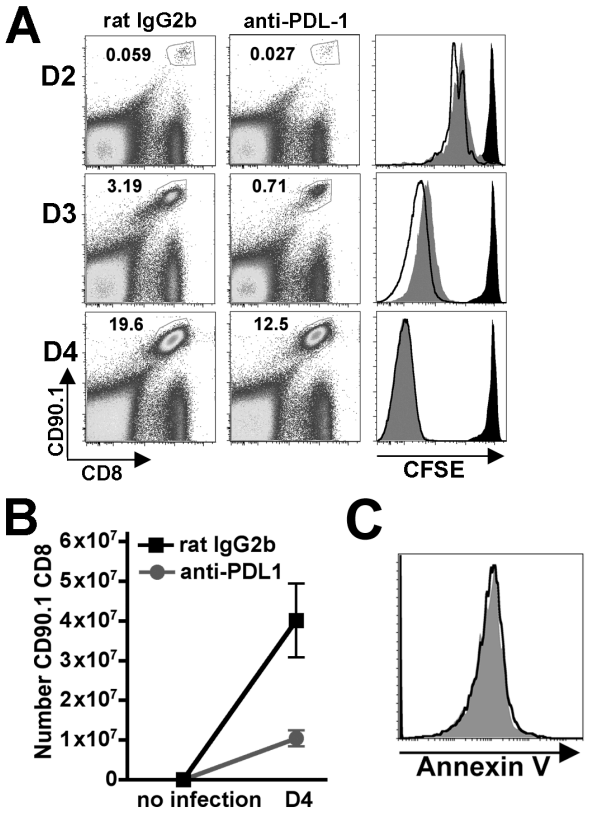

Figure 3.4. - A. Expansion and CFSE dilution in adoptively transferred OT-I (CD90.1+CD8+) cells at the indicated time points after infection with 10^6 Lm ΔactA in anti-PDL-1 antibody treated (gray histograms), control rat IgG2b treated (open histograms), or no infection control mice (black histograms). The numbers in the upper left quadrant indicate percentage of gated cells among total splenocytes. B. Number of OT-I (CD90.1+CD8+) per mouse spleen prior to infection and day 4 after infection in anti-PDL-1 antibody (circle) or rat IgG2b antibody (square) treated mice. C. Annexin V staining among OT-1 (CD90.1+CD8+) cells day 3 after infection in either anti-PDL-1 treated (gray histograms) or control IgG2b treated (open histograms) mice. These data represent 5-7 mice for each experimental group combined from two independent experiments. Bar, standard error.

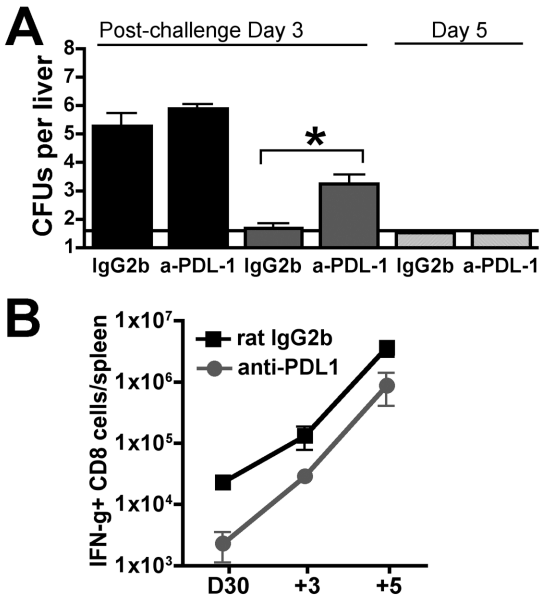

Figure 3.5 - A. Numbers of recoverable Lm CFUs in the liver days 3 and 5 after challenge with 10^5 virulent Lm for the indicated groups of naive mice (solid bar), or mice previously inoculated with Lm ΔactA 30 days prior to challenge (shaded and striped bars). For these experiments, mice were treated with either anti-PDL-1 or control antibody prior to primary infection, and throughout the experiment as described in the Materials and Methods. B. Number of IFN-γ producing CD8 T cells after stimulation with $OVA_{257-264}$ peptide among splenocytes prior to challenge (day 30 after Lm ΔactA infection), and days 3 and 5 after challenge with virulent Lm. These data represent 7 mice for each experimental group combined from two independent experiments. Bar, standard error. * $P < 0.05$.

Chapter 4

Innate IFN-γ is essential for programmed death ligand-1-mediated T cell stimulation following *Listeria monocytogenes* infection[**]

[**] Reprinted from *The Journal of Immunology*. Jared H. Rowe, James M. Ertelt, and Sing Sing Way. "Innate IFN-γ is essential for program death ligand-1 mediated T cell stimulation following *Listeria monocytogenes* infection." **© The American Association of Immunologists. Originally published in *The Journal of Immunology*. 2012. 189:000-000**

SUMMARY

Although best characterized for sustaining T cell exhaustion during persistent viral infection, Programmed death ligand (PDL)-1 also stimulates the expansion of protective T cells after infection with intracellular bacterial pathogens. Therefore, establishing the molecular signals that control whether PDL-1 stimulates immune suppression or activation is important as immune modulation therapies based on manipulating PDL-1 are being developed. Herein, the requirement for PDL-1 blockade initiated before infection with the intracellular bacterium *Listeria monocytogenes* (Lm) in reducing pathogen-specific T cell expansion is demonstrated. In turn, the role of proinflammatory cytokines triggered early after Lm infection in controlling PDL-1-mediated T cell stimulation was investigated using mice with targeted defects in specific cytokines or cytokine receptors. These experiments illustrate an essential role for IL-12 or type I IFNs in PDL-1-mediated expansion of pathogen-specific $CD8^+$ T cells. Unexpectedly, direct stimulation by neither IL-12 nor type I IFNs on pathogen-specific $CD8^+$ cells was essential for PDL-1-mediated expansion. Instead, the absence of early innate IFN-γ production in mice with combined defects in both IL-12 and type I IFN receptor negated the impacts of PDL-1 blockade. In turn, IFN-γ ablation using neutralizing antibodies or in mice with targeted defects in IFN-γ receptor each eliminated the PDL-1-mediated stimulatory impacts on pathogen-specific T cell expansion. Thus, innate IFN-γ is essential for PDL-1-mediated T cell stimulation.

INTRODUCTION

Programmed death ligand-1 (PDL-1, B7-H1) belongs to a growing list of co-stimulation molecules within the B7 family that regulate T cell activation (Brown et al., 2010; Keir et al., 2007; Odorizzi and Wherry, 2012; Sharpe et al., 2007). Best characterized after infection with Lymphocytic choriomeningitis virus (LCMV) and other viral pathogens that cause persistent infection, stimulation via PDL-1 sustains functional exhaustion for otherwise protective viral-specific $CD8^+$ T cells (Barber et al., 2006). In turn, PDL-1 blockade using monoclonal antibodies during persistent infection or with therapeutic vaccination reinvigorates the activation of LCMV-specific $CD8^+$ T cells and accelerates pathogen eradication (Ha et al., 2008). Similarly during hepatitis B or herpes simplex virus infection, PDL-1 neutralization stimulates the activation and IFN-γ production by virus-specific T cells (Jun et al., 2005; Maier et al., 2007). These PDL-1-mediated immune suppressive properties initially described in mouse infection models extend to functional T cell exhaustion for humans infected with viruses that predominantly cause persistent infection. For example, $CD8^+$ T cells with specificity to hepatitis C or human immune-deficiency virus each up-regulate the PDL-1 binding partner, PD-1, with progressively worsening infection (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006; Urbani et al., 2006). Reciprocally, PDL-1 blockade directly *ex vivo* reverses the functional exhaustion, and stimulates proliferation and cytokine production by virus-specific human $CD8^+$ T cells. Furthermore, for rabies virus that primarily cause acute instead of persistent infection, targeted defects in PDL-1 also protects against lethal

infection (Lafon et al., 2008). Taken together, these findings indicate PDL-1 compromises host defense against viral pathogens, and PDL-1 blockade may represent a promising strategy for boosting immunity against these infections.

Interestingly and in striking contrast to immune suppression that occurs during infection with viruses, the interaction between PDL-1 and PD-1 can also stimulate T cell activation and expansion that augments host defense against non-viral pathogens. For example, PDL-1 blockade impairs resistance and impedes the priming of protective $CD8^+$ T cells after infection with the intracellular bacterium *Listeria monocytogenes* (Lm) (Rowe et al., 2008; Seo et al., 2008). In particular, expansion defects for Lm-specific T cells with PDL-1 blockade were apparent throughout primary infection and were associated with delayed re-expansion after secondary infection (Rowe et al., 2008). Similarly, mice with defects in either PDL-1 or PD-1 have blunted expansion and activation of protective $CD4^+$ T cells, and are more susceptible to other intracellular pathogens such as *Salmonella enterica* or *Mycobacterium tuberculosis* (Barber et al., 2011; Lazar-Molnar et al., 2010; Lee et al., 2010). A stimulatory role for PDL-1/PD-1 is further supported by the observation that most PD-1^{hi} $CD8^+$ T cells in healthy humans have an effector memory rather than exhausted phenotype (Duraiswamy et al., 2011). These findings illustrate that depending on the type of infection, the interaction between PDL-1 and PD-1 can provide either immune activation or suppression signals that each play important roles in controlling infection susceptibility. Therefore, establishing the specific infection-induced signals that dictate whether PDL-1 stimulates immune activation or suppression is

important as immune modulation therapies based on manipulating PDL-1 are being developed.

In this study, we investigate how inflammatory cytokines induced by bacterial infection control PDL-1-mediated T cell stimulation. Given the interplay between the cytokines IL-12 and type I IFNs that each control PDL-1/PD-1 expression after infection with viral pathogens (Boasso et al., 2008; Lafon et al., 2008; Ma et al., 2011; Schreiner et al., 2004; Zhang et al., 2011), together with the efficiency whereby the intracellular bacterial pathogen Lm induces the production of these cytokines after infection (O'Connell et al., 2004; O'Riordan et al., 2002; Way et al., 2007), we initially focused on the role of IL-12 and type I IFNs in PDL-1-mediated stimulation of pathogen-specific T cells. Using mice with targeted individual or combined defects in these specific cytokines or their respective receptors, an essential role for either IL-12 or type I IFNs in PDL-1-mediated expansion of Lm-specific T cells is revealed. Unexpectedly however, the requirement for IL-12 and type I IFNs did not require direct stimulation by these cytokines on pathogen-specific T cells, but were instead indirectly mediated by the absence of early IFN-γ production after Lm infection in mice with combined defects in both IL-12 and type I IFN receptor. Together, these results uncover an essential role for innate IFN-γ in PDL-1-mediated T cell stimulation.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) ($CD45.2^+$ $CD90.2^+$; $H\text{-}2K^b$), Ly5.2 ($CD45.1^+$ $CD90.2^+$; $H\text{-}2K^b$), and CD90.1 ($CD45.2^+$ $CD90.1^+$; $H\text{-}2K^b$) mice were purchased from Jackson Laboratory or National Cancer Institute, and used between 6-8 weeks of age. Mice with targeted defects in PD-1, IL-12p40, type I IFN receptor, IL-12 receptor, and IFN-γ receptor, and mice with combined defects in IL-12p40 and type I IFN receptor each on the B6 background have been described (Cousens et al., 1999; Huang et al., 1993; Muller et al., 1994; Nishimura et al., 1998; Way et al., 2007; Wu et al., 1997). OT-1 TCR transgenic mice containing $CD8^+$ cells with specificity to the $OVA_{257\text{-}264}$ $H\text{-}2K^b$ peptide were intercrossed with WT mice (CD90.1), mice with combined defects in both IL-12 receptor and type I IFN receptor (CD90.2), or mice with targeted defects in IFN-γ receptor (CD90.2) (Hogquist et al., 1994; Xiao et al., 2009). All experiments were performed under University of Minnesota IACUC approved protocols.

Listeria infection. Recombinant Lm-OVA stably expresses OVA protein allowing the immune response to this surrogate Lm antigen to be tracked using established cellular immunology tools (Foulds et al., 2002). To bypass differences in susceptibility to virulent Lm-OVA for mice with defects in PDL-1, specific cytokines and/or cytokine receptors, an attenuated Lm-OVA strain containing targeted defects in ΔactA that prevents intracellular and intercellular spread was used (Auerbuch et al., 2004; Brombacher et al., 1999; Carrero et al., 2004; O'Connell et al., 2004; Orgun et al., 2008; Pearce and Shen,

2007; Rowe et al., 2008; Seo et al., 2008; Way et al., 2007). The highly attenuated nature of ΔactA Lm results in a non-productive infection that is rapidly eliminated even in mice lacking cytokines such as IL-12 and IFN-γ required for innate resistance against virulent Lm, and normalizes the pathogen burden in IL-12 and type I IFN receptor-deficient mice where even relatively high inocula are eliminated with similar kinetics compared with control mice (Ertelt et al., 2010; Harty and Bevan, 1995; Orgun et al., 2008; Way et al., 2007). Similarly for anti-PDL-1 antibody treated mice, ΔactA Lm is eliminated within the first 72 hours after infection with kinetics identical to mice treated with isotype control antibody (Rowe et al., 2008). For infection, Lm-OVA ΔactA was grown to early log phase (OD_{600} 0.1) in brain heart infusion media at 37°C, washed, and diluted with saline to a concentration of 10^6 CFUs per 200 μl, and this inoculum was used for intravenous injection as described (Rowe et al., 2008).

Antibodies and flow cytometry. Fluorophore-conjugated antibodies and other reagents for flow cytometry were purchased from BD Bioscience (San Jose, CA) or eBioscience (San Diego, CA). For PDL-1 blockade, anti-mouse PDL-1 (10F.9G2) or rat IgG2b (LTF-2) antibodies were purchased from BioXCell (West Lebanon, NH) and injected intraperitoneally one day before (500 μg/mouse) and four days after infection (250 μg/mouse), or in separate mice beginning only four days after infection (500 μg/mouse) (Barber et al., 2006; Rowe et al., 2008). For IFN-γ neutralization, anti-mouse IFN-γ (XMG1.2) or rat IgG1 (HRPN) antibodies (BioXCell) were injected intraperitoneally one day prior to infection (1.0 mg/mouse). Lm-OVA specific $CD8^+$ T cells were identified

among endogenous splenocytes by staining with H-2K^{b} dimer X (BD Biosciences) loaded with OVA$_{257–264}$ peptide as described (Orgun et al., 2008; Rowe et al., 2008). For cytokine production by antigen-specific CD8^{+} cells, splenocytes isolated directly *ex vivo* were cultured in 96-well round-bottom plates (5 x 10^{6} cells/ml) and stimulated with either OVA$_{257-264}$ peptide (10^{-6} M) or no stimulation in media supplemented with brefeldin A (GolgiPlug, BD Biosciences) at 37ºC for 5 hours as described (Orgun et al., 2008; Rowe et al., 2008; Way et al., 2007). For innate cytokine production, splenocytes isolated directly *ex-vivo* within the first 12, 24, and 48 hours after infection were stained using anti-IFN-γ antibody without additional stimulation. For enumerating PDL-1 and PD-1 expression, splenocytes re-suspended in saline supplemented with albumin (1%) and Fc-block (anti-CD16/32), were cell surface stained using anti-CD11c (clone N418), and anti-CD8 (clone 53.6.7), anti-PDL-1 (clones MIH5 and 1-111A) and anti-PD-1 (clone J43) antibodies.

Adoptive cell transfer. For cell transfers, 1 x 10^{5} CD8^{+} cells from donor WT OT-1 (CD45.2^{+} CD90.1^{+}) mice were mixed at a 1:1 ratio with CD8^{+} cells from either IL-12 receptor-, type I IFN receptor-deficient OT-1 (CD45.2^{+} CD90.2^{+}), or IFN-γ receptor-deficient OT-1 (CD45.2^{+} CD90.2^{+}) TCR transgenic mice, and intravenously transferred into recipient (CD45.1^{+}) mice one day prior to infection. For tracking the expansion of endogenous OVA-specific T cells, one mouse equivalent of CD8^{+} cells (~10^{7} cells) purified by negative selection from PD-1 deficient (CD90.2) mice were adoptively transferred into WT CD90.1 recipient mice treated with anti-PDL-1 or isotype control

antibodies, and infected with Lm-OVA ΔactA the following day.

***Statistic*s.** The number and percent cells were first analyzed and found to be normally distributed, and thereafter differences between groups were evaluated using the Student's *t* test with $p < 0.05$ taken as statistical significance (GraphPad, Prism software).

RESULTS

PDL-1 blockade initiated prior to infection blunts pathogen-specific $CD8^+$ T cell expansion

Given the discordance between PDL-1 blockade prior to, compared with after, *in vivo* stimulation on $CD8^+$ cell expansion recently demonstrated for purified protein administered with poly(I:C) (Pulko et al., 2011), we investigated how the timing of PDL-1 blockade would impact the expansion of pathogen-specific T cells after recombinant Lm infection. Consistent with the results of other studies (Rowe et al., 2008; Seo et al., 2008), PDL-1 blockade initiated prior to Lm-OVA infection (day -1) triggered sharp reductions in the overall expansion of OVA-specific $CD8^+$ T cells. Seven days after infection which corresponds to the peak expansion of endogenous OVA-specific $CD8^+$ T cells, ~70% reductions in OVA-specific $CD8^+$ T cells were identified both by staining with $OVA_{257-264}$ loaded H-$2K^b$ MHC dimer or intracellular cytokine staining after stimulation with $OVA_{257-264}$ peptide for anti-PDL-1 compared with isotype antibody treated mice (Figure 4.1A-D). Furthermore, this reduction in Lm-specific $CD8^+$ T cell expansion with PDL-1 blockade required PD-1, because the impacts of PDL-1 blockade were eliminated for mice with targeted defects in PD-1 in all cells or among PD-1-deficient $CD8^+$ cells after adoptive transfer into PD-1-sufficient recipients (Figure 4.2). Interestingly however, when anti-PDL-1 antibody was administered later after infection (day +4), the impacts of PDL-1 blockade were reversed as OVA-specific $CD8^+$ T cells expanded ~2.5 fold more in anti-PDL-1 compared with isotype control antibody treated

mice (Figure 4.1A-D). Together, these results suggest the stimulatory effects of PDL-1 are controlled by signals triggered within the first few days after Lm infection.

IL-12 or type I IFNs are required for PDL-1-mediated T cell stimulation

Given the efficiency whereby Lm induces IL-12 and type I IFN production (O'Connell et al., 2004; O'Riordan et al., 2002; Way et al., 2007), and the importance of these cytokines in regulating PDL-1/PD-1 expression during viral infections (Boasso et al., 2008; Lafon et al., 2008; Ma et al., ; Schreiner et al., 2004; Zhang et al., 2011), the requirement for IL-12 or type I IFNs in PDL-1-mediated T cell stimulation were investigated with Lm infection. We found PDL-1 blockade in mice with individual defects in either IL-12 or type I IFN receptor caused significant reductions the expansion of Lm-OVA specific $CD8^+$ T cells identified by staining with either $OVA_{257-264}$ loaded H-$2K^b$ MHC dimer or based on IFN-γ production after *in vitro* stimulation with cognate peptide similar to that observed in B6 control mice (Figure 4.3A-D). On the other hand and in sharp contrast to these reductions in Lm-OVA specific $CD8^+$ T cell expansion in B6 or mice with individual defects in IL-12 or type I IFN receptor, the impacts of PDL-1 blockade were eliminated in mice with combined defects in both IL-12 and type I IFN receptor (Figure 4.3A-D). Specifically, $OVA_{257-264}$-specific $CD8^+$ T cells identified using either $OVA_{257-264}$ H-$2K^b$ dimer or intracellular cytokine staining each expanded to a similar extent in anti-PDL-1 or isotype antibody treated mice lacking both IL-12 production and type I IFN responsiveness. These findings illustrate the T cell stimulatory effects of PDL-1

during Lm infection require either IL-12 or type I IFNs, while the combined absence of both extinguishes PDL-1-mediated pathogen-specific T cell expansion.

Given the potential for IL-12 and type I IFNs in controlling PDL-1/PD-1 expression (Boasso et al., 2008; Lafon et al., 2008; Ma et al., 2011; Schreiner et al., 2004; Zhang et al.), we also investigated the possibility that the muted impacts of PDL-1 blockade in mice with combined defects in both IL-12 and type I IFN receptor were due to differences in infection-induced PDL-1 expression. We focused initially on shifts in PDL-1 expression by $CD11c^+$ dendritic cells because these antigen-presenting cells are essential for the priming and expansion of pathogen-specific $CD8^+$ T cells after Lm infection (Jung et al., 2002b). We found although the background level of PDL-1 expression and the kinetics whereby Lm stimulates the up-regulation of PDL-1 expression by $CD11c^+$ cells each did not differ significantly in mice with individual or combined defects in IL-12 and/or type I IFN receptor compared with B6 control mice, the proportion of dendritic cells which up-regulated PDL-1 expression was reduced by ~30% in mice lacking both IL-12 production and type I IFN responsiveness (Figure 4.4). Comparatively, the expression of both PD-1 and PDL-1 were enriched for Lm OVA-specific T cells compared with bulk non-specific $CD8^+$ cells, and the relative expression of each on OVA $dimer^+$ $CD8^+$ cells in mice with individual or combined defects in IL-12 and type I IFN receptor did not differ significantly from B6 control mice (Figure 4.5). Nevertheless, *in vivo* treatment with anti-PDL-1 antibody clone 10F.9G2 efficiently blocked PDL-1 availability because the level of staining with other anti-PDL-1 clones

(MIH5 and 1-111A) was sharply reduced for OVA-specific T cells in anti-PDL-1 compared with isotype antibody treated mice (Figure 4.6). Therefore although the blunted up-regulation of PDL-1 expression by dendritic cells in mice with combined defects in IL-12 and type I IFN receptor may contribute to the negated impacts of PDL-1 blockade, the sharp up-regulation of PDL-1/PD-1 expression from background levels induced by Lm infection in mice with combined defects in both IL-12 and type I IFN receptor may also indicate important non-redundant roles for these cytokines in stimulating PDL-1-mediated pathogen-specific $CD8^+$ T cell expansion.

Cell-intrinsic stimulation by neither IL-12 nor type I IFNs are essential for PDL-1-mediated T cell expansion

Related experiments explored how IL-12 and type I IFNs may control the stimulatory effects of PDL-1. Given the potency whereby IL-12 and type I IFNs can directly stimulate T cell activation (Carrero et al., 2006; Curtsinger and Mescher, 2010; Curtsinger et al., 2005; Haring et al., 2006), we first investigated the requirement for cell-intrinsic stimulation with IL-12 and type I IFNs on antigen-specific T cells in overriding PDL-1-mediated T cell expansion. Specifically, we compared the impacts of PDL-1 blockade initiated one day before Lm-OVA ΔactA infection on the subsequent expansion of antigen-specific $CD8^+$ T cells from IL-12 receptor, type I IFN receptor double deficient (that cannot respond to direct cell-intrinsic stimulation by these cytokines, $CD45.2^+$ $CD90.2^+$) and WT ($CD45.2^+$ $CD90.1^+$) OT-1 TCR transgenic mice each after adoptive transfer into $CD45.1^+$ recipient mice. The discordant expression of CD90.1/90.2

congenic markers allows these two subsets of adoptively transferred antigen-specific $CD8^+$ T cells to be discriminated from each other, and expression of the CD45.2 marker allows both subsets of donor cells to be identified among recipient cells (Figure 4.7A). Using this approach if cell-intrinsic stimulation via either IL-12 or type I IFNs on OVA-specific $CD8^+$ T cells is essential for PDL-1-mediated expansion, the effects of PDL-1 blockade would be eliminated for cells with combined defects in receptors for both IL-12 and type I IFNs, but preserved for WT cells that can respond to stimulation with each of these cytokines. Interestingly and in sharp contrast to this prediction, PDL-1 blockade caused reductions in expansion for both subsets of adoptively transferred antigen-specific $CD8^+$ cells each to a similar extent (~ 50%) (Figure 4.7B,C). Furthermore, the magnitude of these reductions among both subsets of OVA-specific T cells each paralleled the expansion defects among endogenous OVA-specific $CD8^+$ cells with PDL-1 blockade in B6 and mice with individual defects in IL-12 and type I IFN receptor (Figure 4.1 and 4.3) (Rowe et al., 2008; Seo et al., 2008). Together, these results demonstrate the negated impacts of PDL-1 blockade in mice with combined defects both IL-12 and type I IFN receptor are not due to defects in cell-intrinsic stimulation by IL-12 or type I IFNs on $CD8^+$ T cells. Instead, other environmental differences resulting from the combined absence of both IL-12 and type I IFNs override the immune stimulatory effects of PDL-1 following Lm infection.

Lm-induced IFN-γ production drives PDL-1-mediated T cell expansion

Since direct T cell stimulation by IL-12 and type I IFNs are jointly non-essential for PDL-1-mediated expansion of Lm-specific T cells, we investigated the role other cytokines such as IFN-γ known to be stimulated by IL-12 and type I IFNs have in controlling the T cell stimulatory impacts of PDL-1 (Lieberman and Hunter, 2002; Magram et al., 1996; Nomura et al., 2002). Consistent with the results of our prior studies (Orgun et al., 2008), innate IFN-γ production by T and NK cells among bulk splenocytes that peaks within the first 12 hours after Lm-OVA ΔactA infection in B6 mice was completely abolished in mice with combined defects in both IL-12 and type I IFN receptor (Figure 4.8). On the other hand, although early IFN-γ production in IL-12 deficient mice was extinguished more rapidly from peak levels compared with B6 mice, Lm-OVA ΔactA induced indistinguishable levels of IFN-γ production within the first 12 hours after infection in mice with individual defects in IL-12 or type I IFN receptor each compared with B6 mice (Figure 4.8). Moreover, PDL-1 blockade did not significantly impact the magnitude or kinetics of Lm-OVA ΔactA infection-induced IFN-γ production in mice with individual or combined defects in IL-12 or type I IFN receptor each compared with B6 mice (data not shown). Thus, overriding PDL-1-mediated T cell stimulation in mice with combined defects in both IL-12 and type I IFN receptor may reflect differences in production of immune modulatory cytokines such as IFN-γ.

To investigate the requirement for IFN-γ in PDL-1-mediated T cell stimulation, the impacts of IFN-γ neutralization combined with PDL-1 blockade on pathogen-specific $CD8^+$ T cell expansion were enumerated. Remarkably, anti-IFN-γ neutralization

efficiently eliminated the defects in Lm-OVA $CD8^+$ T cell expansion associated with PDL-1 blockade. Specifically, the reductions in OVA-specific T cell expansion tracked using $OVA_{257-264}$ H-$2K^b$ dimer or intracellular cytokine staining associated with PDL-1 blockade were eliminated in mice treated with anti-IFN-γ neutralizing but preserved in mice treated with each respective isotype antibody (Figure 4.9A-D). In a similar fashion, the impacts of PDL-1 blockade on pathogen-specific $CD8^+$ T cell expansion were also eliminated in mice with targeted defects in the IFN-γ receptor, but preserved in IFN-γ receptor sufficient control mice (Figure 4.10A-D). Therefore, although specific cytokine-antibody complexes have the potential to stabilize the biological activity of some cytokines *in vivo* (Boyman et al., 2006; Kamimura and Bevan, 2007; Rubinstein et al., 2006), the paralleled elimination of PDL-1 stimulation using anti-IFN-γ neutralizing antibody and in IFN-γ receptor-deficient mice demonstrates an essential role for IFN-γ in PDL-1-mediated pathogen-specific $CD8^+$ T cell expansion after Lm infection. Together with the absence of IFN-γ production in mice with combined defects in IL-12 and type I IFN receptor (Figure 4.8) (Orgun et al., 2008), these findings suggest IFN-γ produced through either IL-12 or type I IFN-dependent pathways early after Lm infection dictates PDL-1-mediated expansion of pathogen-specific $CD8^+$ T cells.

Given the potential contribution of blunted PDL-1 up-regulation in negating the impacts of PDL-1 blockade in mice with combined defects in IL-12 and type I IFN receptor (Figure 4.4), Lm infection-induced shifts in PDL-1 expression by $CD11c^+$ dendritic cells in IFN-γ receptor-deficient mice were also investigated. We found Lm-OVA ΔactA

triggered sharply increased levels of PDL-1 expression by $CD11c^+$ dendritic cells that peaked 24 hours after infection in IFN-γ receptor deficient mice, and the overall magnitude and tempo of these shifts in PDL-1 expression were identical compared with B6 control mice (Figure 4.11). Similarly, expanded Lm-OVA-specific $CD8^+$ T cells in both IFN-γ receptor deficient and B6 control mice were enriched for PD-1 and PDL-1 expression compared with bulk non-specific $CD8^+$ cells (Figure 4.12). Thus, the muted impacts of PDL-1 blockade on antigen-specific $CD8^+$ T cell expansion in IFN-γ receptor deficient mice are not explained by differences in infection induced PDL-1/PD-1 expression.

Cell-intrinsic stimulation by IFN-γ is non-essential for PDL-1-mediated T cell expansion

The requirement for IFN-γ in PDL-1-mediated expansion of antigen-specific $CD8^+$ T cells following Lm infection led us to further investigate the importance of IFN-γ responsiveness by $CD8^+$ T cells, and how PDL-1 blockade may control the expansion of these cells. Using a similar adoptive transfer strategy for investigating the requirement for cell intrinsic IL-12 and type I IFN stimulation on OVA-specific $CD8^+$ cell expansion (Figure 4.7), the impact of PDL-1 blockade initiated one day before Lm-OVA ΔactA infection on the subsequent expansion of antigen-specific $CD8^+$ T cells from IFN-γ-receptor-deficient ($CD45.2^+$ $CD90.2^+$) compared with WT ($CD45.2^+$ $CD90.1^+$) OT-1 TCR transgenic mice each after adoptive transfer into CD45.1 recipient mice was evaluated. We found PDL-1 blockade in recipient mice prior to Lm-OVA ΔactA infection caused

reductions in the expansion for both subsets of adoptively transferred OT-1 $CD8^+$ T cells each to a similar extent (Figure 4.13A-C). Furthermore, the overall magnitude of these reductions (~ 50%) were similar to the reductions among endogenous OVA specific $CD8^+$ T cells and among adoptively transferred $CD8^+$ cells from TCR transgenic mice with defects in the receptors for both IL-12 and type I IFNs (Figures 4.3 and 4.7). Taken together, these results indicate that although IFN-γ produced early after Lm infection is essential for PDL-1-mediated $CD8^+$ T cell expansion, and direct cell intrinsic IFN-γ stimulation on effector T cells has been described to be important for their expansion in other infections (Sercan et al., 2006; Sercan et al., 2010; Whitmire et al., 2005a; Whitmire et al., 2005b), IFN-γ stimulation on $CD8^+$ T cells is non-essential for their expansion following Lm infection.

DISCUSSION

The balance between immune stimulation and suppression signals that together control T cell activation and expansion is intricately regulated. Although PDL-1 has been mostly characterized to mediate suppression and functional T cell exhaustion during persistent viral infections (Barber et al., 2006; Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006; Urbani et al., 2006), the interaction between PDL-1 and PD-1 can also stimulate T cell activation most notably after infection with intracellular bacterial pathogens. For example, PD-1-deficient mice have increased pathogen burden and readily succumb to even relatively low inocula of aerosol MTb (Lazar-Molnar et al., 2010). Moreover during MTb infection, PD-1 expression marks highly proliferative pathogen-specific T cell precursors that replenish protective cytokine producing effector $CD4^+$ cells (Lazar-Molnar et al., 2010; Reiley et al., 2010). Similarly, PDL-1 disruption using mice with targeted defects in this molecule or blocking antibodies administered prior to infection, augments infection susceptibility and impedes the expansion of pathogen-specific T cells after infection with other intracellular bacterial pathogens such as *Salmonella enterica* and *Listeria monocytogenes* (Lee et al., 2010; Rowe et al., 2008; Seo et al., 2008). Accordingly, PDL-1 stimulation has the potential to provide either immune activation or suppression signals, and these discordant roles are most likely controlled by differences in the cytokine milieu or other infection-induced environmental differences between intracellular bacterial compared with viral pathogens. Our finding that PDL-1-mediated T cell stimulation that occurs with PDL-1 blockade initiated before

Lm infection is reversed with blockade initiated four days after infection (Fig. 1) further illustrates discordant roles for PDL-1 at early and later time points within the same infection. These results are consistent with the discordant impacts resulting from PDL-1 blockade initiated before compared with after *in vivo* stimulation with purified protein plus poly(I:C) on T cell expansion (Pulko et al., 2011), and together reinforce the potential importance of the cytokine milieu that shifts dramatically within the first few days after acute infection or immunization on the ensuing immune response through PDL-1.

By initiating PDL-1 blockade prior to infection in mice with targeted defects in cytokines such as IL-12 and type I IFNs that are readily induced by Lm and other intracellular bacterial pathogens, we sought to identify how PDL-1-mediated T cell stimulation is controlled. Although IL-12 and type I IFNs have each been described to control PDL-1/PD-1 expression in various other contexts (Boasso et al., 2008; Lafon et al., 2008; Ma et al., ; Schreiner et al., 2004; Zhang et al., 2011), we found mice with individual defects in each cytokine (or cytokine receptor) showed no significant differences in PDL-1 expression by $CD11c^+$ antigen presenting cells or PD-1/PDL-1 expression by antigen-specific $CD8^+$ T cells (Figures 4.3 and 4.5). Interestingly however, Lm infection-induced up-regulation of PDL-1 expression was blunted in mice with combined defects in both IL-12 and type I IFNs. Although the reduced levels of PDL-1 expression in mice lacking both IL-12 production and type I IFN receptor may contribute to the negated impacts of PDL-1 blockade, the requirement for IFN-γ in PDL-1-mediated T cell expansion of Lm-

OVA specific $CD8^+$ T cells suggests these results are more likely explained by the absence of IFN-γ triggered early after Lm infection in these mice. In turn, the comparable magnitude and tempo whereby PDL-1 expression is up-regulated in IFN-γ receptor-deficient and control mice indicates the requirement for IFN-γ in PDL-1-mediated T cell expansion are not simply due to differences in PDL-1 expression. Instead, other IFN-γ-induced molecules such as nitric oxide that are up-regulated with PDL-1 blockade and suppress T cell proliferation may explain the requirement for IFN-γ in PDL-1-mediated T cell stimulation (Beckerman et al., 1993; Yamazaki et al., 2005). This notion is supported by the sharp up-regulation of reactive nitrogen intermediates after Lm infection that occurs in an IFN-γ–dependent fashion (Beckerman et al., 1993; Boockvar et al., 1994), and suggest establishing how PDL-1 controls nitric oxide production after in vivo infection are important areas for future investigation.

The requirement for IFN-γ in PDL-1-mediated T cell expansion during infection with intracellular bacterial pathogens is consistent with the essential role this cytokine plays in host defense against these infections (Flynn et al., 1993; Harty and Bevan, 1995; Hess et al., 1996). Our results suggest that during infection with these pathogens, early IFN-γ, in addition to activating innate host defense, also promotes the expansion of protective pathogen-specific T cells via PDL-1. This association between intracellular pathogens that stimulate a Tc1/Th1-dominated response and the reversal of PDL-1 from immune suppression to activation signals are also consistent with T-bet-mediated repression of PD-1 expression that stimulates the activation of viral specific $CD8^+$ T cells (Kao et al.,

2011). Reciprocally, for other pathogens like LCMV clone 13 or *M. bovis* BCG that cause persistent infection and where early IFN-γ is less critical for innate host defense, PDL-1 stimulates T cell suppression instead of activation signals (Barber et al., 2006; Kamijo et al., 1993; Ou et al., 2001; Sakai et al., 2010). A notable exception here is *Toxoplasma gondii* infection where IFN-γ is critically required for innate protection, and yet PDL-1 actively inhibits protective $CD8^+$ T cell responses (Bhadra et al., 2011; Suzuki et al., 1988). Whether this is unique for *Toxoplasma* or more generalizable for other parasitic pathogens remains undefined, but also represent important areas for future investigation. Finally, given the discordant roles in host defense resulting from PDL-1/PD-1 disruption, developing PDL-1 blockade for therapeutically boosting immunity against some infections need to be carefully weighed against the potential for increased susceptibility to infection with other types of pathogens. The finding that early IFN-γ is essential for PDL-1-mediated T cell stimulation suggests PDL-1 disruption will most severely impede host defense against infections where IFN-γ is produced early and presumably essential for innate resistance. Our ongoing studies are aimed at further investigating the interplay between PDL-1 and IFN-γ in stimulating pathogen-specific adaptive responses using other models of experimental infection, and dissecting the molecular basis whereby IFN-γ redirects PDL-1 stimulation into T cell activation signals.

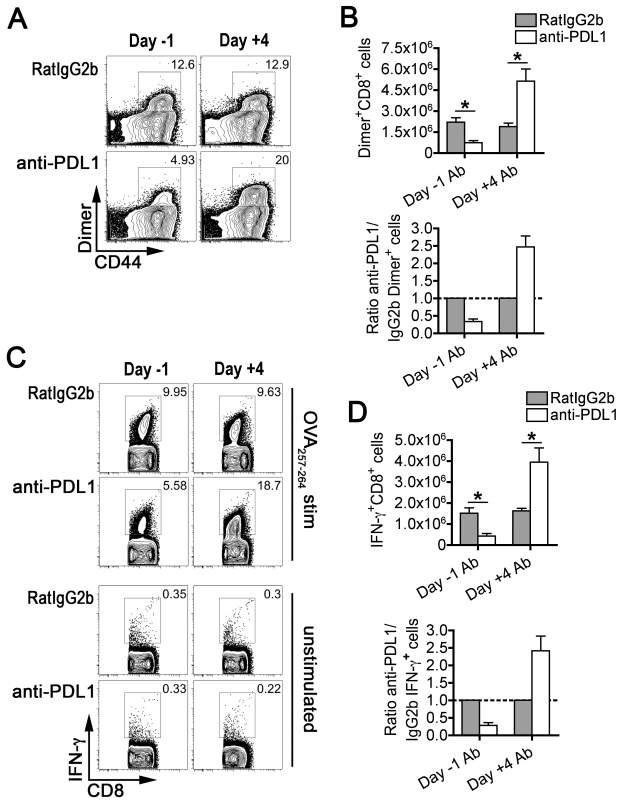

Figure 4.1 - PDL-1 blockade initiated prior to infection blunts pathogen-specific CD8$^+$ T cell expansion. A. Percent OVA-specific CD8$^+$ splenocytes identified by H-2K^b $OVA_{257–264}$ dimer staining seven days after Lm-OVA ΔactA infection for B6 mice treated with anti-PDL-1 or isotype control (IgG2b) antibody initiated either one day before (day -1) or four days after (day +4) infection. B. Total number (top) and ratio (bottom) of OVA dimer$^+$ CD8$^+$ T cells for the mice described in panel A. C. Percent IFN-γ producing CD8$^+$ splenocytes seven days after Lm-OVA ΔactA infection, and $OVA_{257–264}$ peptide stimulation (top) or no stimulation controls (bottom) for B6 mice treated with anti-PDL-1 or isotype control antibody initiated either one day before (day -1) or four days after (day +4) infection. D. Total number (top) and ratio (bottom) of IFN-γ producing CD8$^+$ T cells for the mice described in panel C. These data reflect 7-12 mice from three independent experiments each with similar results. Bar, one standard deviation. * $p < 0.05$.

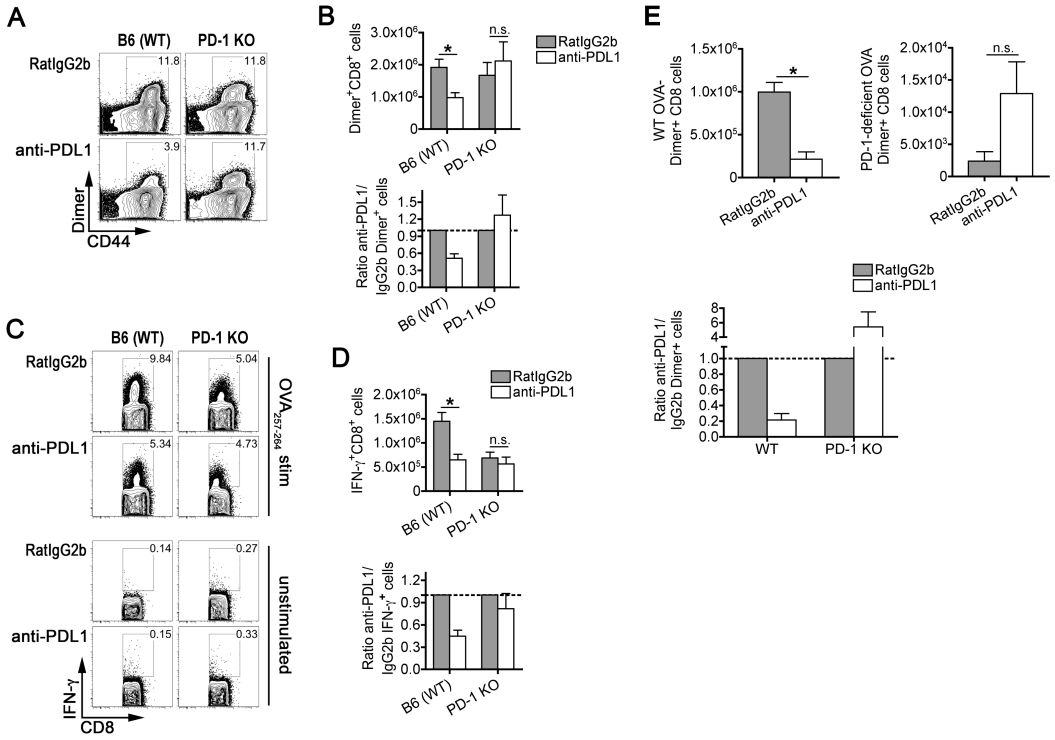

Figure 4.2 - Reductions in antigen-specific CD8$^+$ T cell expansion with PDL-1 blockade are eliminated in PD-1-deficient mice. A. Percent OVA-specific CD8$^+$ splenocytes identified by staining with H-2K^b OVA$_{257–264}$ dimer seven days after Lm-OVA ΔactA infection for B6 (WT) or PD-1-deficient mice each treated with anti-PDL-1 or rat IgG2b isotype control antibody one day prior to infection. B. Total number (top) and ratio (bottom) of OVA dimer$^+$ CD8$^+$ cells for the mice described in panel A. C. Percent IFN-γ producing CD8$^+$ splenocytes seven days after Lm-OVA ΔactA infection, and stimulation *in vitro* with OVA$_{257–264}$ peptide (top) or no stimulation controls (bottom) for B6 (WT) or PD-1-deficient mice treated with anti-PDL-1 or isotype control antibody one day before infection. D. Total number (top) and ratio (bottom) of IFN-γ producing CD8$^+$ T cells for the mice described in panel C. E. Total number (top) and ratio (bottom) of WT and PD-1-deficient OVA-Dimer$^+$ CD8$^+$ splenocytes seven days after Lm-OVA ΔactA infection in CD90.1 recipient mice adoptively transferred PD-1 deficient (CD90.2) CD8$^+$ cells treated with anti-PDL-1 or rat IgG2b isotype control antibody one day prior to infection. These data reflect 7-12 mice per group from two to three independent experiments each with similar results. Bar, one standard deviation. * $p < 0.05$.

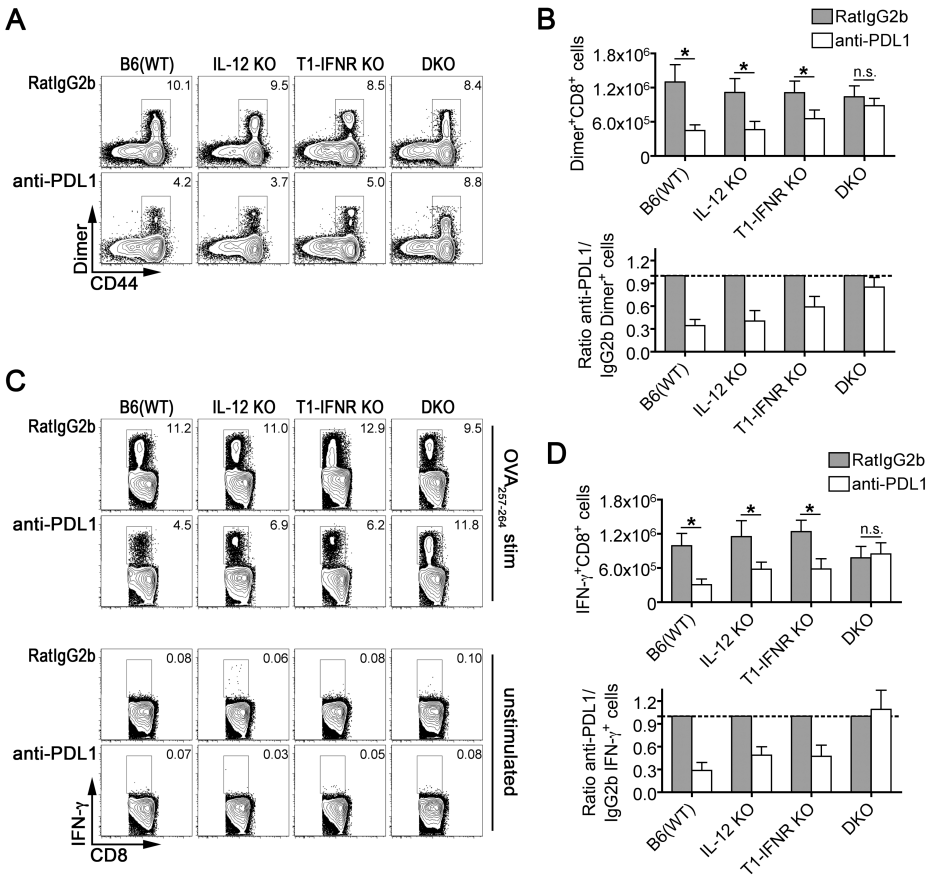

Figure 4.3 - IL-12 or type I IFN receptor is required for PDL-1-mediated expansion of pathogen-specific CD8$^+$ T cells. A. Percent OVA-specific CD8$^+$ splenocytes identified by H-2K^b OVA$_{257–264}$ dimer staining seven days after Lm-OVA ΔactA infection for each group of mice treated with either anti-PDL-1 or isotype control (IgG2b) antibody one day prior to infection. B. Total number (top) and ratio (bottom) of OVA-specific dimer$^+$ CD8$^+$ T cells for the mice described in panel A. C. Percent IFN-γ producing CD8$^+$ splenocytes seven days after Lm-OVA ΔactA infection, and OVA$_{257–264}$ peptide stimulation (top) or no stimulation controls (bottom) for each group of mice treated with either anti-PDL-1 or isotype antibody one day prior to infection. D. Total number (top) and ratio (bottom) of IFN-γ producing CD8$^+$ T cells for the mice described in panel C. These data reflect 7-12 mice per group from three independent experiments each with similar results. Bar, one standard deviation, * $p < 0.05$.

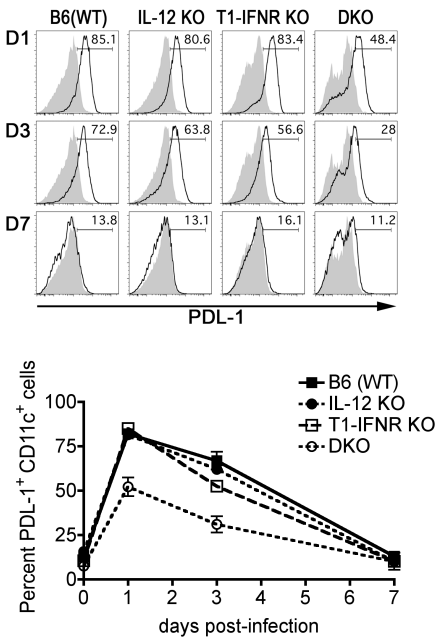

Figure 4.4 - Infection-induced shifts in PDL-1 expression by CD11c$^+$ antigen presenting cells. Representative plots (top) and composite data (bottom) illustrating PDL-1 expression by CD11c$^+$ splenocytes for each group of mice at the indicated time points after Lm-OVA ΔactA infection (line histogram) compared with uninfected controls (shaded histogram). These data reflect 6-8 mice per group from three independent experiments each with similar results. Bar, one standard deviation.

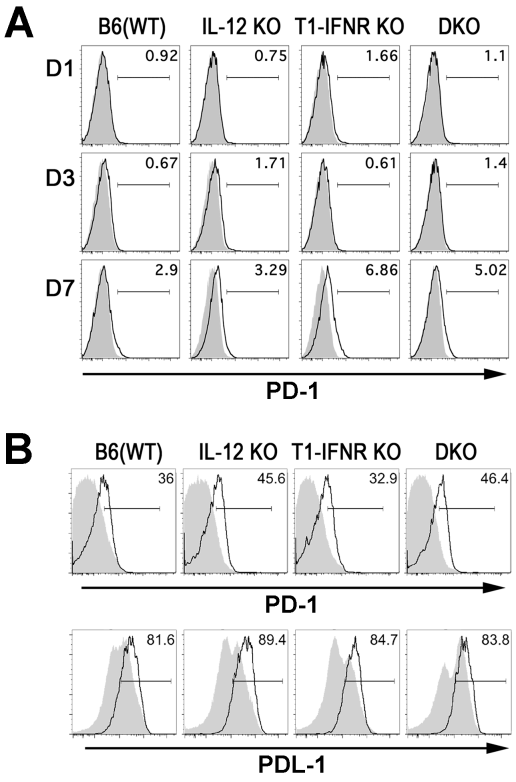

Figure 4.5 - Infection-induced PD-1 and PDL-1 expression among CD8$^+$ cells in mice with individual or combined defects in IL-12 or type I IFN receptor compared with B6 control mice. A. PD-1 expression on bulk CD8$^+$ splenocytes for each group of mice at the indicated time points after infection with 10^6 Lm-OVA ΔactA (line histogram) compared with uninfected controls (shaded histogram). B. PD-1 and PDL-1 expression by H-2K^b OVA$_{257–264}$ dimer$^+$ (line histogram) compared with bulk CD8$^+$ T cells (shaded histogram) for each group of mice seven days after Lm-OVA ΔactA infection. These data reflect 6-8 mice per group from three independent experiments each with similar results. Bar, one standard deviation.

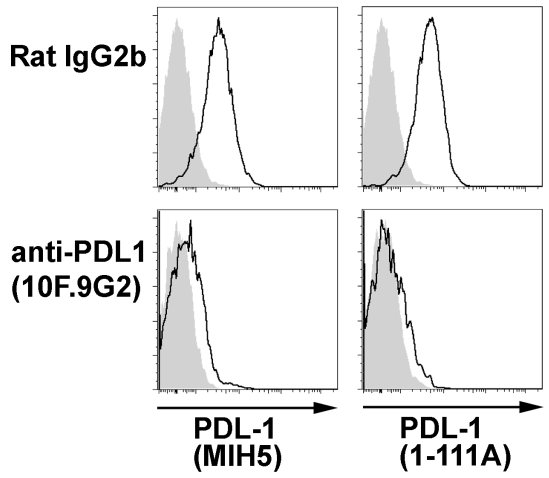

Figure 4.6 - PDL-1 blockade with anti-PDL-1 clone 10F.9G2 *in vivo* eliminates the availability of PDL-1 on antigen-specific CD8$^+$ T cells. Relative intensity of PDL-1 staining using other anti-PDL-1 clones (line histograms, MIH5 or 1-111A) compared with staining using isotype control antibody (shaded histograms, IgG2a) on H-2K^b OVA$_{257–264}$ dimer$^+$ CD8$^+$ T cells seven days after infection with 10^6 Lm-OVA ΔactA. These data reflect 6 mice per group from two independent experiments each with similar results.

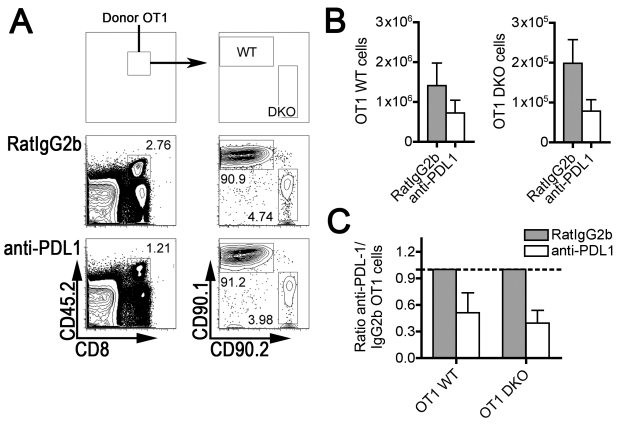

Figure 4.7 - T cell-intrinsic stimulation by neither IL-12 nor type I IFNs are essential for PDL-1-mediated pathogen-specific CD8$^+$ T cell expansion. A. Percent donor (CD45.2$^+$) CD8$^+$ cells among either WT (CD90.1$^+$) or IL-12 receptor, type I IFN-receptor DKO (CD90.2$^+$) OT-1 cells three days after Lm-OVA ΔactA infection, for cells adoptively transferred into CD45.1 recipient mice treated with either anti-PDL-1 or isotype control (IgG2b) antibody one day before infection. Total number (B) and ratio (C) of adoptively transferred OVA-specific CD8$^+$ T cells from WT or IL-12 receptor, type I IFN-receptor DKO OT-1 transgenic mice for the mice described in panel A. These data reflect 6-8 mice per group from three independent experiments. Bar, one standard deviation.

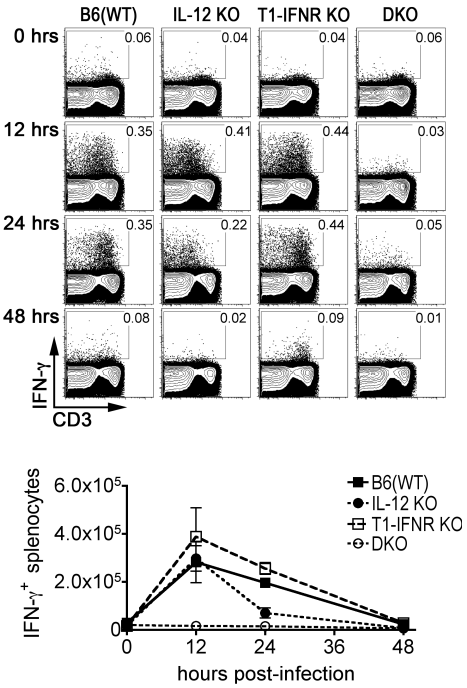

Figure 4.8 - IL-12 or type I IFNs are essential for innate IFN-γ production after Lm infection. Representative FACS plots illustrating percent (top) and composite data demonstrating total number (bottom) of IFN-γ producing splenocytes for each group of mice at the indicated time points after Lm-OVA ΔactA infection. These data reflect 6-8 mice per group from three independent experiments each with similar results. Bar, one standard deviation.

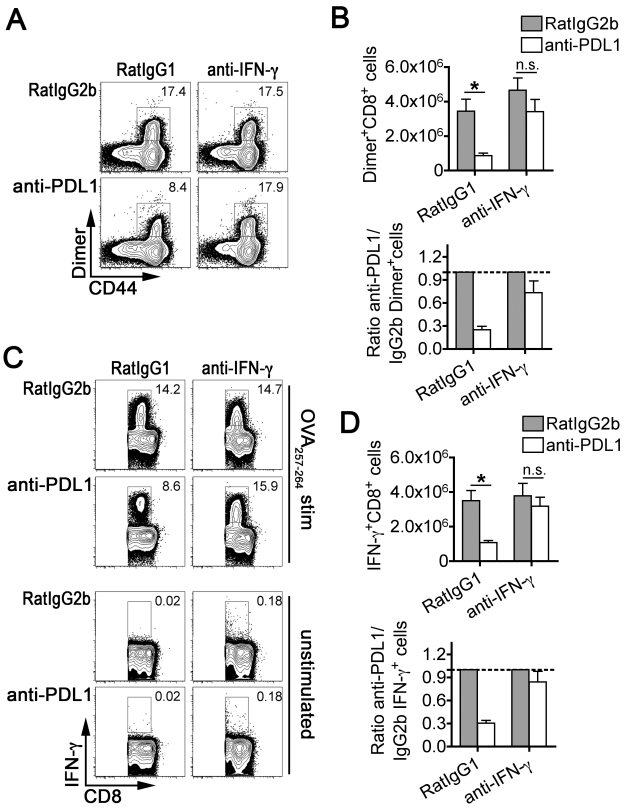

Figure 4.9 - IFN-γ neutralization eliminates PDL-1-mediated expansion defects for pathogen-specific CD8$^+$ T cells. A. Percent OVA-specific CD8$^+$ splenocytes identified by H-2K^b OVA$_{257–264}$ dimer staining seven days after Lm-OVA ΔactA infection for B6 mice treated with anti-PDL-1, and/or anti-IFN-γ, or each respective isotype control antibody one day prior to infection. B. Total number (top) and ratio (bottom) of OVA dimer$^+$ CD8$^+$ T cells for the mice described in panel A. C. Percent IFN-γ producing CD8$^+$ splenocytes seven days after Lm-OVA ΔactA infection, and OVA$_{257–264}$ peptide stimulation (top) or no stimulation controls (bottom) for each group of mice, treated with anti-PDL-1, and/or anti-IFN-γ, or each respective isotype control antibody one day prior to infection. D. Total number (top) and ratio (bottom) of IFN-γ producing CD8$^+$ T cells for the mice described in panel C. These data reflect 7-12 mice per group from three independent experiments each with similar results. Bar, one standard deviation. * $p <$ 0.05.

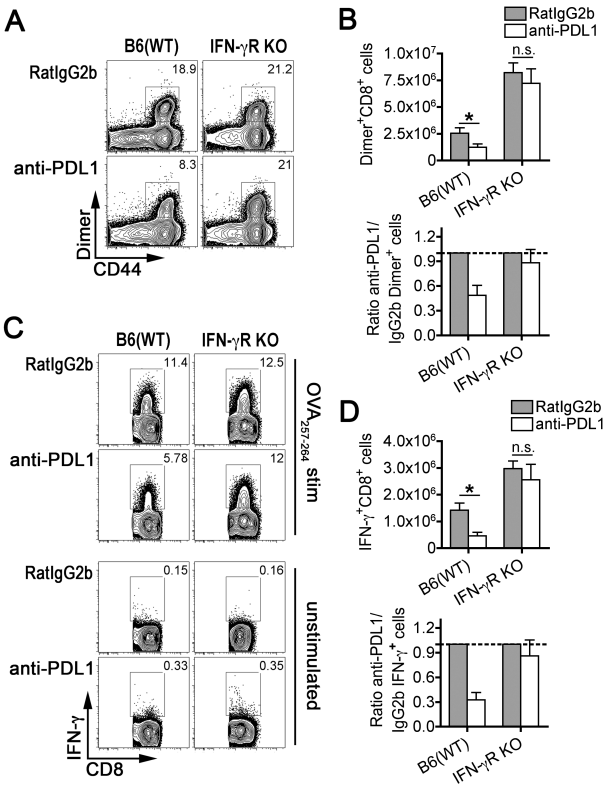

Figure 4.10 - Expansion defects for pathogen-specific CD8$^+$ T cells with PDL-1 blockade are eliminated in IFN-γ receptor-deficient mice. A. Percent OVA-specific CD8$^+$ splenocytes identified by H-2K^b OVA$_{257–264}$ dimer staining seven days after Lm-OVA ΔactA infection for B6 (WT) or IFN-γ-receptor-deficient mice treated with either anti-PDL-1 or isotype control (IgG2b) antibody one day prior to infection. B. Total number (top) and ratio (bottom) of OVA dimer$^+$ CD8$^+$ T cells for the mice described in panel A. C. Percent IFN-γ producing CD8$^+$ splenocytes seven days after Lm-OVA ΔactA infection, and OVA$_{257–264}$ peptide stimulation (top) or no stimulation controls (bottom) for each group of mice treated with anti-PDL-1 or isotype (IgG2b) antibody one day prior to infection. D. Total number (top) and ratio (bottom) of IFN-γ producing CD8$^+$ T cells for the mice described in panel C. These data reflect 7-10 mice per group from three independent experiments each with similar results. Bar, one standard deviation. * $p <$ 0.05.

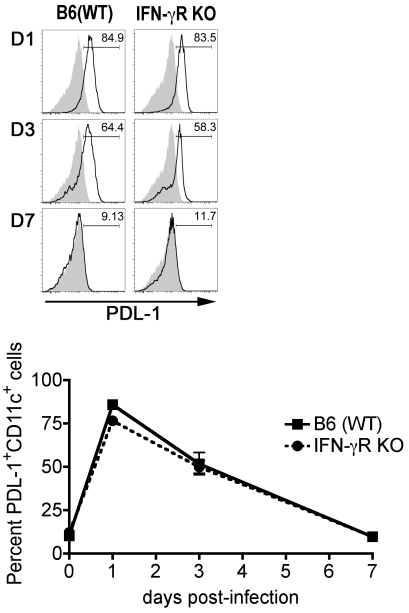

Figure 4.11 - IFN-γ receptor plays non-essential roles for infection-induced up-regulation of PDL-1 expression. Representative plots (top) and composite data (bottom) illustrating PDL-1 expression by $CD11c^+$ splenocytes for each group mice at the indicated time points after Lm-OVA ΔactA infection (line histogram) compared with uninfected controls (shaded histogram). These data reflect 6-8 mice per group from three independent experiments each with similar results. Bar, one standard deviation.

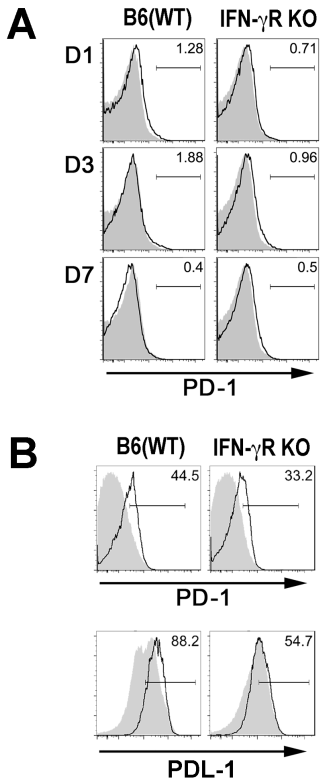

Figure 4.12 - Infection-induced PD-1 and PDL-1 expression among CD8$^+$ cells in IFN-γ receptor-deficient compared with B6 control mice. A. PD-1 expression by bulk CD8$^+$ splenocytes for each group of mice at the indicated time points after infection with 10^6 Lm-OVA ΔactA (line histogram) compared with uninfected controls (shaded histogram). B. PD-1 and PDL-1 expression by H-2K^b $OVA_{257–264}$ dimer$^+$ (line histogram) compared with bulk CD8$^+$ T cells (shaded histogram) for each group of mice seven days after Lm-OVA ΔactA infection. These data reflect 6-8 mice per group from three independent experiments each with similar results. Bar, one standard deviation.

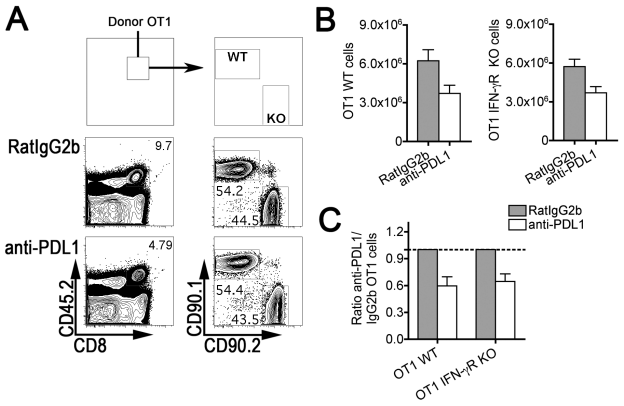

Figure 4.13 - T cell-intrinsic stimulation by IFN-γ is non-essential for PDL-1-mediated expansion of pathogen-specific CD8$^+$ T cells. A. Percent donor (CD45.2$^+$) CD8$^+$ T cells among WT (CD90.1$^+$) or IFN-γ receptor-deficient (CD90.2$^+$) OT-1 cells three days after Lm-OVA ΔactA infection, for cells adoptively transferred into CD45.1 recipient mice treated with either anti-PDL-1 or isotype control (IgG2b) antibody one day before infection. Total number (B) and ratio (C) of adoptively transferred OVA-specific CD8$^+$ T cells from WT or IFN-γ receptor-deficient OT-1 transgenic mice for the mice described in panel A. These data reflect 6 mice per group from three independent experiments each with similar results. Bar, one standard deviation.

Chapter 5

Foxp3^{+} regulatory T cell expansion required for sustaining pregnancy compromises host defense against prenatal bacterial pathogens[††]

[††] Reprinted from *Cell Host & Microbe*. Jared H. Rowe, James M. Ertelt, Marijo N. Aguilera, Michael A. Farrar, and Sing Sing Way. "Foxp3^{+} regulatory T cell expansion required for sustaining pregnancy comprimises host defense against prenatal bacterial pathogens."

SUMMARY

Although pregnancy confers unique susceptibility to infection, the pregnancy-associated immune defects that erode host defense remain largely undefined. Herein, we demonstrate the expansion of immune-suppressive Foxp3^{+} regulatory T cells (Tregs) that occurs physiologically during pregnancy or when experimentally induced in transgenic mice each caused enhanced susceptibility to prenatal pathogens including *Listeria* and *Salmonella* species. Reciprocally, infection susceptibility was uniformly reduced with Treg-ablation. Importantly however, the sustained expansion of maternal Tregs was essential for maintaining immune tolerance to the developing fetus because even partial transient ablation of Foxp3-expressing cells fractured maternal tolerance to fetal antigen and triggered fetal resorption. Interestingly, Foxp3 cell-intrinsic defects in the immune suppressive cytokine IL-10 alone were sufficient to override Treg-mediated infection susceptibility, while IL-10 was non-essential for sustaining pregnancy. Thus, maternal Treg expansion required for sustaining pregnancy creates naturally occurring holes in host defense that confers prenatal infection susceptibility.

INTRODUCTION

The pregnancy-associated susceptibility to intracellular pathogens has been classically attributed to a shift in helper T cell differentiation from a Th1 to Th2-dominated response required for maintaining pregnancy (Krishnan et al., 1996; Piccinni et al., 1998; Wegmann et al., 1993). However, the more recent identification other distinct $CD4^+$ T cell lineages has blurred the dichotomy between Th2 responses that sustain pregnancy, and Th1 cells that promote rejection of the developing fetus by activating cellular immunity (Saito et al., 2010). These include Th17 cells that provide protection against extracellular pathogens by promoting inflammation, and immune-suppressive regulatory T cells (Tregs) that maintain peripheral tolerance by restraining the activation of self-reactive immune cells (Curtis and Way, 2009; Littman and Rudensky, 2010). In parallel with the identification of these additional T cell subsets, the requirement for expanded immune tolerance during pregnancy has become more specifically linked with the expansion of maternal Tregs. For example, circulating maternal $Foxp3^+$ Tregs expand and peak mid-gestation to approximately 50% increased levels in human pregnancy (Santner-Nanan et al., 2009; Somerset et al., 2004). Reciprocally, defects in Treg expansion are associated with specific pregnancy complications such as preeclampsia or spontaneous abortion each related to maternal intolerance to the developing fetus (Prins et al., 2009; Santner-Nanan et al., 2009; Sasaki et al., 2004).

This association between maternal Tregs and pregnancy outcomes has also been

experimentally explored in mouse models where $CD25^+CD4^+$ cells are required for maintaining healthy pregnancy (Aluvihare et al., 2004; Kahn and Baltimore, 2010). However, since previously employed approaches that cause the near complete ablation of these cells do not allow the importance of expanded Tregs from steady state pre-pregnancy levels in sustaining pregnancy to be addressed, and CD25 expression does not discriminate between bona fide Tregs and activated T cells, the actual requirement for expanded maternal $Foxp3^+$ Tregs during pregnancy remains undefined. More importantly, given the fluid balance between immune suppression and stimulation controlled by Tregs, the expansion of $Foxp3^+$ cells during pregnancy may also create holes in host defense that confers susceptibility to pathogens with a predilection for prenatal infection. In this regard, while the effects of Treg ablation on infection susceptibility have been investigated in numerous models of parasitic, bacterial, fungal, and viral infection (Belkaid, 2007; Suvas and Rouse, 2006), the impacts on host defense resulting from the more physiological expansion of these cells remain undefined.

$Foxp3^+$ Tregs can use numerous molecules to mediate context specific immune suppression (Sakaguchi et al., 2009; Shevach, 2009; Vignali et al., 2008). For example, Tregs are enriched for CTLA-4 expression, and sustained CTLA-4 ablation in $Foxp3^+$ cells causes non-specific T cell activation and systemic autoimmunity (Friedline et al., 2009; Wing et al., 2008). Interestingly, while CTLA-4 ablation in $Foxp3^+$ cells reproduces some features of Treg deficiency, it does not recapitulate the more rapid onset of fatal systemic autoimmunity in mice with naturally-occurring or targeted defects in all

Tregs due to Foxp3 deficiency (Fontenot et al., 2003; Khattri et al., 2003). Comparatively, ablation of IL-10 in $Foxp3^+$ Tregs results in minimal systemic autoimmunity, but instead causes inflammation limited to sites with contact to the external environment (Rubtsov et al., 2008). This discordance in phenotype after sustained ablation of specific molecules in Foxp3-expressing cells illustrates unique, non-overlapping and specialized roles for individual Treg-expressed molecules. Accordingly, identifying how $Foxp3^+$ cells mediate suppression in each context opens up the exciting possibility of dissociating the beneficial and harmful impacts of Tregs. Specifically, given the parallels between maternal Treg expansion and healthy uncomplicated pregnancy, together with the detrimental role Tregs play in host defense against infection, dissociating the Treg-associated molecules required for each may unlock therapeutic approaches that boost immunity against infection without compromising immune tolerance required for sustaining pregnancy.

To address these questions, we established a pregnancy model using mice with divergent MHC that recapitulates the natural variation between maternal and fetal antigen, and progressive expansion of maternal $Foxp3^+$ Tregs during human pregnancy. Using this model, we demonstrate maternal Tregs impair host defense causing susceptibility to pathogens including *Listeria* and *Salmonella* species with a defined predilection for prenatal infection. In turn, the sustained expansion of maternal $Foxp3^+$ Tregs was also essential for maintaining healthy pregnancy because even partial transient ablation to levels found in non-pregnant controls triggered sharply increased rates of fetal resorption

and fractured tolerance to fetal antigen. Using mice containing Tregs with targeted defects in defined molecules implicated in suppression, we further demonstrate an essential role for Treg IL-10 in suppressing host defense against infection, while maternal IL-10 is non-essential for maintaining pregnancy. These results illustrate naturally occurring holes in host defense associated with physiological shifts in maternal Tregs, and identify Treg-associated molecules that dissociate the detrimental impacts of expanded Tregs on infection susceptibility from those required for sustaining pregnancy.

MATERIALS AND METHODS

Mice. B6, Balb/c, SJL CD45.1^{+}, IL-10-deficient (Kuhn et al., 1993), and IFN-γ receptor-deficient (Huang et al., 1993) mice were each purchased from The Jackson Laboratory. Foxp3^{DTR} and Foxp3^{GFP} mice were generously provided by Dr. Alexander Rudensky (Fontenot et al., 2005b; Kim et al., 2007). Foxp3^{DTR} mice were backcrossed > 15 generations to B6 mice, and intercrossed with CD45.1^{+} mice. Actin-OVA mice (Ehst et al., 2003) were backcrossed > 10 generations to Balb/c mice. OT-1 and OT-II TCR transgenic mice were maintained on a Rag1-deficient CD90.1 (B6) background. CA-STAT5b mice (Burchill et al., 2003) were intercrossed with Foxp3^{DTR} mice to generate CA-STAT5b Foxp3^{DTR} mice. CTLA-4-deficient mice have been described (Tivol et al., 1995; Waterhouse et al., 1995). Mice with combined defects in IL-10 and CTLA-4 were generated by intercrossing mice with individual defects (Figure S5). For Treg ablation, mice were treated with an initial dose of DT (25 μg/kg) followed by subsequent daily doses of 5 μg/kg. All experiments were performed using University of Minnesota IACUC approved protocols.

Antibodies and flow cytometry. Fluorophore-conjugated antibodies and other reagents for cell surface, intracellular, and intranuclear staining were purchased from eBioscience or BD-Biosciences. Intracellular cytokine staining was performed for bulk *ex vivo* splenocytes (containing OVA-specific CD8^{+} T cells and antigen presenting cells) after stimulation with OVA$_{257-264}$ peptide or no stimulation control in media containing

GolgiPlug (BD Biosciences). Antibodies for IL-10 receptor neutralization (1B1.3A) (Brooks et al., 2006; Ejrnaes et al., 2006) and rat IgG1 isotype control were purchased form BioXcell, and inoculated intraperitoneally (1 mg/mouse) into pregnant mice at mid-gestation.

Cell transfers. Purified $CD8^+$ T cells (10^5) from OT-I or $CD4^+$ T cells (10^6) from OT-II TCR transgenic mice were adoptively transferred into $Foxp3^{WT/WT}$, $Foxp3^{DTR/DTR}$, or $Foxp3^{DTR/WT}$ females impregnated with Actin-OVA or non-OVA-expressing Balb/c males at mid-gestation (E10.5) with the initiation of DT. To measure cytotoxicity, splenocytes from congenic ($CD45.2^+$) mice were stained with either high (1 μM) or low (50 nM) CFSE, pulsed with $OVA_{257-264}$ peptide (50 nM CFSE) or no peptide control (1 μM CFSE), mixed at a 1:1 ratio, adoptively transferred into pregnant mice four days after the initiation of DT at mid-gestation, and harvested 8 hours after transfer.

Listeria and Salmonella Infections. Lm strain 10403s (Portnoy et al., 1988) and ST strain SL1344 (Johanns et al., 2010a) were each grown to early log phase (OD_{600} 0.1) in brain heart infusion media at 37°C, washed and diluted with saline to 200 μl, and injected intravenously (5×10^3 CFUs for Lm, and 1×10^2 CFUs for ST). For enumerating susceptibility, the number of recoverable bacteria in the spleen or liver three days after infection were quantified by organ homogenization, and plating serial dilutions onto agar plates as described (Rowe et al., 2008). For enumerating fetal invasion, each placental-fetal unit was individually dissected, homogenized in saline, and cultured on agar plates.

Bone marrow chimera and Treg reconstitutions. Bone marrow cells were harvested from the tibias and femurs from donor mice, intravenously transferred into sub-lethally irradiated (725 rads) $Foxp3^{DTR}$ mice, and allowed to reconstitute for 8-10 weeks. Thereafter, mice were treated with DT for 10 days, then infected with Lm to evaluate infection susceptibility. For reconstituting $Foxp3^{+}$ cells in non-irradiated $Foxp3^{DTR}$ mice, donor Tregs from B6, CA-STAT5b, or IL-10-deficient mice were adoptively transferred into $Foxp3^{DTR}$ mice (5×10^5 donor $Foxp3^{+}$ $CD4^{+}$ cells per mouse) with the initiation of daily DT, and infected with Lm 15 days later.

Statistical analysis. The number and percent live pups, resorbed concepti, cell numbers, and recoverable log10 bacterial CFUs were first analyzed and found to be normally distributed. Thereafter, differences between each group were analyzed using the unpaired Student's t-test (Prism, GraphPad) with $p < 0.05$ taken as statistical significance.

RESULTS

Expanded maternal Foxp3$^+$ Tregs dictate infection susceptibility during pregnancy

To reproduce the heterogeneity between maternal and fetal antigens during human pregnancy, we established synchronized matings between MHC mis-matched strains of inbred mice (Balb/c H-2^d males with B6 H-2^b females) that more fully recapitulates the expanded repertoire of non-self fetal antigen encountered by the maternal immune system. In this model, Foxp3$^+$ Tregs expand in a synchronized fashion to ~50% increased levels by mid-gestation compared with non-pregnant controls or after syngeneic mating (Figure 5.1A). This magnitude of maternal Treg expansion is consistent with that reported in human and other models of mouse allogeneic pregnancy, and increased compared with syngeneic pregnancy where the only source of antigen-heterogeneity are those encoded by the Y-chromosome (Aluvihare et al., 2004; Santner-Nanan et al., 2009; Somerset et al., 2004). Interestingly, the expansion of maternal Tregs is not due to IFN-γ production against the allogeneic fetus because Foxp3$^+$ cell expansion is also primed to a similar magnitude in IFN-γ receptor-deficient mice (12.2±0.3% Foxp3$^+$ cells in non-pregnant compared with 17.0±0.6% at mid-gestation). These direct parallels between the magnitude of maternal Foxp3$^+$ cell expansion and the degree of heterogeneity between maternal and fetal antigen suggest expanded Tregs play an important role in maintaining immune tolerance to the developing fetus during pregnancy.

Given the pivotal role Foxp3^{+} Tregs play in controlling the balance between immune suppression that maintains peripheral tolerance and immune activation required for optimal host defense against infection (Belkaid, 2007; Suvas and Rouse, 2006), we speculated the expansion of immune suppressive Tregs during pregnancy may also confer susceptibility to infection. In turn, this host defense defect would likely be exploited by pathogens that have a defined predisposition for infection during pregnancy. Among human pathogens, the intracellular bacterium *Listeria monocytogenes* (Lm) has a striking predilection for disseminated infection in pregnant women (Gellin et al., 1991; Mylonakis et al., 2002). Consistent with these epidemiological features, pregnant mice at mid-gestation compared with non-pregnant controls were markedly more susceptible to Lm infection (Figure 5.1B). To investigate the contribution of maternal Tregs on infection susceptibility, female Foxp3^{DTR} mice on the B6 background were substituted for mating with Balb/c males. These transgenic mice co-express with Foxp3 the high-affinity human diphtheria toxin (DT) receptor allowing targeted ablation of maternal Foxp3-expressing cells with low-dose DT (Kim et al., 2007). We found the elimination of maternal Tregs beginning mid-gestation reversed the pregnancy-associated susceptibility to Lm infection (Figure 5.1B). This Treg-mediated defect in host defense was not limited only to Lm because susceptibility to *Salmonella typhimurium* (ST) during pregnancy was similarly reversed by the ablation of maternal Foxp3^{+} cells (Pejcic-Karapetrovic et al., 2007) (Figure 5.1C). Together, these results indicate maternal Foxp3^{+} Tregs contribute to pregnancy-associated infection susceptibility.

Foxp3$^+$ Tregs impair host defense against infection

Given the expansion of fetal tissue that occurs in parallel with maternal Foxp3$^+$ Tregs, infection susceptibility during pregnancy may also reflect more target tissue susceptible to direct invasion by *Listeria* and *Salmonella* species (Bakardjiev et al., 2006; Le Monnier et al., 2007; Pejcic-Karapetrovic et al., 2007). In agreement, we found Lm infection mid-gestation caused dose dependent increased rates of fetal invasion (Figure 5.2). Moreover, since the products of conception are not resorbed immediately after Foxp3$^+$ cell-ablation, the precise impacts of expanded Tregs on infection susceptibility cannot be investigated exclusively using pregnant mice. Accordingly, to more definitively interrogate the impacts of Foxp3$^+$ Tregs on host defense, we compared infection susceptibility of non-pregnant mice after Treg manipulation using complementary gain- and loss- of function approaches.

Foxp3 expression in T cells is stimulated by STAT5b promoter binding, and mice containing a constitutively active isoform of STAT5b (CA-STAT5b) have expanded Foxp3$^+$ Tregs (Burchill et al., 2003) (Figure 5.3, 5.4A). Consistent with the notion that expanded Tregs promote infection susceptibility, significantly more recoverable CFUs were found in CA-STAT5b compared with B6 control mice after Lm infection (Figure 5.4B). Since aberrations in other immune cells (e.g. CD8$^+$ T and CD19$^+$ B cells) in CA-STAT5b mice may also contribute to infection susceptibility, related experiments more specifically investigated the contribution of expanded Foxp3$^+$ Tregs in these mice. First, CA-STAT5b mice were intercrossed with Foxp3^{DTR} mice allowing for the ablation of

expanded Foxp3^{+} Tregs with DT. We found DT treatment in CA-STAT5b Foxp3^{DTR} mice efficiently ablated expanded Foxp3^{+} cells and restored resistance to levels comparable to Treg un-manipulated controls (Figure 5.4A,B). In turn, DT treatment in Foxp3^{DTR} compared with Foxp3^{WT} mice also caused significant reductions in recoverable Lm CFUs congruent with the effects of Treg-ablation on infection susceptibility for pregnant mice. As a complementary approach, we exploited the efficiency whereby adoptively transferred Tregs not susceptible to DT repopulates this ablated cell compartment by using Tregs from CA-STAT5b or control mice to reconstitute Treg-ablated Foxp3^{DTR} mice sustained on low-dose DT. We found Treg-ablated mice reconstituted with CA-STAT5b compared with WT Tregs contained ~2-fold increased Foxp3^{+} cells (23% compared with 11%) due to cell-intrinsic STAT5b stimulation that drives Foxp3^{+} cell expansion (Figure 5.C). Foxp3^{+} CD4^{+} cells in mice reconstituted with CA-STAT5b or WT Tregs were each > 99% donor derived, while < 1% Foxp3^{-} cells were donor derived based on expression of the CD45.2 congenic marker. After Lm infection, Treg-ablated Foxp3^{DTR} mice reconstituted with expanded CA-STAT5b compared with WT Tregs contained significantly more recoverable CFUs that paralleled the increased susceptibility of CA-STAT5b mice (Figure 5.4D). Thus, expanded Foxp3^{+} Tregs impair host defense and confer susceptibility to disseminated Lm infection.

Sustained expansion of maternal Foxp3^{+} Tregs is required for maintaining pregnancy

The infection susceptibility associated with the physiological expansion of maternal Foxp3^{+} cells during allogeneic pregnancy suggests the proposed role these cells play in facilitating antigenic diversity is more important for species survival outweighing transient defects in host defense against infection. In this regard, although the importance of maternal Tregs during pregnancy has been described using strategies that cause the near complete ablation of these cells based on CD25 expression (Aluvihare et al., 2004; Kahn and Baltimore, 2010), the specific requirement for maternal Foxp3^{+} cells expanded from steady state levels found in non-pregnant controls in maintaining pregnancy remains undefined. To investigate this question, we exploited the X-linked inheritance of *foxp3* where random chromosome inactivation in Foxp3$^{DTR/WT}$ heterozygous females results in distinct Foxp3^{DTR} and Foxp3^{WT} Treg subsets. We found the initiation of DT beginning mid-gestation in Foxp3$^{DTR/WT}$ mice caused more modest (~35%) reductions in Foxp3^{+} Tregs, compared with the expected 50%, that closely approximates the level found in non-pregnant controls (Figure 5.5A and 5.1A). Even with sustained DT, these reductions were transient as Foxp3^{+} cells rapidly rebounded to levels found in pregnant Foxp3$^{WT/WT}$ controls. These findings are each consistent with the continuous refilling of this partially depleted compartment with Foxp3^{WT} Tregs that are not susceptible to ablation in Foxp3$^{DTR/WT}$ mice (Kim et al., 2007). Interestingly, these partial transient reductions in maternal Tregs achieved with DT in Foxp3$^{DTR/WT}$ mice were nevertheless sufficient to trigger sharply increased rates of fetal resorption (10-fold, $p < 0.001$) with reciprocal reductions in the number of live pups born (70% reduction, $p < 0.001$) each compared with Treg-sufficient pregnancies (Figure 5.5B,C). By extension, DT treatment in

pregnant Foxp3$^{DTR/DTR}$ mice caused the sustained near-complete ablation of Tregs, and more profound rates of fetal resorption with reciprocal elimination of live pups born (Figure 5.5A-C).

Importantly, these detrimental pregnancy outcomes were not due to non-specific effects related to DT because each group of mice received the same dosing of this reagent beginning mid-gestation; nor were they caused by inherent defects in Foxp3$^{DTR/DTR}$ or Foxp3$^{DTR/WT}$ mice because without DT, the number of live pups born for each was indistinguishable from Treg-sufficient controls (Figure 5.5C). Furthermore, given the potential for DT-mediated ablation of fetal Foxp3^{+} cells in Foxp3^{DTR} pregnancies, we examined Foxp3 expression in E10.5 to E16.5 embryos using both anti-Foxp3 antibody and Foxp3^{GFP} reporter mice (Fontenot et al., 2005b). In each method, no detectable Foxp3 expression *in utero* above background levels was observed (Figure 5.6). The absence of *in utero* Foxp3 expression is consistent with the birth of males hemizygous for defects in Foxp3 at the expected Mendelian ratios (Brunkow et al., 2001), the ability of donor Tregs adoptively transferred after birth to rescue Foxp3-deficient mice from systemic autoimmunity (Fontenot et al., 2003), and the very minuscule levels of Foxp3 expression in one day old mice (Fontenot et al., 2005a). Moreover, the observed rates of *in utero* fetal resorption triggered by DT in Foxp3$^{DTR/WT}$ (57%) and Foxp3$^{DTR/DTR}$ (90%) pregnancies significantly exceed the expected percentage of Foxp3^{DTR} pups after allogeneic mating with Foxp3$^{WT/WT}$ males. Accordingly, the effects of DT treatment in Foxp3$^{DTR/WT}$ and Foxp3$^{DTR/DTR}$ pregnancies most likely reflect the manipulation of

maternal and not fetal Tregs. Since the majority of expanded Foxp3^{+} Tregs during pregnancy are also CD25^{+} (Figure 5.1A), these results are consistent with the importance of CD25^{+} CD4^{+} T cells in maintaining pregnancy (Aluvihare et al., 2004; Kahn and Baltimore, 2010). However, our use of reagents that manipulate Tregs based on Foxp3-expression and cause the partial ablation of these cells to pre-pregnancy levels more specifically establishes the importance of sustained maternal Treg expansion during pregnancy.

Expanded maternal Tregs sustain tolerance to fetal antigen

The requirement for sustained expansion of Foxp3^{+} cells during pregnancy suggests these cells provide uninterrupted active immune tolerance to the developing fetus. To investigate this hypothesis, the impacts of Treg ablation on the priming and activation of maternal T cells with specificity to the fetus were enumerated. To more precisely track the response to fetal antigen, we substituted mice where ovalbumin (OVA) is expressed in all cells on the Balb/c background for mating with non OVA-expressing females on the B6 background (Ehst et al., 2003). This strategy allows T cells among maternal immune cells with specificity to defined peptides within the surrogate fetal-(OVA) antigen to be tracked using well-characterized immunological tools (Erlebacher et al., 2007). We found Treg-ablation beginning mid-gestation triggered similar increased rates of fetal resorption in Foxp3$^{DTR/WT}$ (57%) and Foxp3$^{DTR/DTR}$ (96%) pregnancies that was associated with robust expansion of OVA-specific CD4^{+} and CD8^{+} T cells compared with Foxp3$^{WT/WT}$ pregnancies (Figure 5.7A). Importantly, the expansion of these OVA-specific T cells was

specific to the developing fetus and not caused by non-specific effects related to $Foxp3^+$ cell manipulation because only background levels were found in Treg-ablated females impregnated with non-OVA expressing males (Figure 5.8). Furthermore, maternal Tregs also suppress the activation of fetal-(OVA)-specific T cells because $CD8^+$ T cells from pregnancies with partial ($Foxp3^{DTR/WT}$) or near complete ($Foxp3^{DTR/DTR}$) Treg ablation produce significantly more IFN-γ after $OVA_{257-264}$ peptide stimulation, and eliminated adoptively transferred $OVA_{257-264}$ peptide-coated target cells more efficiently each compared with Treg-sufficient ($Foxp3^{WT/WT}$) pregnancies (Figure 5.7B,C). Thus, fetal resorption and fractured tolerance to fetal antigen triggered by even partial reductions in maternal $Foxp3^+$ Tregs in mice recapitulates human pregnancy complications related to fetal intolerance (e.g. spontaneous abortion, preeclampsia) each associated with blunted expansion of maternal Tregs (Prins et al., 2009; Santner-Nanan et al., 2009; Sasaki et al., 2004).

Treg cell-intrinsic IL-10 confers infection susceptibility

Given the divergent roles for expanded maternal Tregs that are on one hand required for sustaining pregnancy, but also impair host defense that confers infection susceptibility, we explored if targeted ablation of individual Treg-associated molecules would allow these protective and harmful effects to be dissociated. In this regard, although numerous Treg-associated molecules that can each mediate immune suppression *in vitro* have been identified (Sakaguchi et al., 2009; Shevach, 2009; Vignali et al., 2008), the relative importance for each in dictating the multi-faceted role $Foxp3^+$ cells play *in vivo* remain

incompletely defined. We focused initially on CTLA-4 and IL-10 because each play dominant roles for Treg suppression in other *in vivo* contexts (Asseman et al., 1999; Belkaid et al., 2002; Friedline et al., 2009; Rubtsov et al., 2008; Wing et al., 2008). To evaluate the requirement for Treg expression of each molecule, bone marrow cells from CTLA-4-deficient, IL-10-deficient or WT mice were adoptively transferred into sub-lethally irradiated Foxp3^{DTR} CD45.1^{+} recipient mice. After reconstitution and before DT treatment, these mixed chimera mice contain Tregs with targeted defects in either CTLA-4 or IL-10 each derived from donor bone marrow cells and WT Tregs from Foxp3^{DTR} recipient mice. However, after DT treatment, Tregs derived from recipient Foxp3^{DTR} mice are eliminated leaving behind only those derived from donor CD45.2^{+} bone marrow cells that rapidly expand to refill this partially ablated compartment (Figure 5.9A). Using this approach that allows the more synchronized ablation of each Treg-associated molecule, mice containing only CTLA-4-deficient compared with WT Tregs were found to have expanded Foxp3^{+} cells consistent with the role of CTLA-4 in limiting Treg proliferation (Kolar et al., 2009; Tang et al., 2008). In turn, mice with expanded CTLA-4 deficient compared with WT Tregs were also more susceptible to Lm infection (Figure 5.9B). Comparatively, while IL-10-deficient and WT Tregs each expand to a similar extent in irradiated Foxp3^{DTR} mice, those containing only IL-10-deficient Tregs compared with mice containing either WT or CTLA-4-deficient Tregs were markedly more resistant to Lm infection (Figure 5.9A,B).

To further investigate the requirement for IL-10 and CTLA-4 expression by Tregs in host defense against infection, mixed chimera mice containing $Foxp3^{+}$ cells with targeted defects in both molecules were generated by using bone marrow cells from mice with combined defects in both IL-10 and CTLA-4 (DKO) for reconstituting sub-lethally irradiated $Foxp3^{DTR}$ mice. Although the rate of recombination between these two genes separated by 39.8 centi-Morgan on chromosome 1 was somewhat lower than expected (Figure 5.10A), mice with defects in both IL-10 and CTLA-4 could be generated, and began to appear runty with a "scurfy" phenotype beginning 23 to 27 days of life that was indistinguishable from CTLA-4-deficient mice (Tivol et al., 1995; Waterhouse et al., 1995). After adoptive transfer into irradiated $Foxp3^{DTR}$ mice, bone marrow cells from these DKO mice led to expanded levels of $Foxp3^{+}$ Tregs comparable to reconstitution with bone marrow from CTLA-4-deficient mice (Figure 5.9A). However, despite the expansion of $Foxp3^{+}$ Tregs due to cell-intrinsic defects in CTLA-4, these mice were significantly more resistant to Lm infection with significantly reduced CFUs compared with mice containing CTLA-4-deficient (IL-10-sufficient) Tregs (Figure 5.9B). Importantly, these impacts on infection susceptibility are not explained by potential differences in non-specific immune activation because donor bone marrow derived Tregs from each group of mice compared with Treg-ablated mice without donor bone marrow suppressed activation (percent $CD44^{hi}CD62L^{lo}$) among bulk T cells to the same extent (Figure 5.10B). Together, these results indicate that while CTLA-4 controls host defense against Lm indirectly by limiting Treg-expansion, ablation of IL-10 overrides the

infection susceptibility associated with both expanded CTLA-4-deficient and normal levels of WT Tregs.

Related experiments sought to investigate whether resistance against Lm infection in mice containing exclusively IL-10-deficient Tregs could be attributed to the mixed population of WT and IL-10 deficient non-Treg cells derived from donor bone marrow in irradiated $Foxp3^{DTR}$ mice. Donor Tregs from IL-10-deficient or B6 control mice were used to repopulate non-irradiated Foxp3-cell ablated $Foxp3^{DTR}$ mice; and with sustained DT for seven to ten days, >99% of $Foxp3^{+}$ Tregs and <1% non-Tregs were donor derived based on CD45.2 expression (Figure 5.11A). After Lm infection, mice reconstituted with IL-10-deficient compared with WT Tregs were more resistant containing significantly reduced numbers of recoverable CFUs (Figure 5.11B). Similar to reconstituted Tregs in irradiated mice, adoptively transferred IL-10 and WT Tregs each suppressed non-specific T cell activation to a similar extent compared with Treg-ablated mice without donor cells (Figure 5.12). Taken together, these results demonstrate a critical role for $Foxp3^{+}$ Tregs and IL-10 production by these cells in suppressing host defense against Lm infection.

IL-10 is dispensable for sustaining pregnancy, but impairs host defense against prenatal infection

Given the requirement for maternal $Foxp3^{+}$ Tregs in sustaining pregnancy, we examined whether parallel requirements exist for maternal IL-10 by enumerating pregnancy outcomes in IL-10-deficient compared with B6 control mice after allogeneic mating with

Balb/c males. In sharp contrast to pregnancy loss that occurs with even partial transient Treg ablation, IL-10-deficient compared with IL-10-sufficient pregnancies had indistinguishable normal numbers of live pups born at term (Figure 5.13A). The non-essential role for maternal IL-10 in sustaining allogeneic pregnancy together with the importance of Treg IL-10 in promoting susceptibility to Lm infection suggest IL-10 neutralization may be used for boosting immunity against infection without compromising pregnancy outcomes. Consistent with this hypothesis, we found pregnant IL-10-deficient compared with pregnant B6 controls were significantly less susceptible to Lm infection (Figure 5.13B). These results are in agreement with the increased resistance against Lm for IL-10-deficient non-pregnant mice (Dai et al., 1997), and extend the importance of this cytokine to infection susceptibility during pregnancy. As a complementary approach, we enumerated the impacts of IL-10 receptor neutralization on pregnancy outcomes and resistance to Lm infection. Similar to findings using IL-10-deficient mice, anti-IL-10 receptor compared with isotype control antibody treatment at mid-gestation had no significant impacts on the number of live births after allogeneic pregnancy, but caused sharp reductions in the number of recoverable Lm CFUs after infection (Figure 5.13C,D). Together, these results establish that although IL-10 is dispensable for sustaining pregnancy, it impairs host defense against prenatal Lm infection.

DISCUSSION

Regulatory T cells control the fluid balance between immune suppression that maintains peripheral tolerance and immune stimulation required for optimal host defense against infection. Herein, we demonstrate pregnancy triggers the physiological expansion of $Foxp3^+$ Tregs that shifts this balance allowing maternal tolerance to the developing fetus to be maintained. Using tools that manipulate Tregs based on Foxp3 expression and cause the partial ablation of these cells, the blunted expansion of maternal Tregs observed in human pregnancy complications such as spontaneous abortion or preeclampsia where these cells are reduced to pre-pregnancy levels, but not eliminated is more closely recapitulated (Prins et al., 2009; Santner-Nanan et al., 2009; Sasaki et al., 2004). We find the sustained expansion of $Foxp3^+$ Tregs was imperative because even transient partial ablation to levels found in non-pregnant mice was sufficient to cause sharply increased rates of fetal resorption, reductions in the number of live pups born, and the expansion and activation of maternal T cells with specificity to the fetus. Given the parallel requirements for tryptophan catabolism through indoleamine 2,3-dioxygenase (IDO) in sustaining pregnancy, and the potency whereby Tregs stimulate tryptophan catabolism (Fallarino et al., 2003; Munn et al., 1998), blunted expansion or transient ablation of maternal Tregs may complicate pregnancy by disrupting this pathway. This is consistent with the up-regulation of IDO during uncomplicated human pregnancy compared with spontaneous abortion cases (Miwa et al., 2005). Our ongoing studies are aimed at dissecting the molecular basis for how maternal Tregs sustain pregnancy, and the

pregnancy-associated signals that drive the expansion of maternal Tregs. In this regard, while maternal Tregs suppress IFN-γ production by fetal-specific T cells and IFN-γ is a potent inducer of IDO expression (Taylor and Feng, 1991), IFN-γ responsiveness was non-essential for Treg expansion illustrating other unidentified signals stimulate shifts in maternal $Foxp3^+$ cells during allogeneic pregnancy.

The physiological expansion of Tregs during pregnancy and non-antigen-specific fashion whereby these cells mediate suppression suggest other detrimental or potentially protective immune responses may be suppressed as well. For example for patients with rheumatoid arthritis or autoimmune hepatitis, reduction in disease severity or disease remission occurs during pregnancy (Buchel et al., 2002; Ostensen and Villiger, 2007). On the other hand, given the intricately regulated balance between immune stimulation and suppression, sustained expansion of immune tolerance during pregnancy may also cause defects in host defense especially against pathogens such as *Listeria monocytogenes* and *Salmonella typhimurium* with a predilection for prenatal infection (Gellin et al., 1991; Mylonakis et al., 2002; Pejcic-Karapetrovic et al., 2007). Accordingly, we investigated the impacts of expanded Tregs on infection susceptibility in complementary *in vivo* models that include allogeneic pregnancy, CA-STAT5b transgenic mice, and mice with Treg-intrinsic defects in CTLA-4. In each condition where Tregs are expanded, increased infection susceptibility was uniformly identified. Furthermore, in each model of expanded Tregs where host defense against infection is impaired, secondary defects that include the ablation of expanded Tregs or specific Treg-intrinsic molecules were used to

confirm the overall importance of Tregs, or to identify Treg-associated molecules that mediate infection susceptibility. Although our experiments were not designed to evaluate the additional incremental impacts whereby direct invasion into the products of conception cause susceptibility to disseminated Lm infection (Bakardjiev et al., 2006; Le Monnier et al., 2007), the direct links between Tregs manipulated using these complementary gain- and loss-of function approaches in pregnant and non-pregnant mice on susceptibility to Lm infection establish that expanded Tregs required for sustaining pregnancy also impair host defense against this important prenatal pathogen. These findings suggest the expansion of maternal Tregs that facilitates increased genetic diversity between maternal and paternal antigen is more important for species survival outweighing transient defects in host defense.

The deleterious impacts of expanded Tregs on host defense against Lm is consistent with the accelerated pathogen eradication caused by ablation of $CD25^+$ or $Foxp3^+$ Tregs during infection with an increasingly wide assortment of other pathogens (Belkaid, 2007; Johanns et al., 2010a; Suvas and Rouse, 2006). However, while prior studies have primarily used loss-of-function approaches for investigating the role of Tregs in host defense, this study uses complementary approaches that mimic the physiological prenatal expansion of these cells to demonstrate how increased $Foxp3^+$ Tregs dictates infection susceptibility during pregnancy. An important area for future investigation is to explore if similar impacts on host defense are found after infection with other pathogens using gain-of-function approaches for $Foxp3^+$ Tregs. In this regard, although the number of

pathogens where Tregs have been implicated to suppress host defense far exceeds the limited number with defined predilection for prenatal infection, the results of these studies will likely have boarder implications for understanding infection susceptibility in aging and other contexts where the physiological expansion of Tregs also occurs (Gregg et al., 2005).

Numerous Foxp3$^+$ cell-associated molecules have been shown to mediate the suppressive properties of Tregs (Sakaguchi et al., 2009; Shevach, 2009; Vignali et al., 2008). Recently, intercrossing mice where the Cre-recombinase and *foxp3* are co-expressed with other mice where each Treg-associated molecule of interest is flanked by loxP sites have revealed unique and discordant roles for defined Treg-associated molecules in controlling specific aspects of peripheral tolerance (Rubtsov et al., 2008; Wing et al., 2008). Although ideal for identifying Treg-associated molecules that with sustained ablation cause autoimmunity, this strategy that results in the ablation of each molecule in Foxp3$^+$ cells throughout development is not suitable for interrogating their role in host defense against infection where the synchronized ablation of each molecule in Tregs is required. Accordingly, for interrogating the relative importance of Treg-associated molecules in controlling host defense against Lm infection, we exploited the efficiency whereby donor Tregs from mice with targeted defects in each Treg-associated suppression molecule expands to fill the partially ablated cellular compartment in mixed chimera mice, or completely empty compartment in Treg-ablated mice. These approaches that allow more synchronized ablation of specific Treg-associated molecules in Foxp3$^+$ cells identified

important roles for Treg IL-10 in suppressing host defense against Lm infection. Interestingly however, elimination of Treg IL-10 in neither non-irradiated Treg-reconstituted mice nor irradiated mice containing expanded CTLA-4-deficient Tregs restored resistance to levels found in Treg-ablated mice. These findings indicate other Treg-associated molecules likely play functionally redundant roles and/or act synergistically with IL-10 in impairing host defense against Lm infection.

The importance of IL-10 in suppressing immunity against Lm infection during pregnancy is in agreement with the previously reported importance of this cytokine in promoting susceptibility to other pathogens (Belkaid et al., 2002; Brooks et al., 2006; Ejrnaes et al., 2006). Together with controlling inflammation at mucosal surfaces (Asseman et al., 1999; Rubtsov et al., 2008), our results extend the context specific role of Treg IL-10 to include suppressing host defense components after acute pathogenic infection during pregnancy. Given the non-essential roles for maternal IL-10 in maintaining pregnancy even after allogeneic mating, these results demonstrate IL-10 can dissociate the detrimental impacts of expanded Tregs on infection susceptibility from the beneficial effects required for sustaining pregnancy. Accordingly, it is tantalizing to consider that the benefits of transiently neutralizing IL-10 for boosting host defense during complicated prenatal infections would not be harmful for pregnancy, and may therefore outweigh the potentially detrimental impacts on peripheral tolerance.

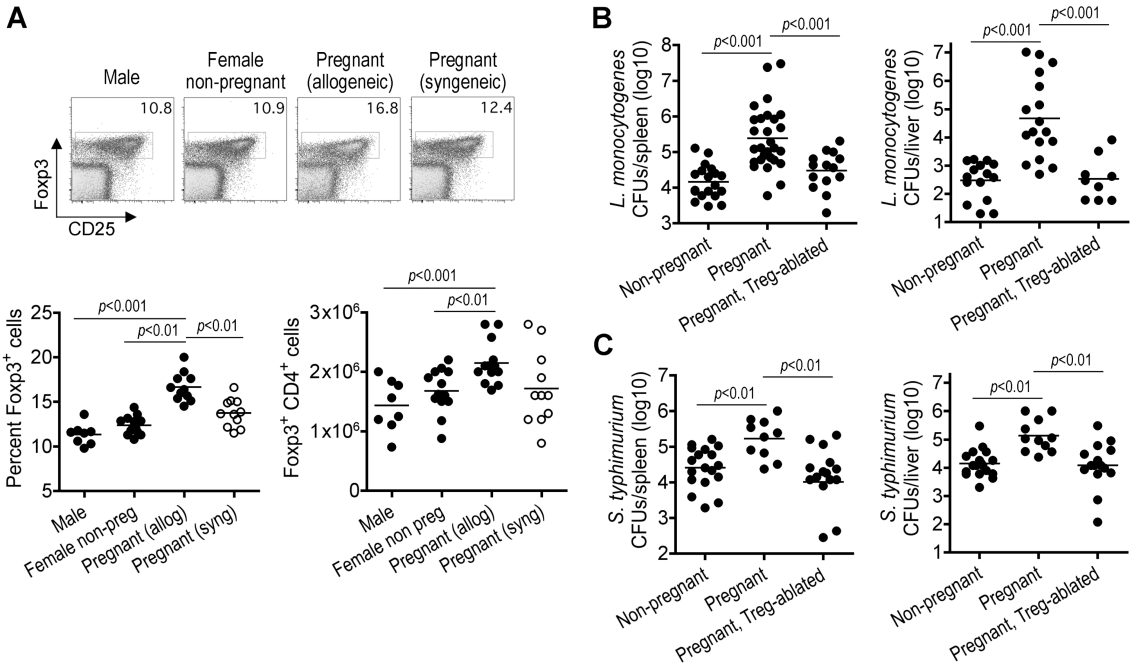

Figure 5.1 - Expanded maternal Foxp3$^+$ Tregs dictate infection susceptibility during pregnancy. (A) Percent and total number Foxp3$^+$ among CD4$^+$ splenocytes in male, virgin female, or pregnant B6 (H-2^b) females at mid-gestation after allogeneic mating with Balb/c (H-2^d) or syngeneic mating with B6 males. (B, C) Recoverable CFUs three days after infection in non-pregnant, pregnant Treg-sufficient, or pregnant Treg-abated Foxp3$^{DTR/DTR}$ females after allogeneic mating, and initiated on DT beginning mid-gestation and infected with *Listeria monocytogenes* (B) or *Salmonella typhimurium* (C) one day later.

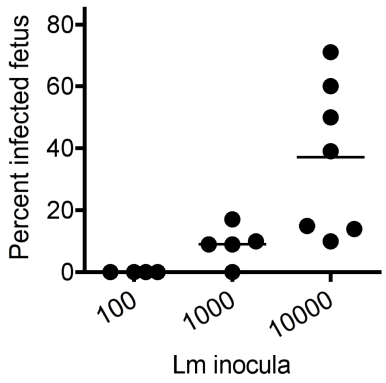

Figure 5.2 - Dose-dependent rates of *in-utero* fetal invasion after virulent Lm infection during allogeneic pregnancy. Percent fetus with recoverable Lm five days after infection with escalating inocula beginning mid-gestation (E10.5) for B6 females after mating with Balb/c males.

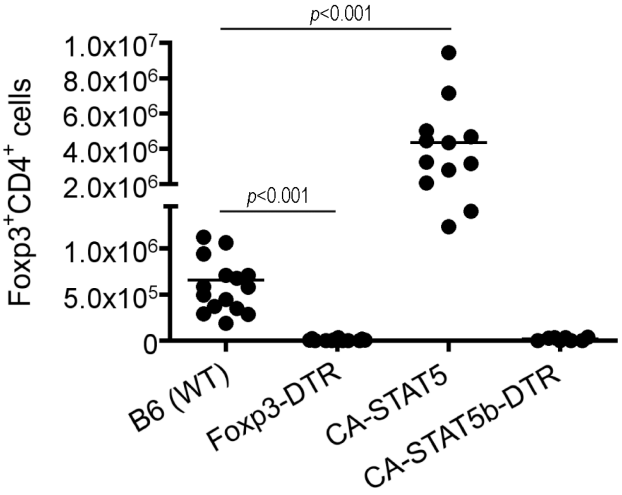

Figure 5.3 - Number of Foxp3^{+} Tregs among splenocytes in B6 (WT), Foxp3^{DTR}, CA-STAT5b, and CA-STAT5b Foxp3^{DTR} mice one day after DT treatment. Each symbol represents an individual mouse for results combined from three independent experiments.

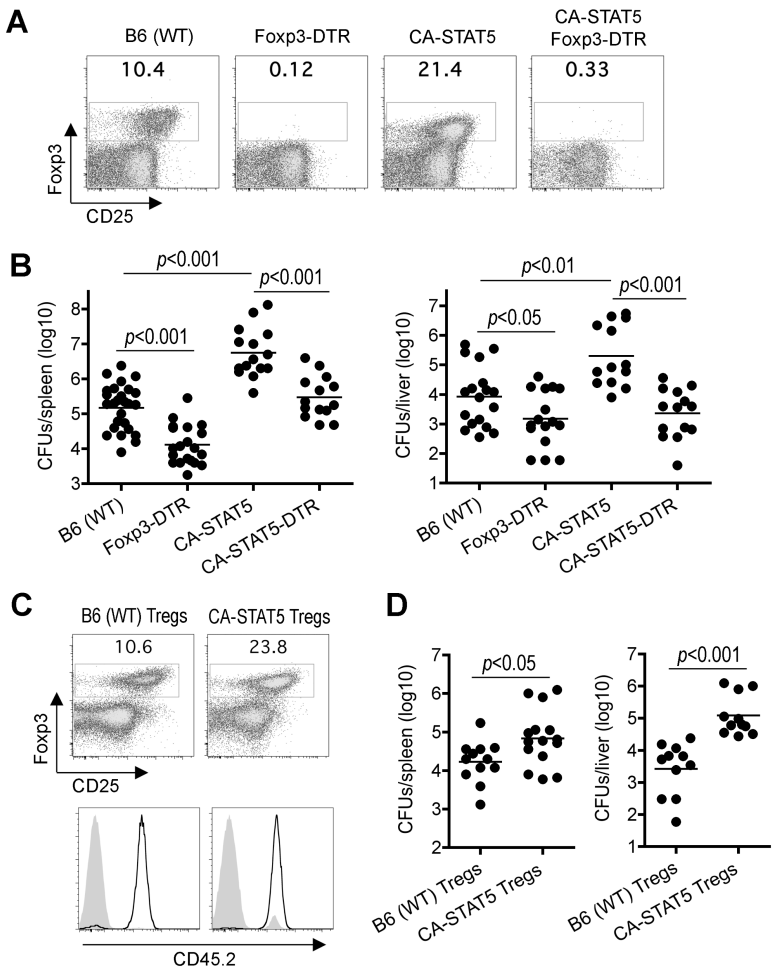

Figure 5.4 - Expanded Foxp3$^+$ Tregs impair host defense against Lm infection. (A) Percent Foxp3$^+$ among CD4$^+$ splenocytes after DT treatment in B6 (WT), Foxp3^{DTR}, CA-STAT5b, or CA-STAT5b Foxp3^{DTR} mice. (B) Recoverable CFUs three days after Lm infection for each group of mice initiated on DT treatment one day prior to infection. (C) Percent Foxp3$^+$ Tregs day 14 after the initiation of sustained DT in Foxp3^{DTR} CD45.1$^+$ mice reconstituted with donor Tregs (CD45.2$^+$) from WT or CA-STAT5b mice (top). CD45.2 expression by Foxp3$^+$ (line) or Foxp3$^-$ (shaded) CD4$^+$ T cells (bottom). (D) Recoverable CFUs three days after Lm infection for the mice described in (C).

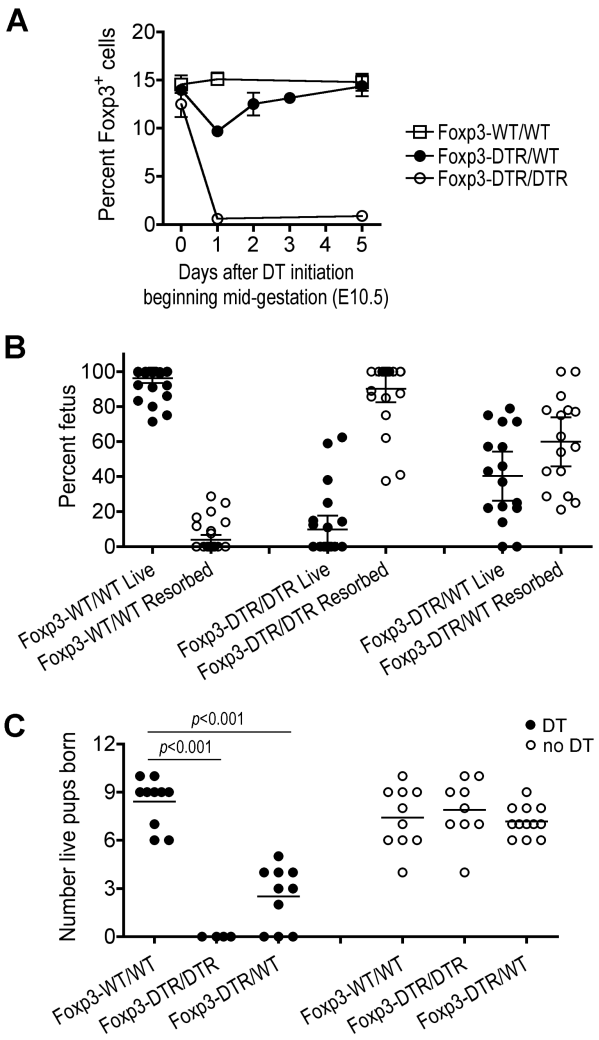

Figure 5.5 - Sustained expansion of maternal Foxp3$^+$ Tregs is required for maintaining pregnancy. (A) Percent Foxp3$^+$ among CD4$^+$ splenocytes during allogeneic pregnancy in Foxp3$^{WT/WT}$, Foxp3$^{DTR/WT}$, or Foxp3$^{DTR/DTR}$ mice after the initiation of DT treatment beginning mid-gestation (E10.5). Bar, one standard error. (B) Percent live or resorbed fetuses among total placental-fetus in individual pregnancies for each group of mice day 5 after the initiation of DT beginning mid-gestation. Bar, mean and 95% confidence interval. (C) Number of live pups born after allogeneic mating for each group of female mice either treated with DT for four consecutive days beginning mid-gestation (filled) or without DT treatment (open).

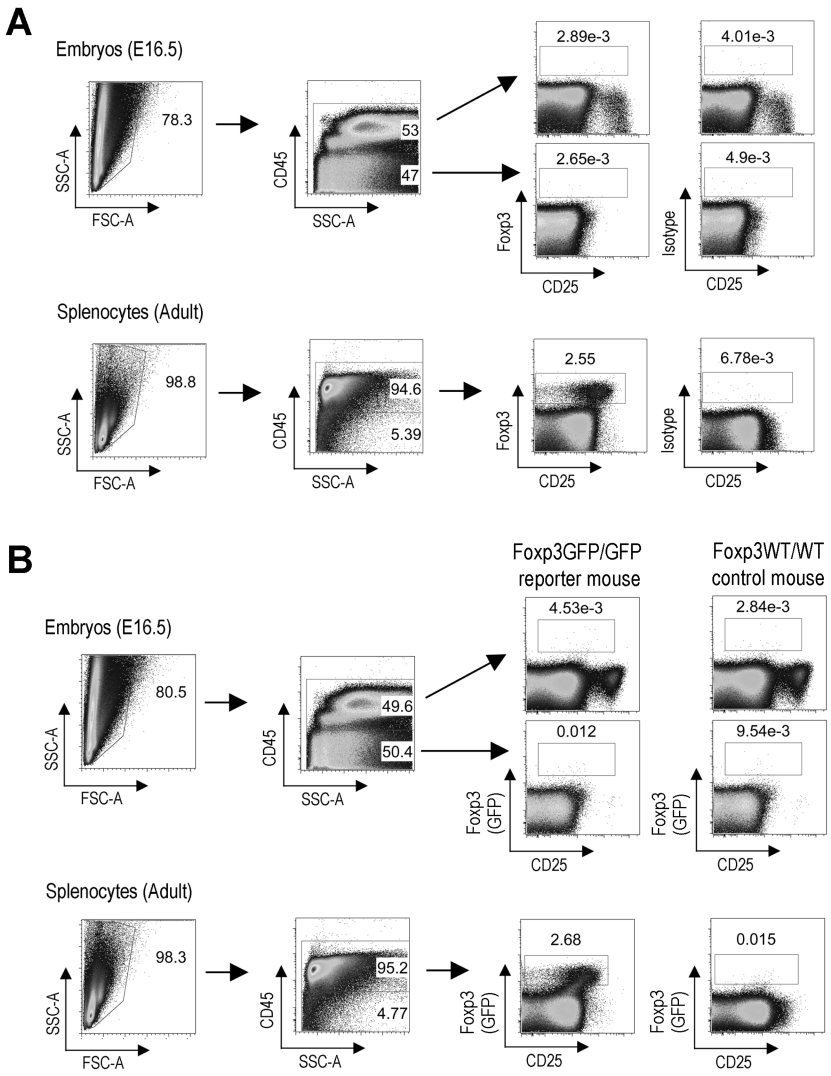

Figure 5.6 - Lack of Foxp3-expression among E16.5 mouse embryos. FACS plots illustrate the gating strategy used to analyze nucleated cells from E16.5 embryos or splenocytes from adult mice. First based on expression of the hematopoietic marker (CD45), and then Foxp3-expression after staining with anti-Foxp3 antibody compared with isotpye (rat IgG2a) control (A) or using Foxp3^{GFP} reporter compared with Foxp3^{WT} control mice (B). For staining, mouse embryos were individually dissected, digested with collagenase (2.5 mg/ml, 40 minutes 37ºC), and individual cells separated through a Percoll gradient (mononuclear cells isolated between 40 and 60%). This analysis depicts 10^6 events per sample representative of 6-8 mouse embryos from three separate experiments.

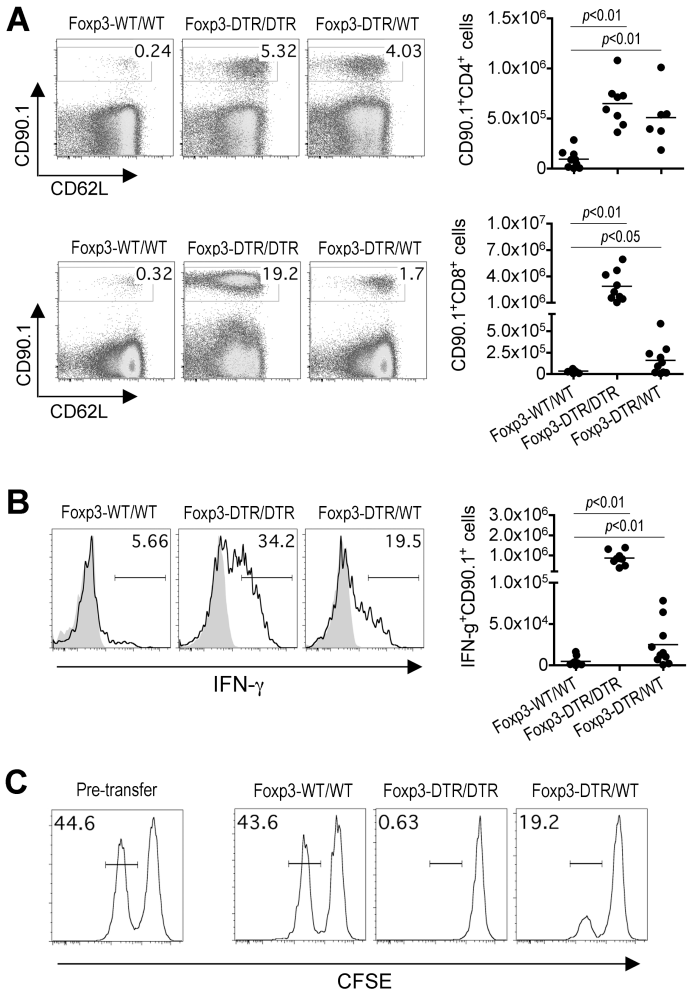

Figure 5.7 - Expanded maternal Tregs sustain tolerance to fetal antigen. (A) Percent and number of adoptively transferred CD90.1$^+$ fetal ($OVA_{323-339}$)-specific CD4$^+$ (top) or ($OVA_{257-264}$)-specific CD8$^+$ (bottom) cells among splenocytes in Foxp3$^{WT/WT}$, Foxp3$^{DTR/DTR}$, or Foxp3$^{DTR/WT}$ females impregnated by Actin-OVA transgenic males five days after the initiation of DT beginning mid-gestation (E10.5). (B) Percent and number of IFN-γ-producing CD90.1$^+$ CD8$^+$ T cells after *in vitro* $OVA_{257-264}$ peptide stimulation (line) or un-stimulated controls (shaded). (C) Percent $OVA_{257-264}$ peptide-pulsed (CFSE^{lo}) relative to untreated control cells (CFSE^{hi}) pre-transfer, or after adoptive transfer into each group of females impregnated by Actin-OVA males initiated on DT treatment beginning mid-gestation.

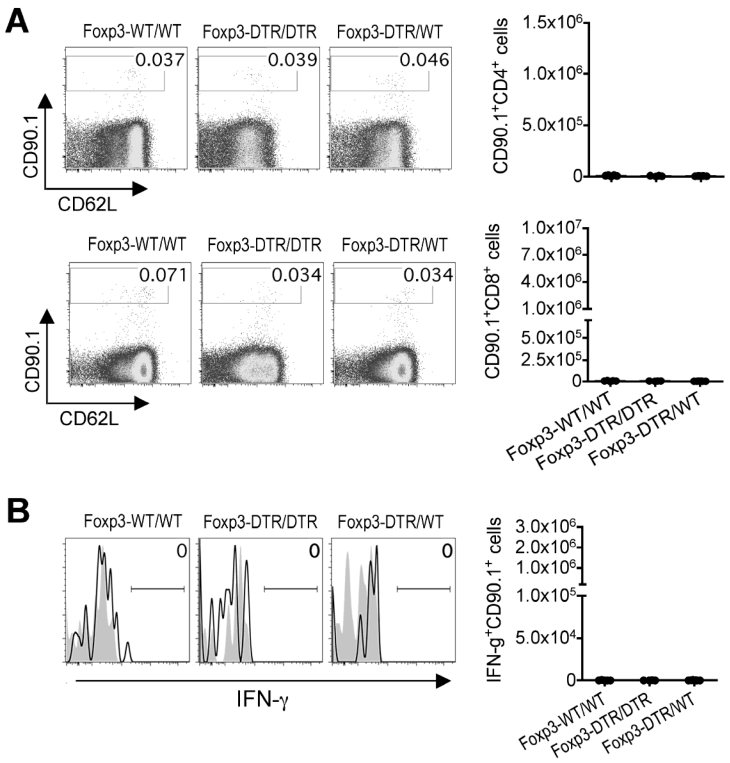

Figure 5.8 - Expansion of OVA-specific CD4$^+$ and CD8$^+$ T cells during pregnancy requires fetal-(OVA) antigen. (A) Percent and number of adoptively transferred CD90.1$^+$ fetal ($OVA_{323-339}$)-specific CD4$^+$ (top) or ($OVA_{257-264}$)-specific CD8$^+$ (bottom) cells among splenocytes in Foxp3$^{WT/WT}$, Foxp3$^{DTR/DTR}$, or Foxp3$^{DTR/WT}$ mice impregnated by non-OVA expressing Balb/c males five days after the initiation of DT treatment beginning mid-gestation (E10.5). (B) Percent and number of IFN-γ-producing CD90.1$^+$ CD8$^+$ T cells after $OVA_{257-264}$ peptide stimulation (line) or un-stimulated controls (shaded) for cells recovered from each group of mice.

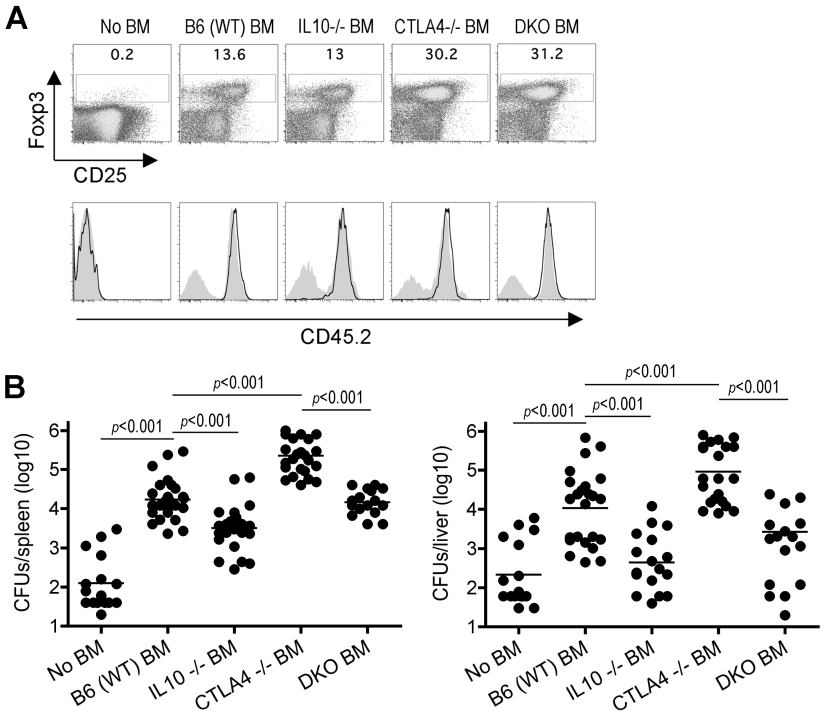

Figure 5.9 - Treg-mediated infection susceptibility requires cell-intrinsic IL-10. (A) Percent Foxp3^{+} among CD4^{+} splenocytes day 10 after the initiation of sustained DT in irradiated Foxp3^{DTR} CD45.1^{+} mice without donor bone marrow, or reconstituted with bone marrow from WT, IL-10-deficient, CTLA-4-deficient mice, or mice with combined defects in both IL-10 and CTLA-4 (DKO) (top). CD45.2 expression by Foxp3^{+} (line) or Foxp3^{-} (shaded) CD4^{+} cells (bottom). (B) Recoverable CFUs three days after Lm infection for the mice described in (A).

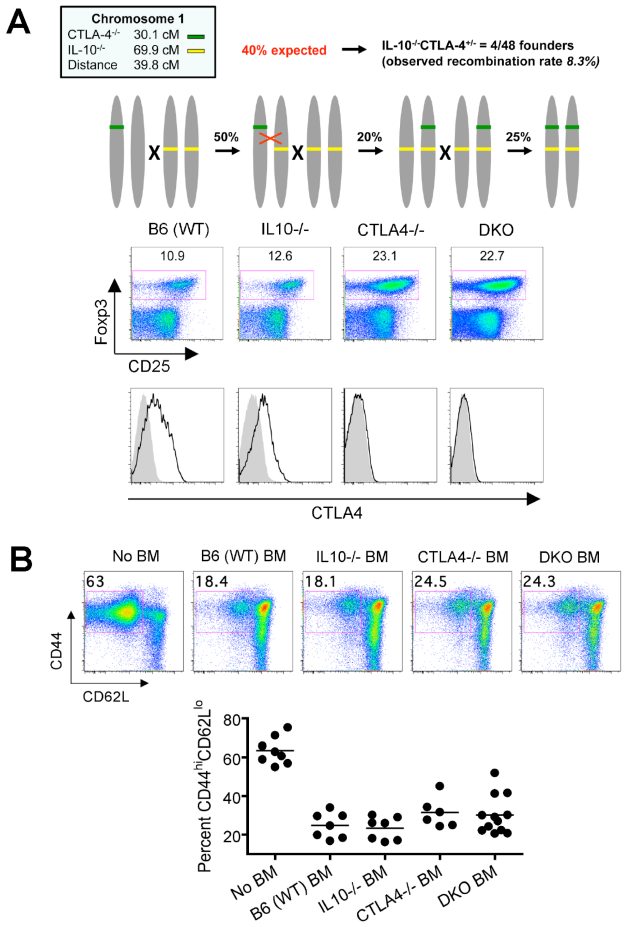

Figure 5.10 - Foxp3^{+} cells derived from donor bone marrow suppress non-specific T cell activation in Treg-ablated mice. A. Generation of mice with combined deficiency in both IL-10 and CTLA-4. Mating strategy demonstrating expected versus observed frequencies of homologous recombination between the genes encoding IL-10 and CTLA-4 on mouse chromosome 1 (top). FACS plots indicate percent Foxp3^{+} among CD4^{+} splenocytes from B6 (WT), IL-10-deficient, CTLA-4-deficient, and mice with combined defects in both IL-10 and CTLA-4 (DKO). CTLA-4 expression in Foxp3^{+} (line) and Foxp3^{-} CD4^{+} cells (shaded) for each mouse strain (bottom). B. Percent activated (CD44^{hi} CD62L^{lo}) CD8^{+} T cells in sub-lethally irradiated Foxp3^{DTR} mice reconstituted with bone marrow cells from B6 (WT), IL-10-deficient, CTLA-4-deficient mice, or mice with targeted defects in both IL-10 and CTLA-4 (DKO), or no donor bone marrow each with sustained DT treatment for 12 days.

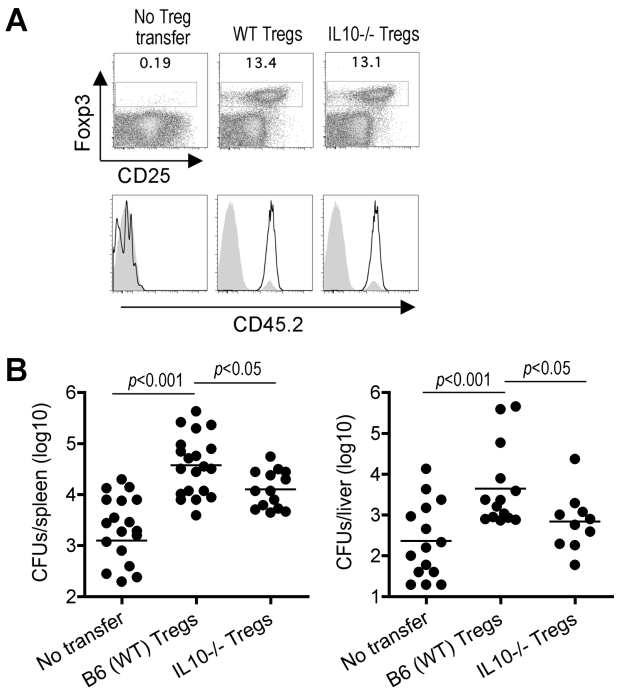

Figure 5.11 - Treg IL-10 impairs resistance against Lm infection. (A) Percent Foxp3^{+} among CD4^{+} splenocytes day 12 after the initiation of sustained DT in non-irradiated Foxp3^{DTR} CD45.1^{+} mice without donor Tregs, or reconstituted with donor Tregs from WT or IL-10-deficient mice (top). CD45.2 expression by Foxp3^{+} (line) or Foxp3^{-} (shaded) CD4^{+} cells (bottom). (B) Recoverable CFUs three days after Lm infection for the mice described in (A).

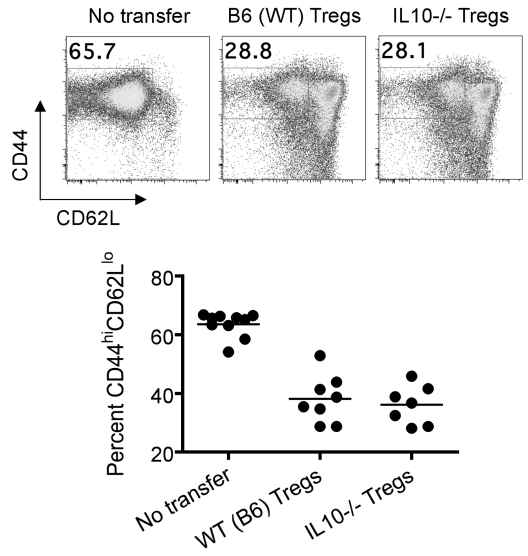

Figure 5.12 - Adoptively transferred Tregs suppress non-specific T cell activation in Treg-ablated mice. FACS plots (top) and composite analysis (bottom) illustrating percent activated ($CD44^{hi}$ $CD62L^{lo}$) $CD8^{+}$ T cells in $Foxp3^{DTR}$ mice with sustained DT treatment for 12 days without donor Tregs (no transfer) or reconstituted Tregs from B6 (WT) or IL-10-deficient mice.

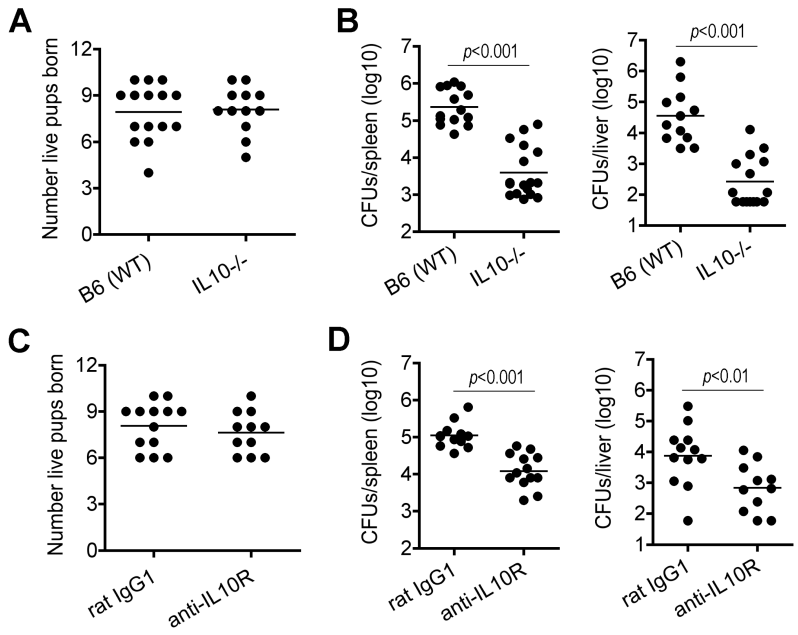

Figure 5.13 - Maternal IL-10 is dispensable for sustaining pregnancy, but impairs host defense against prenatal Lm infection. (A) Number of live pups born for IL-10-deficient or B6 (WT) mice after allogeneic mating with Balb/c males. (B) Recoverable CFUs three days after Lm infection at mid-gestation (E10.5) for IL-10-deficient or B6 (WT) pregnant mice. (C) Number of live pups born for B6 mice after allogeneic mating with Balb/c males treated with anti-IL-10 receptor or isotype control (rat IgG1) antibody beginning mid-gestation. (D) Recoverable CFUs in pregnant B6 mice three days after Lm infection and treatment with anti-IL-10 receptor or isotype control antibody at mid-gestation.

Chapter 6

Listeria monocytogenes cytoplasmic entry induces fetal wastage by disrupting maternal Foxp3^{+} regulatory T cell-sustained fetal tolerance

SUMMARY

Although the intracellular bacterium *Listeria monocytogenes* has an established predilection for disseminated infection during pregnancy that often results in spontaneous abortion or stillbirth, the specific host-pathogen interaction that dictate these disastrous complications remain poorly defined. Herein, we demonstrate systemic maternal *Listeria* infection during pregnancy fractures fetal tolerance and triggers fetal wastage in a dose-dependent fashion. *Listeria* was recovered from the majority of concepti after high-dose infection illustrating the potential for *in utero* invasion. Interestingly with reduced inocula, fetal wastage occurred without *in utero* invasion, and instead paralleled reductions in maternal $Foxp3^+$ regulatory T cell suppression with reciprocal expansion and activation of effector T cells with fetal-specificity. Using mutants lacking virulence determinants required for *in utero* invasion, we establish *Listeria* cytoplasmic entry is essential for disrupting fetal tolerance that triggers maternal T cell-mediated fetal resorption. Thus, infection-induced reductions in maternal $Foxp3^+$ regulatory T cell suppression with ensuing disruptions in fetal tolerance play critical roles in pathogenesis of immune-mediated fetal wastage.

INTRODUCTION

Listeria monocytogenes (Lm) is a ubiquitous human pathogen with a unique predisposition for invasive infection during pregnancy that represents an important cause of spontaneous abortion, stillbirth, and neonatal infection (Gellin and Broome, 1989; Schuchat et al., 1991). Although many Lm-specific proteins required for cell entry and maintaining residence within infected cells have been identified, and some play important roles in placental cell invasion (Cossart, 2011; Portnoy et al., 2002), the interplay between Lm and maternal immune cells that sustain fetal tolerance in the pathogenesis of infection-induced fetal injury has not been well-characterized. Recently, the physiological accumulation of immune suppressive maternal $Foxp3^{+}$ regulatory T cells (Tregs) during gestation was shown to compromise host defense against Lm and other pathogens that cause prenatal infection (Rowe et al., 2011). Nevertheless, despite increased infection susceptibility, the sustained expansion of maternal Tregs was more essential because even transient partial ablation to baseline levels was sufficient to disrupt fetal tolerance and trigger fetal resorption. These findings in mice recapitulate the blunted expansion of maternal Tregs with spontaneous abortion and other complications associated with fractured fetal tolerance in human pregnancy (Guerin et al., 2009; Santner-Nanan et al., 2009; Sasaki et al., 2007; Sasaki et al., 2004; Yang et al., 2008). Thus, healthy pregnancy requires the sustained accumulation of immune-suppressive maternal Tregs that maintains tolerance to the developing fetus.

In addition to these quantitative changes, fluctuations in Treg suppressive potency also occur. These shifts fine-tune the delicate fluid balance between immune stimulation and suppression. In particular within the first few days after infection in non-pregnant mice with Lm or other pathogens that primarily cause acute infection, progressive reductions in either Treg number or their suppressive potency have each been described (Ertelt et al., 2011b; Kastenmuller et al., 2011b). In turn, infection-induced reductions in Treg suppression unleash the activation of immune effectors that efficiently eradicate infection (Rowe et al., 2012). Importantly however, how pregnancy-expanded Tregs impact infection-induced shifts in suppression, and reciprocally how infection-induced shifts in maternal Treg suppression impact fetal tolerance are each undefined. Given the substantial overlap in pregnancy complications (e.g. spontaneous abortion, stillbirth) associated with prenatal Lm infection and defects in fetal tolerance induced by experimental or naturally-occurring defects in maternal Treg accumulation, we sought to investigate if infection-induced reductions in maternal Treg suppression might disrupt fetal tolerance and cause fetal wastage. Immune-mediated fetal injury that may occur without *in utero* pathogen invasion could explain the only modest fraction of concepti with recoverable Lm born to mothers with invasive infection (Mylonakis et al., 2002), and spur new approaches for improving pregnancy outcomes.

To address these questions, we investigated maternal Tregs and the maintenance of fetal tolerance using escalating dosages of virulent Lm for infection during pregnancy. Foxp3^{GFP} reporter mice allowed maternal Tregs to be purified based on their lineage-

defining marker (Fontenot et al., 2005b), and a mating strategy using ovalbumin (OVA)-expressing males allowed the maternal response to this surrogate fetal antigen to be precisely characterized (Ehst et al., 2003; Erlebacher et al., 2007; Rowe et al., 2011). To more specifically evaluate the contribution of immune-mediated fetal wastage in isolation, pregnancy outcomes, maternal Tregs, and fetal tolerance were also compared using Lm mutants lacking defined virulence determinants required for *in utero* invasion. Together, these experiments demonstrate Lm entry into the cell cytoplasm disrupts fetal tolerance sustained by maternal $Foxp3^{+}$ Tregs that triggers immune-mediated fetal wastage.

MATERIALS AND METHODS

Ethics statement. All experiments involving the use of animals were performed using University of Minnesota IACUC-approved protocols.

Mice. C57BL/6 (B6, H-2^b), Balb/c (H-2^d), and B6.PL-*Thy1* (CD90.1) mice were purchased from The National Cancer Institute or The Jackson Laboratory. $Foxp3^{GFP}$ mice backcrossed to B6 mice, OVA-expressing backcrossed to Balb/c mice, and OT-I TCR transgenic mice maintained on a CD90.1 background have been described (Ehst et al., 2003; Erlebacher et al., 2007; Fontenot et al., 2005b; Hogquist et al., 1994). The timing of pregnancy was determined by visualization of a copulation plug (embryonic day 0.5) after introducing virgin female with male mice.

Listeria Infections. Lm strains 10403s (WT), 1942 (ΔactA), and 2319 (ΔLLOΔPLC) were each grown to early log phase (OD_{600} 0.1) in brain heart infusion media at 37 °C, washed and diluted with saline to 200 µl, and injected intravenously via the lateral tail vein (Curtis et al., 2010; O'Riordan et al., 2002; Orgun and Way, 2008). The inoculum was verified for each infection by plating serial dilutions onto agar plates. For enumerating invasion, each placental-fetal unit or the liver was individually dissected, homogenized in saline containing 0.05% Triton X and cultured onto agar plates as described (Rowe et al., 2011).

Antibodies, Flow Cytometry, and Cell Transfer. Fluorophore-conjugated antibodies and other reagents for cell surface, intracellular cytokine, and intranuclear Foxp3 staining were purchased from eBioscience or BD Biosciences. Cytokine production was evaluated for OVA-specific CD8 T cells after stimulating splenocytes with $OVA_{257-264}$ peptide in media containing GolgiPlug (BD Biosciences). For cell transfers, purified $CD8^+$ cells (10^5) isolated from OT-I TCR transgenic (CD90.1) mice were injected intravenously into recipient (CD90.2) mice one day prior to Lm infection at midgestation as described (Rowe et al., 2011). For T cell depletion, purified anti-mouse CD4 (GK1.5) and anti-mouse CD8 (2.43) antibodies (BioXcell) were administered intraperitoneally (500 μg each antibody per mouse) one day prior to LmΔactA infection at midgestation.

Treg suppression assay. For enumerating Treg suppressive potency, CD4 cells were first enriched by negative selection (Miltenyi Biotec) from $Foxp3^{GFP}$ reporter mice, followed by sorting for the GFP^+ CD4 subset (Fontenot et al., 2005b). In each experiment, GFP^+ Tregs were verified to be >98% pure by staining for Foxp3 expression. Responder $CD8^+$ T cells isolated from naïve CD90.1 mice were labeled with CFSE (5 μM for 10 min at room temperature), and co-cultured in triplicate in 96-well round bottom plates (1×10^4 responder cells per well) with purified GFP^+ Tregs at the indicated ratios. The relative suppressive potency of Tregs in each experiment was calculated by comparing responder cell proliferation (CFSE dilution) after co-culture with GFP^+ Tregs from uninfected control mice as described (Ertelt et al., 2011b; Johanns et al., 2010a).

Statistical Analysis. The number of live pups, resorbed concepti, cell numbers, and percent cytokine producing cells were first analyzed and found to be normally distributed. Thereafter, differences between groups were analyzed using an unpaired Student's t test (Prism, Graph Pad) with $p < 0.05$ taken as statistical significance.

RESULTS

Listeria infection induces dose-dependent fetal resorption

To investigate the pathogenesis of prenatal listeriosis, pregnancy outcomes after infection with escalating dosages of virulent Lm beginning midgestation were evaluated. In this regard, although Lm has been described to stimulate fetal resorption and *in utero* invasion during syngeneic pregnancy (Barber et al., 2005; Krishnan et al., 2010; Le Monnier et al., 2007; Poulsen et al., 2011), this mating scheme does not recapitulate the natural heterogeneity between maternal and paternal antigens, and more pronounced expansion of maternal Tregs (Aluvihare et al., 2004; Kahn and Baltimore, 2010; Rowe et al., 2011). To bypass these limitations, pregnancy outcomes using MHC mismatched strains of inbred mice (Balb/c H-2^d males with B6 H-2^b females) were enumerated. We found Lm infection midgestation (E10.5) caused dose-dependent reductions in the number of live pups born at term ~10 days thereafter (Figure 1A). Compared with uninfected pregnancies (8.0 ± 0.6 live pups), the number of live pups was reduced by 54% and 88% [3.7 ± 0.9 (10^3 CFUs); 1.0 ± 0.5 (10^4 CFUs)] for mice infected with each respective Lm dosage (Figure 6.1A). Furthermore, no live pups were born for the majority of pregnant mice (9 of 13) infected with 10^4 Lm. Thus, Lm infection during allogeneic pregnancy triggers fetal loss.

To more comprehensively evaluate infection-induced fetal injury, the rates of *in utero* fetal resorption in pregnant mice at an earlier time point after infection (day 5 post-

infection, E15.5) were enumerated. Consistent with dose-dependent reductions in number of live pups born at term, progressively increased rates of fetal wastage were found with escalating dosages of Lm used for infection (Figure 6.1B). Interestingly, by culturing each individual resorbed placental-fetal unit, dose-dependent increased rates of *in utero* Lm invasion that were distinct from the overall resorption frequencies were also identified. In particular, while the majority of resorbed fetuses (87%) contained recoverable Lm after infection with the highest inocula (10^4 CFUs), the recovery of bacteria among resorbed fetuses declined sharply with reduced inocula (14% for 10^3 CFUs, and 0% for 10^2 CFUs) (Figure 6.1C). Together, these results demonstrate although Lm has the potential for *in utero* invasion especially after high-dose infection, infection-induced fetal injury can also occur without direct *in utero* pathogen invasion.

Listeria blunts maternal Treg suppression and disrupts fetal tolerance

Given the importance of expanded maternal Foxp3$^+$ Tregs in maintaining pregnancy (Aluvihare et al., 2004; Kahn and Baltimore, 2010; Rowe et al., 2011), and infection-induced reductions in Treg suppression that unleash immune activation in non-pregnant mice (Ertelt et al., 2011b; Kastenmuller et al., 2011b; Rowe et al., 2012), we investigated if similar reductions in maternal Treg suppression occur with infection during pregnancy. We found Lm infection at midgestation did not significantly impact either the number or percent Foxp3$^+$ among CD4 cells, suggesting quantitative reductions in maternal Tregs do not occur in this context (Figure 6.2). Next, to investigate the potential for infection-induced qualitative shifts in maternal Treg suppressive potency, Foxp3^{GFP} reporter mice

on the B6 background were substituted for mating with Balb/c males so that maternal Foxp3$^+$ Tregs could be purified by FACS directly *ex vivo*. Consistent with prior studies in non-pregnant mice (Ertelt et al., 2011b; Fontenot et al., 2005b), maternal Tregs were isolated from pregnant mice with equally high purity by sorting for GFP$^+$ CD4 cells (Figure 6.3A). By enumerating the efficiency whereby these GFP$^+$ Tregs suppress the proliferation of responder cells in co-culture, no significant difference in suppressive potency were found for Tregs recovered from pregnant compared with non-pregnant control mice (Figure 6.4).

By contrast with escalating Lm dosages used for infection beginning midgestation, progressive reductions in maternal Treg suppressive potency were identified because the proliferation of responder T cells isolated from naïve mice was more pronounced after co-culture with maternal GFP$^+$ Tregs from infected compared with uninfected control mice (Figure 6.3B). To evaluate the magnitude of these infection-induced reductions in Treg suppressive potency, we titrated the ratio of GFP$^+$ Tregs to responder cells in co-culture and found two-fold increased ratios of Tregs were required from pregnant mice infected with 10^3 Lm to achieve the same level of suppression as Tregs cells recovered from uninfected mice (Figure 6.3B). Comparatively, GFP$^+$ Tregs recovered from mice infected with 10^4 Lm suppressed responder cell proliferation even less efficiently; requiring two- to four-fold more Tregs to achieve the same level of suppression compared with GFP$^+$ Tregs recovered from uninfected controls, while Tregs recovered from mice infected with 10^2 Lm suppressed responder cell proliferation to an

intermediate degree compared with Tregs isolated from uninfected mice and those infected with increased Lm inocula (Figure 6.3B). Thus, Lm infection during pregnancy stimulates dose-dependent reductions in maternal Treg suppressive potency which are comparable to reductions in $Foxp3^+$ cell suppressive potency with Lm infection in non-pregnant mice (Ertelt et al., 2011b).

Since the sustained expansion of maternal $Foxp3^+$ Tregs are essential for maintaining tolerance to paternal antigens expressed by the developing fetus (Aluvihare et al., 2004; Rowe et al., 2011), we further investigated how these infection-induced reductions in maternal Treg suppressive potency might impact fetal tolerance. To identify maternal cells with fetal specificity, transgenic male mice engineered to express OVA in all cells behind the β-actin promoter were substituted for mating with B6 females so that maternal T cells responsive to peptides within the surrogate fetal-OVA antigen can be tracked using established immunological tools (Ehst et al., 2003; Erlebacher et al., 2007; Rowe et al., 2011). Following Lm infection at midgestation, we found maternal CD8 T cells with fetal-OVA specificity became activated in a dose-dependent fashion (Figure 6.5). Specifically, compared with the few OVA-specific cells recovered from uninfected pregnant mice that produced only background levels of IFN-γ, fetal-OVA-specific T cells expanded over 50-fold and efficiently produced IFN-γ in pregnant mice infected with 10^4 Lm (Figure 6.5). Although the degree of expansion and cytokine production each progressively diminished with reduced Lm inocula, both remained significantly elevated compared with background levels found in uninfected pregnant mice. Together, these

results demonstrate Lm infection during pregnancy blunts maternal Treg suppression and disrupts fetal tolerance. Furthermore, since even transient partial reductions in maternal Treg numbers cause fetal wastage and disrupt tolerance (Rowe et al., 2011), fetal resorption that occurs without *in utero* invasion with lower Lm inocula are likely triggered by infection-induced reductions in maternal Treg suppressive potency.

Cytoplasmic entry is essential for *Listeria*-induced fetal resorption

To more specifically investigate the pathogenesis of immune-mediated fetal injury, we compared pregnancy outcomes after infection with attenuated Lm containing defects in defined virulence determinants required for productive infection that do not cause fetal invasion (Cossart, 2011; Le Monnier et al., 2007; Portnoy et al., 2002). These include LmΔLLOΔPLC that cannot escape from the endocytic vacuole and enter into the cell cytoplasm; and LmΔactA that enters the cell cytoplasm, but cannot recruit actin required for intra- and inter-cellular spread (O'Riordan et al., 2002). In particular, these mutants were chosen because their ability to stimulate protective T cells *in vivo* that requires overriding Treg suppression is drastically discordant; LmΔactA readily primes the expansion of protective T cells, whereas LmΔLLOΔPLC does not (Bahjat et al., 2006; Curtis et al., 2010; Orgun and Way, 2008). Consistent with robust immune activation that occurs with Lm entry into the cell cytoplasm (Brzoza et al., 2004), LmΔactA infection midgestation caused sharp reductions in the number of live pups with reciprocal increased rates of fetal resorption compared with uninfected controls (Figure 6.6). By contrast, the number of live pups and frequency of fetal resorption did not differ

significantly between pregnant mice infected with LmΔLLOΔPLC and non-infected controls (Figure 6.6). Importantly, these differences in pregnancy outcomes could not be attributed to potential differences in relative attenuation because more LmΔLLOΔPLC compared with LmΔactA was recovered from the liver representing another tissue susceptible to Lm invasion (Figure 6.7). Thus, cytoplasmic entry is essential for Lm-induced fetal wastage.

Listeria cytoplasmic entry blunts maternal Treg suppression and disrupts fetal tolerance

Given the reductions in Treg suppressive potency associated with fetal resorption and fractured fetal tolerance after virulent Lm infection (Figures 6.1, 6.3, and 6.5), we investigated if differences in fetal wastage induced by LmΔactA and LmΔLLOΔPLC also paralleled discordance efficiencies in dampening maternal Treg suppression and disrupting fetal tolerance. We found Tregs could be purified after LmΔactA and LmΔLLOΔPLC infection in pregnant $Foxp3^{GFP}$ reporter mice similar to WT Lm or uninfected control mice (Figure 6.8A compared with Figure 6.3A). Using purified GFP^+ Tregs, we found LmΔactA infection midgestation triggered ~2-fold reductions in suppressive potency for maternal Tregs compared with GFP^+ cells recovered from uninfected pregnant controls (Figure 6.8B). Interestingly, the magnitude of these reductions in Treg suppressive potency were almost identical to cells recovered from mice infected with low or intermediate WT Lm dosages that induce fetal resorption without apparent *in utero* invasion. By contrast, the suppressive potency for maternal

GFP$^+$ Tregs after LmΔLLOΔPLC infection did not differ significantly compared with cells from uninfected controls (Figure 6.8B). Thus, reductions in maternal Treg suppressive potency directly parallel fetal wastage induced by LmΔactA and LmΔLLOΔPLC.

To further establish how these reductions in maternal Treg suppression induced by attenuated Lm impact fetal tolerance, the activation of maternal T cells with specificity to the surrogate fetal-OVA antigen were also enumerated after infection in pregnant B6 mice mated with OVA-expressing Balb/c males. Similar to WT Lm, LmΔactA inoculated midgestation primed the robust expansion and IFN-γ production by fetal-OVA specific CD8 T cells (Figure 6.9). Comparatively, LmΔLLOΔPLC failed to stimulate expansion and IFN-γ production above background levels found in uninfected control mice. These findings directly parallel the relative efficiency whereby each attenuated Lm dampens maternal Treg suppressive potency and induces fetal wastage (Figures 6.6 and 6.8). Lastly, to more definitively establish infection-induced immune-mediated fetal injury, the impacts of maternal CD4 and CD8 T cell depletion prior to Lm infection on pregnancy outcomes were evaluated. These experiments exploited the highly attenuated nature of LmΔactA that is eliminated even in immune-compromised mice to investigate how depletion of effector and regulatory T cells together impact infection-induced fetal wastage. Remarkably, we found the rate of LmΔactA-induced fetal wastage was sharply reduced in T cell-depleted compared with T cell-sufficient pregnancies [11.2 ± 3.5% resorption in T cell depleted mice (n=9); 62.7 ± 9.5% resorption in T cell-sufficient mice

(n=15), $P = 0.0005$]. Taken together, these results demonstrate Lm cytoplasmic entry is essential for disrupting fetal tolerance that triggers maternal T cell-mediated fetal resorption.

DISCUSSION

The intracellular bacterium Lm represents an important infectious cause of pregnancy loss and stillbirth (Gellin and Broome, 1989; Schuchat et al., 1991). Herein, we investigate the host pathogen interaction that triggers these unfortunate outcomes, with particular focus on how prenatal infection impacts fetal tolerance sustained by expanded maternal Foxp3^{+} Tregs. Our results demonstrate infection-induced dampening Treg suppression that unleashes immune activation required for optimal host defense (Rowe et al., 2012), in the context of prenatal infection when sustained tolerance to fetal antigen is essential, plays a pivotal role in the pathogenesis of fetal wastage. The importance of immune-mediated fetal injury is shown by the increased frequency of fetal resorption with reciprocal reduction in the number of live pups following infection with low or intermediate doses of virulent Lm where bacteria are not found in the majority of resorbed fetuses. Similarly for attenuated Lm that do not cause fetal invasion (Le Monnier et al., 2007), fetal wastage and disrupted fetal tolerance occurs only for strains that retain the ability to prime protective T cells through entry into the cell cytoplasm and dampen maternal Treg suppressive potency. Furthermore, depletion of effector T cells along with Tregs prior to cytoplasmic Lm infection prevents infection-induced fetal wastage. Together with fetal injury that is induced by systemic treatment with various TLR ligands [e.g. LPS, poly(I:C)] (de Fougerolles and Baines, 1987; Gendron et al., 1990) shown to modulate Treg suppression *in vitro* or after *in vivo* stimulation in non-pregnant mice (Caramalho et al., 2003; de Fougerolles and Baines, 1987; Gendron et al.,

1990; Kubo et al., 2004; Pasare and Medzhitov, 2003; Sakaguchi, 2003), these results establish the importance of infection or inflammation- induced disruption of maternal Treg suppression in the pathogenesis of fetal wastage. Although we used intravenous inoculation to recapitulate disseminated infection that occurs with Lm during pregnancy, crucial next steps based on these results are to evaluate if systemic disruption of fetal tolerance is essential, or if local disruption induced by pathogens that primarily reside in the vaginal or cervical (e.g. bacterial vaginosis, *Ureaplasma* and *Chlamydia* sp.) mucosa are also sufficient to trigger fetal injury.

On the other hand, since Lm and other prenatal pathogens can cause *in utero* fetal invasion, immune-mediated fetal injury alone does not fully address the pathogenesis of these infections. In this regard, an important clue from our results is that although virulent Lm-induced fetal resorption and invasion are each dose-dependent, these processes can be readily dissociated based on the inocula of Lm used for infection. Accordingly, we propose a model whereby low-dose maternal infection dampens Treg suppression that unleash the immune activation enough to rapidly eliminate the pathogen so that immune-mediated fetal injury occurs almost exclusively (Figure 6.10). Comparatively, with higher-dose infection, blunted maternal Treg suppression that promotes immune activation does not eradicate infection as efficiently. In turn with ongoing disruptions in fetal tolerance, remaining pathogen drawn to inflammation at the uterine-placental interface promotes invasion into the placental-fetal unit. Although clearly an over-simplification, this model suggests overriding maternal Treg suppression is the pivotal

step in dictating whether fetal wastage occurs regardless of *in utero* pathogen invasion. Therefore, important areas for further investigation are to identify the cellular receptors and cytokines that specifically respond to cytoplasmic Lm and other inflammatory stimuli that dictate reductions in maternal Treg suppression.

Finally, given the increasingly established heterogeneity and functional specialization among $Foxp3^+$ cells that utilize distinct cell-associated and secreted molecules to mediate context specific immune suppression (Shevach, 2009; Vignali et al., 2008; Wing and Sakaguchi, 2010), establishing the Treg-associated molecule(s) that sustain fetal tolerance and distinguishing them from those required for host defense against infection have direct implications for developing therapies for dissociating the beneficial and detrimental impacts of expanded maternal Tregs. In this regard, while IL-10 is non-essential for sustaining fetal tolerance under non-inflammatory conditions, it likely plays important roles in maintaining pregnancy under inflammatory conditions known to trigger fetal wastage (Gendron et al., 1990; Robertson et al., 2007; White et al., 2004). Accordingly, our ongoing studies are aimed at establishing the importance of other Treg-associated molecules in maintaining fetal tolerance and sustaining pregnancy. Given the sharply increased rates of fetal wastage and pregnancy loss that occurs with prenatal Lm infection, the specific Treg-associated molecules essential for maintaining pregnancy may overlap with those required for optimal protection against prenatal infection. Nevertheless, given the importance of maternal Tregs in both sustaining fetal tolerance and host defense against prenatal infection, we propose establishing how these cells work

in each context represent critical next steps towards new therapeutic approaches for improving pregnancy outcomes.

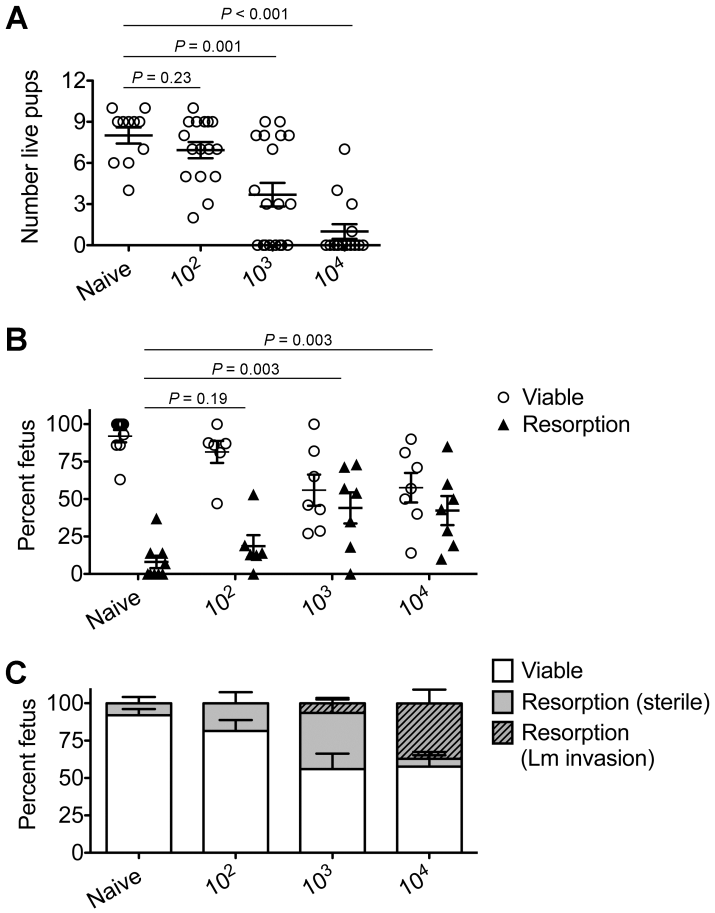

Figure 6.1 - *Listeria monocytogenes* infection during allogeneic pregnancy triggers dose-dependent rates of fetal wastage. (A) Number of live pups born with WT Lm inoculated midgestation (E10.5) at the indicated dosages for pregnant B6 females mated with Balb/c males. (B) Percent viable and resorbed fetuses five days after Lm WT infection at midgestation. (C) Percent viable and resorbed fetuses with recoverable Lm for the mice described in B. Each point represents results from an individual mouse combined from three independent experiments each with similar results.

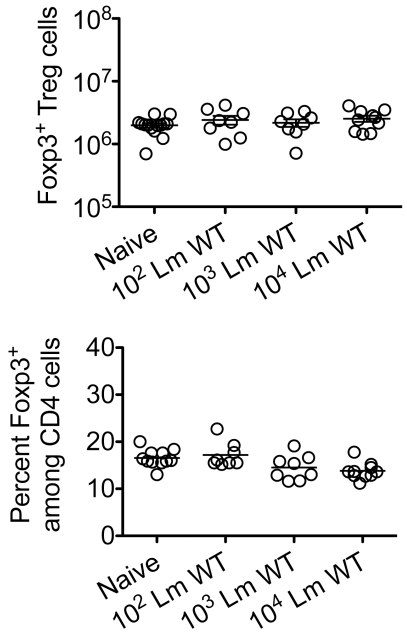

Figure 6.2 - *Listeria* infection during pregnancy does not induce quantitative changes in maternal Foxp3$^+$ regulatory CD4 T cells. Absolute number (top) and percent Foxp3$^+$ among CD4 T cells (bottom) three days after WT Lm infection at midgestation in B6 females mated with Balb/c males. Each point represents results from an individual mouse combined from two independent experiments each with similar results.

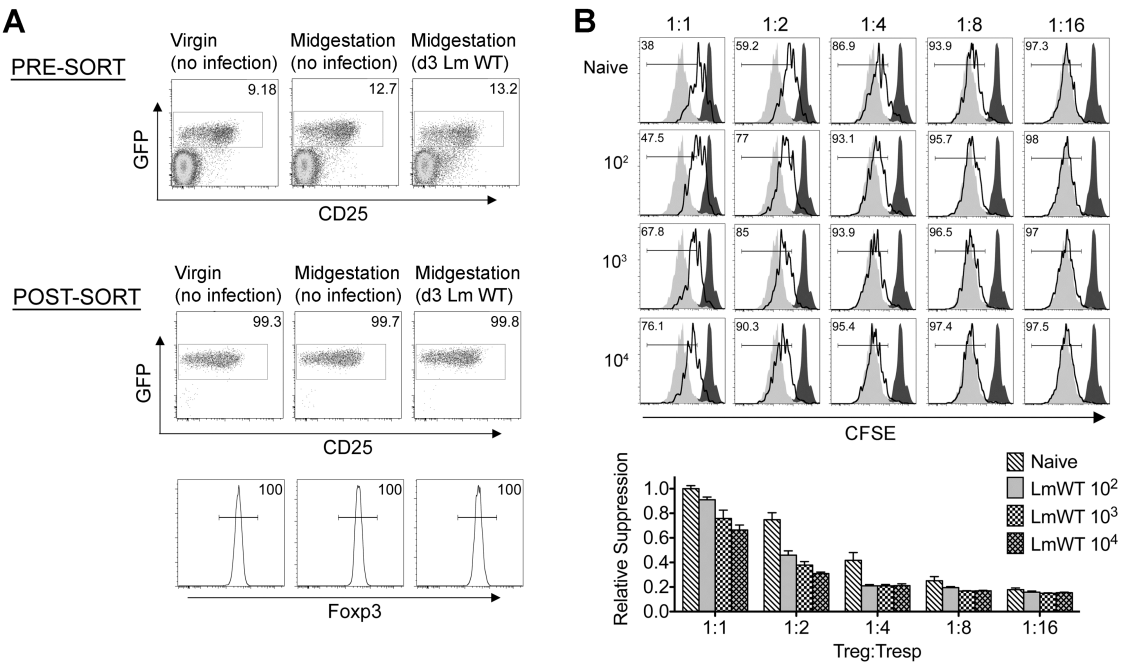

Figure 6.3 - ***Listeria monocytogenes*** **infection during pregnancy dampens maternal Foxp3$^+$ regulatory cell suppressive potency.** (A) Percent GFP$^+$ or Foxp3$^+$ among CD4 cells in virgin, pregnant mice midgestation without infection, or three days after infection with 10^4 WT Lm. (B) Representative plots demonstrating proliferation (CFSE dilution) among responder (Tresp) CD8$^+$ CD90.1$^+$ cells after co-culture with each ratio of GFP$^+$ Tregs isolated from Lm-infected mice and stimulation with anti-CD3 antibody (black line), compared with no Treg (gray filled) or no stimulation (black filled) controls (top). Relative suppression of responder cell proliferation (CFSE dilution) after co-culture with GFP$^+$ Tregs for the mice described above normalized to suppression by GFP$^+$ cells from uninfected controls at a 1:1 Treg:Tresp ratio (bottom). These data reflect six to eight mice per group representative of three independent experiments each with similar results.

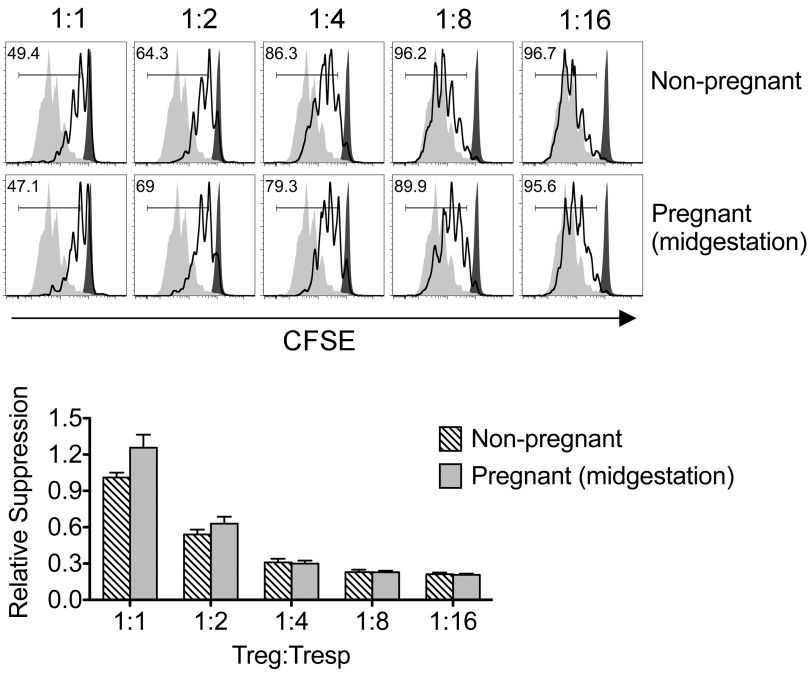

Figure 6.4. Pregnancy does not induce shifts in maternal regulatory T cell suppressive potency. Representative plots demonstrating proliferation (CFSE dilution) among responder CD90.1$^+$ CD8$^+$ cells (Tresp) after co-culture with each ratio of GFP$^+$ Tregs isolated from virgin or pregnant B6 mice midgestation after mating with Balb/c males, and stimulation with anti-CD3 antibody (black line), compared with no Treg (gray filled) or no stimulation (black filled) controls (top). Relative suppression of responder cell proliferation (CFSE dilution) after co-culture with GFP$^+$ Tregs for the mice described above normalized to suppression by GFP$^+$ cells from non-pregnant controls at a 1:1 Treg:Tresp ratio (bottom).

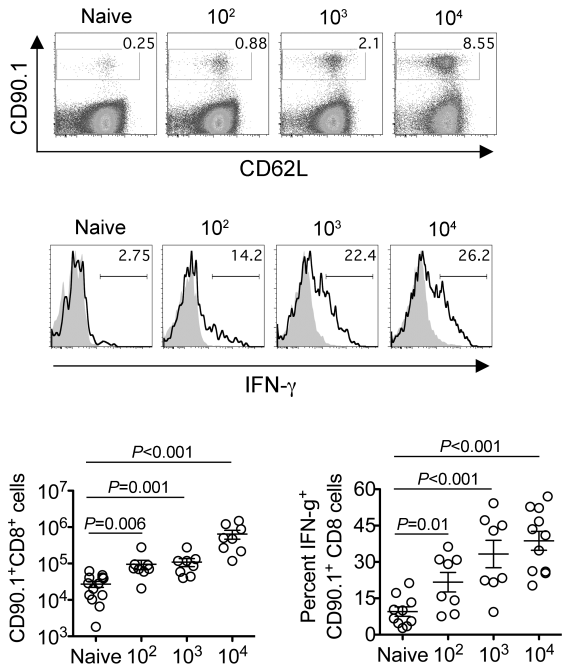

Figure 6.5 - *Listeria monocytogenes* infection during pregnancy disrupts fetal tolerance. Representative FACS plots (top) and composite data (bottom) illustrating expansion and IFN-γ production by fetal-OVA-specific $CD8^+$ T cells among maternal splenocytes in mice impregnated by Actin-OVA males five days after Lm infection at midgestation. For IFN-γ production, cells were stimulated with $OVA_{257-264}$ peptide (black line) or no peptide controls (gray filled). Each point represents results from an individual mouse combined from three independent experiments each with similar results.

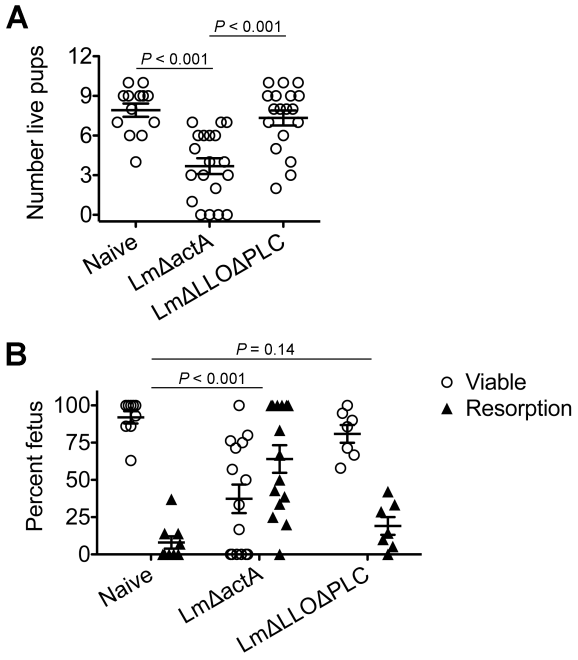

Figure 6.6 - Cytoplasmic entry is essential for *Listeria* infection-induced fetal wastage. (A) Number of live pups born with LmΔactA (10^7 CFUs) or LmΔLLOΔPLC (10^8 CFUs) infection at midgestation (E10.5) in B6 females mated with Balb/c males. (B) Percent viable and resorbed fetuses five days after LmΔactA (10^7 CFUs) or LmΔLLOΔPLC (10^8 CFUs) infection at midgestation. Each point represents results from an individual mouse combined from three independent experiments each with similar results.

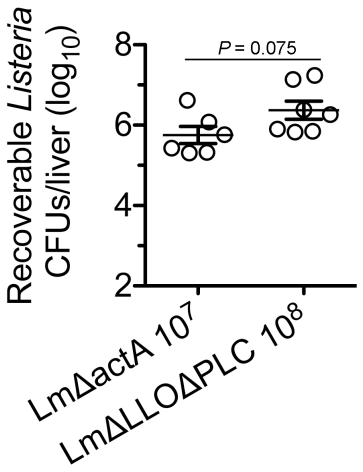

Figure 6.7 - Comparable *in vivo* bacterial burden after LmΔactA and LmΔLLOΔPLC infection. Number of recoverable CFUs in the liver one day after infection with 10^7 LmΔactA compared with 10^8 LmΔLLOΔPLC.

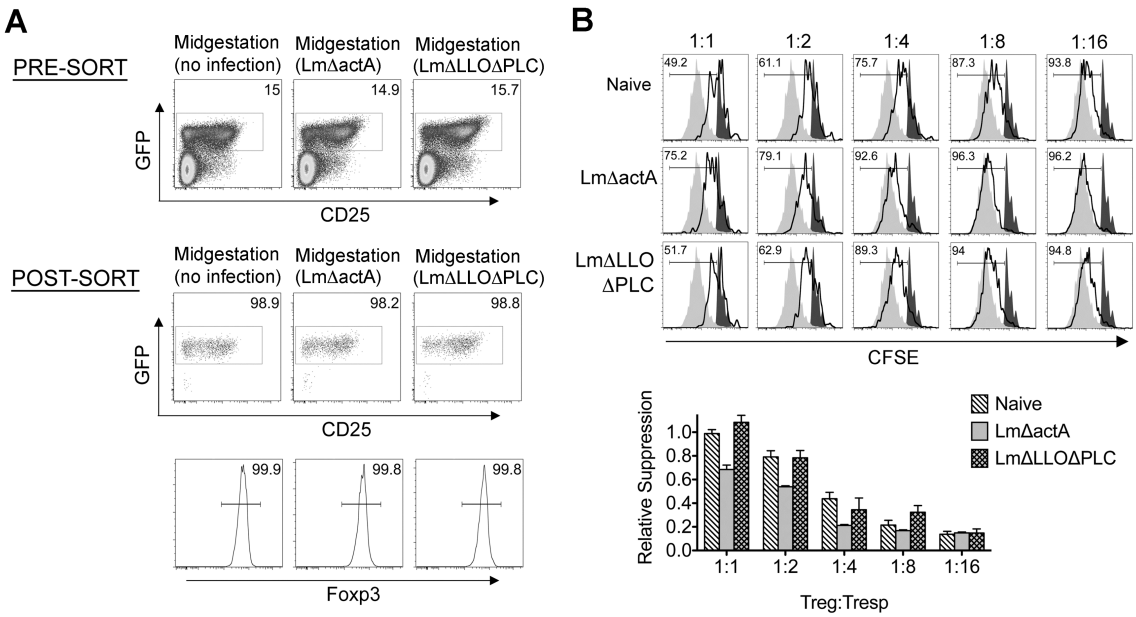

Figure 6.8 *Listeria* cytoplasmic entry dampens maternal Treg suppressive potency. (A) Percent GFP$^+$ or Foxp3$^+$ among CD4 cells in pregnant mice midgestation after infection with LmΔactA (10^7 CFUs) or LmΔLLOΔPLC (10^8 CFUs). (B) Representative plots demonstrating proliferation (CFSE dilution) among responder (Tresp) CD8$^+$ CD90.1$^+$ cells after co-culture with each ratio of GFP$^+$ Tregs isolated from LmΔactA (10^7 CFUs) or LmΔLLOΔPLC (10^8 CFUs) infected mice, and stimulation with anti-CD3 antibody (black line), compared with no Treg (gray filled) or no stimulation (black filled) controls (top). Relative suppression of responder cell proliferation (CFSE dilution) after co-culture with GFP$^+$ Tregs for cells from mice described above normalized to suppression by GFP$^+$ cells from uninfected controls at a 1:1 Treg:Tresp ratio (bottom). These data reflect six to eight mice per group representative of three independent experiments each with similar results.

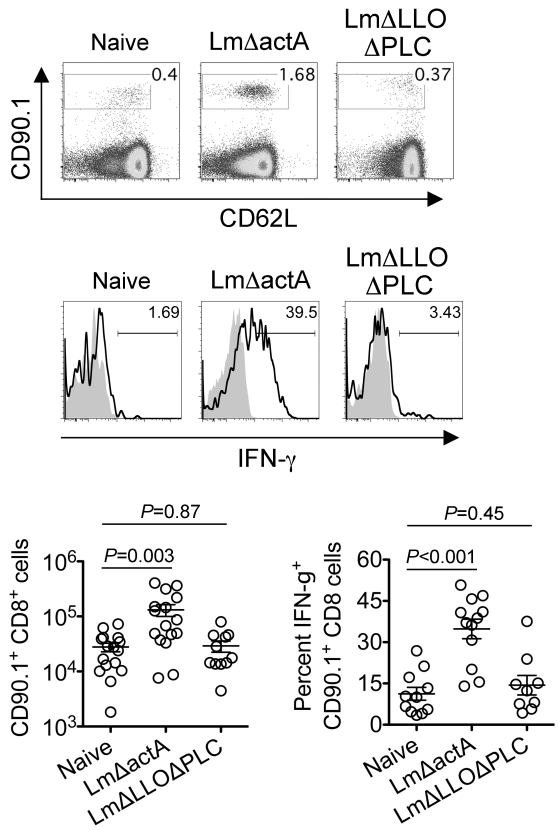

Figure 6.9 *Listeria* cytoplasmic entry disrupts fetal tolerance with infection during pregnancy. Representative FACS plots (top) and composite data (bottom) illustrating expansion and IFN-γ production by fetal-OVA-specific $CD8^+$ T cells among maternal splenocytes in mice impregnated by Actin-OVA males five days after LmΔactA (10^7 CFUs) or LmΔLLOΔPLC (10^8 CFUs) infection at midgestation. For IFN-γ production, cells were stimulated with $OVA_{257-264}$ peptide (black line) or no peptide controls (gray filled). Each point represents results from an individual mouse combined from three independent experiments each with similar results.

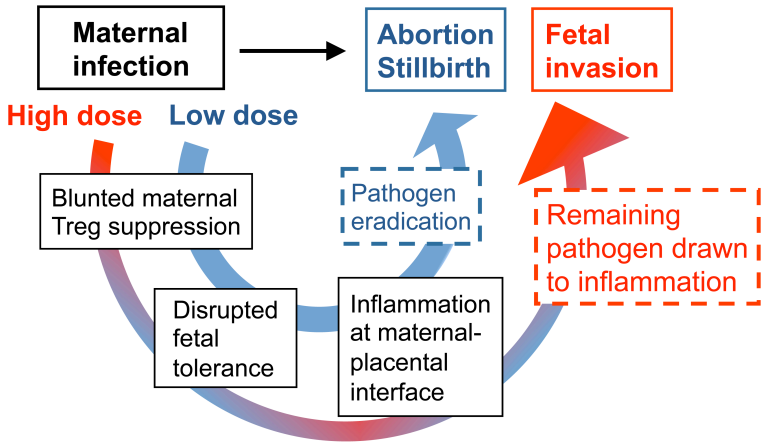

Figure 6.10 - Proposed model for immune-mediated fetal wastage and *in utero* pathogen invasion with maternal infection during pregnancy. After low dose infection, reductions in maternal regulatory T cell suppressive potency unleash immune activation enough to rapidly eliminate the pathogen. However, given the requirement for sustained regulatory T cell suppression, these reductions in suppressive potency also disrupt fetal tolerance that results in immune-mediated fetal wastage. By comparison with relatively higher dose infection, blunted maternal regulatory T cell suppression that promotes immune activation does not eradicate infection as efficiently. In turn with ongoing disruptions in fetal tolerance, remaining pathogen drawn to inflammation at the uterine-placental interface promotes invasion into the placental-fetal unit.

Chapter 7

Pregnancy imprints regulatory memory that sustains tolerance to fetal antigen

SUMMARY

Pregnancy results in the expansion of maternal regulatory T cells (Tregs) throughout the course of pregnancy that are specific for fetal-derived peptides. The accumulation of fetal-specific Foxp3$^+$ Tregs throughout gestation is driven by both the expansion of pre-existing "natural" Tregs (nTregs and the peripheral induction of naïve CD4$^+$ T cells into Foxp3-expressing Tregs (iTregs). We find that each of these populations contribute to about half of the expanded pool of maternal Tregs specific for the fetus. Interestingly, following parturition and removal of fetal antigens, the pool of expanded CD4$^+$ T cells specific for the fetus was maintained and Foxp3$^+$ nTregs and iTregs were enriched by 2-fold. These fetal-specific "memory" Foxp3$^+$ Tregs were able to re-expand more efficiently than naïve fetal-specific Tregs during secondary pregnancies. Furthermore, this memory population of Foxp3$^+$ Tregs restricted the function of fetal-specific effector cells following infection with *Listeria monocytogenes* expressing the same antigen as the fetus. These results suggest that pregnancy primes the fetal-specific expansion and subsequent memory formation of highly functional immune suppressive Tregs.

INTRODUCTION

Pregnancy is an intricately orchestrated process where immune effectors with fetal specificity are selectively silenced. This requires the sustained expansion of immune suppressive maternal Foxp3$^+$ regulatory T cells (Tregs), because even transient partial ablation triggers fetal-specific T cell activation and pregnancy loss (Aluvihare et al., 2004; Kahn and Baltimore, 2010; Rowe et al., 2011). In turn, many idiopathic pregnancy complications including preeclampsia proposed to stem from disrupted fetal tolerance are associated with blunted maternal Treg expansion (Prins et al., 2009; Santner-Nanan et al., 2009; Sasaki et al., 2004). Importantly however, the antigen-specificity and cellular origin of maternal Tregs remain undefined. Here we show maternal CD4 cells with fetal-specificity become highly enriched (>50%) for Foxp3$^+$ expression by parturition using tetramer-based enrichment that allows the identification of rare endogenous T cells (Moon et al., 2007). Interestingly after delivery, fetal-specific Tregs persisted at elevated levels, maintained tolerance to pre-existing fetal antigen, and rapidly re-accumulated during secondary pregnancy. Furthermore, although induction of Foxp3 expression and proliferation of pre-existing Foxp3$^+$ cells each contributed to Treg expansion during primary pregnancy, accelerated fetal-specific Treg accumulation during secondary pregnancy was driven almost exclusively by proliferation of Foxp3$^+$ cells retained from prior pregnancy. Thus, pregnancy imprints antigen-specific Foxp3$^+$ CD4 cells that sustain regulatory memory to fetal antigen. These findings establish the scientific basis for reduced rates of preeclampsia and other complications in secondary compared with

primary pregnancy that is partner-specific, and eliminated with a new partner in subsequent pregnancy (Trupin et al., 1996). We anticipate these results will spark further investigation on maternal regulatory T cell antigen-specificity that unlocks new strategies for improving pregnancy outcomes.

The accumulation of maternal Tregs during pregnancy parallels the need for expanded peripheral tolerance to encompass "non-self" paternal antigens expressed by the fetus (Aluvihare et al., 2004; Kahn and Baltimore, 2010; Prins et al., 2009; Santner-Nanan et al., 2009; Sasaki et al., 2004). However, one consequence of sustained $Foxp3^+$ cell expansion is impaired host defense and susceptibility to pathogens causing prenatal infections (Rowe et al., 2011). Given the increasingly recognized importance of Treg specificity in regulating the fluid balance between immune activation that maintains host defense and immune suppression that prevents autoimmunity (Hsieh et al., 2012; Josefowicz et al., 2012; Lathrop et al., 2011; Shafiani et al., 2010; Suffia et al., 2006; Wing and Sakaguchi, 2010), we reasoned establishing maternal $Foxp3^+$ cell specificity to fetal antigen could unravel ways to dissociate beneficial from detrimental impacts of expanded Tregs. Furthermore, extending this analysis for maternal Tregs with fetal-specificity after delivery to investigate the potential for regulatory memory recently demonstrated for $Foxp3^+$ cells responsive to an induced self antigen could reveal why rates of preeclampsia and other complications are increased during first pregnancies, and the partner-specific beneficial effect successful pregnancy imparts on subsequent pregnancies (Rosenblum et al., 2011; Trupin et al., 1996). To address these questions, we

developed an allogeneic mating strategy where the I-A^b $2W1S_{52-68}$ peptide becomes a surrogate fetal antigen using male mice (H-2^d; Balb/c) engineered to co-express this peptide with β-actin to impregnate non-2W1S-expressing females (H-2^b; C57Bl/6) (Moon et al., 2011). The high precursor frequency of CD4 cells with specificity for $2W1S_{52-68}$ peptide in C57Bl/6 mice allows even relatively rare endogenous maternal Tregs to this surrogate fetal antigen to be identified using MHC class II tetramer enrichment techniques (Moon et al., 2007).

MATERIAL AND METHODS

Mice. Balb/c (H-2^d), C57Bl/6 (H-2^b), and CD45.1 and CD90.1 mice each on the C57Bl/6 background were purchased from The Jackson Laboratory. 2W1S-expressing and $Foxp3^{DTR/DTR}$ mice have each been described (Kim et al., 2007; Moon et al., 2011). 2W1S males were backcrossed 5 generations onto the Balb/c background and verified before use to be H-$2^{d/d}$. $Foxp3^{DTR/DTR}$ mice on the C57Bl/6 background were intercrossed with CD45.1 mice. Males were introduced to virgin or postpartum females for 24 hours, and mated mice visualized by a copulation plug representing E0.5. Pups were removed within 24 hours after delivery to prevent the potential transfer of fetal antigen through breast-feeding. For sterilization, female mice were sub-lethally irradiated (100 rads). For infection, mice were intravenously inoculated with recombinant *Listeria monocytogenes* engineered to stably express $2W1S_{52\text{-}68}$ peptide, Lm-2W1S (10^4 CFUs per mouse) (Ertelt et al., 2009), and harvested 5 days thereafter. All mice were used in accordance with University of Minnesota IACUC approved protocols.

Tetramer enrichment and flow cytometry. PE or APC conjugated MHC class II I-A^b $2W1S_{52\text{-}68}$ tetramer, and their use with anti-fluorophore conjugated beads for enrichment have been described (Moon et al., 2007; Moon et al., 2011). For enumerating total $2W1S^+$ cells per mouse, all nucleated cells from the spleen and lymph nodes (axillary, brachial, cervical, inguinal, mesenteric, pancreatic, and para-aortic/uterine) were collected, enriched using I-A^b $2W1S_{52\text{-}68}$ tetramer, and stained for cell-surface CD4,

CD8α, CD25, CD45.1, CD45.2, CD44, CD11b, CD11c, B220, F4/80, intracellular IFN-γ, or intranuclear Foxp3, Helios, Ki67, or Tbet expression using fluorophore-conjugated antibodies and permeabilization reagents (BD PharMingen or eBioscience). For stimulation, cells were cultured with PMA and ionomycin for 5 hours in media supplemented with Brefeldin A before tetramer staining.

Cell transfer and ablation. CD4 cells in the spleen and lymph nodes were purified by negative selection (Miltenyi Biotech), and one mouse equivalent of donor cells was intravenously transferred into recipient mice. For ablation of donor $Foxp3^{+}$ cells derived from $Foxp3^{DTR/DTR}$ mice, recipient $Foxp3^{WT/WT}$ mice were treated with purified DT (two doses 8 hours apart, 0.5 μg/dose). For $Foxp3^{+}$ cell reconstitution requiring sustained Treg ablation in $Foxp3^{DTR/DTR}$ recipient mice, DT was administered daily (0.5 μg first dose, followed by 0.1 μg/dose thereafter) following transfer of purified donor CD4 cells as described (Rowe et al., 2011).

Statistics. The number and percent cells were first analyzed and found to be normally distributed, and the difference between separate groups of mice were analyzed using an unpaired Student's *t* test, and the difference between individual cell subsets within the same mouse were analyzed using the paired Student's *t* test each with $P < 0.05$ taken as statistical significance.

RESULTS

Using this approach, fetal I-A^b 2W1S-specific maternal CD4 cells ($2W1S^+$) were found to sharply up-regulate CD44 expression, progressively accumulate throughout pregnancy, and persist at ~10-fold increased levels through day 30 post partum each compared with non-pregnant controls (Figure 7.1A). The expansion of $2W1S^+$ cells was specific to mating with 2W1S-expressing males because they did not accumulate in females impregnated by non-2W1S-expressing males (Figure 7.2). Since seminal fluid also contains cells of paternal origin (Robertson, 2010), $2W1S^+$cells in female mice rendered infertile with low-dose irradiation before mating were also evaluated. We found although mating without pregnancy stimulated modest $2W1S^+$ cell expansion and CD44 up-regulation in an antigen-specific fashion, the magnitude was sharply reduced compared with impregnated females (Figure 7.2). Thus, the expansion of maternal $2W1S^+$ cells during pregnancy reflects an antigen-specific response to cells of fetal origin.

Given the importance of maternal Tregs in maintaining fetal tolerance (Aluvihare et al., 2004; Munoz-Suano et al., 2011), Foxp3 expression among fetal 2W1S-specific cells was evaluated. We found $2W1S^+$ compared with $2W1S^-$ CD4 cells became significantly enriched for Foxp3 expression beginning midgestation that persisted through day 30 after parturition (Figure 7.1A,B). Most notably, ~50% of $2W1S^+$ cells were $Foxp3^+$ compared with ~16% among $2W1S^-$ CD4 cells late gestation through the first 48 hours post-partum (E18.5 to PP2) (Figure 7.1B). Furthermore, $2W1S^+Foxp3^+$ cells, and to a lesser extent

2W1S^{+}Foxp3^{-} cells, up-regulated the proliferation marker Ki67 that paralleled increasing mass of fetal cells throughout pregnancy (Figure 7.1C). Reciprocally at later time points after expulsion of the fetus and other products of conception (PP14 to PP30), Ki67 expression among both 2W1S^{+}Foxp3^{+} and 2W1S^{+}Foxp3^{-} cells was reduced (Figure 7.1C). Interestingly despite decreased Ki67 expression, 2W1S^{+}Foxp3^{+} cells persisted >30-fold and Foxp3 expression was sustained at ~20% (Figure 7.1A,B). Thus, maternal Tregs with specificity to fetal antigen accumulate during pregnancy and persist following parturition.

To investigate the origin of fetal-specific Tregs, Helios expression that marks natural Tregs programmed in the thymus from those peripherally induced from Foxp3^{-} CD4 cells was enumerated (Thornton et al., 2010). We found 2W1S^{+}Foxp3^{+} cells in pregnant mice progressively down-regulated Helios that nadired to ~40% Helios^{hi} Tregs at late gestation compared with the few 2W1S^{+}Foxp3^{+} cells in non-pregnant mice that were uniformly Helios^{hi} (Figure 7.1D). Comparatively, Helios expression among 2W1S^{-}Foxp3^{+} cells did not shift significantly suggesting CD4 cells with fetal specificity become induced Foxp3^{+} cells (Figure 7.1D). However, since some peripherally induced Foxp3^{+} Tregs can also express Helios (Gottschalk et al., 2012), we more specifically investigated whether Foxp3^{-} cells convert into Foxp3^{+} Tregs by tracking adoptively transferred CD4 cells from Foxp3$^{DTR/DTR}$ donors ablated of Tregs with diphtheria toxin (DT) in Foxp3$^{WT/WT}$ recipient mice (Figure 7.1E) (Kim et al., 2007). At midgestation after mating with 2W1S-expressing males, 2W1S^{+}Foxp3^{+} cells were maintained among Treg-ablated donor

Foxp3$^{DTR/DTR}$ cells indicating conversion of fetal-specific Foxp3^{-} cells into Foxp3^{+} cells occurs during pregnancy (Figure 7.1E). This conversion was pregnancy-specific and not due to incomplete ablation among donor cells because Foxp3^{+} cells were undetectable among Treg-ablated donor cells in unmated control mice (Figure 7.3). Interestingly however, the percentage of Foxp3^{+} cells among Treg-ablated donor cells was significantly reduced (by ~50%) compared with 2W1S^{+}Foxp3^{+} cells among Treg-sufficient donor cells in control mice without DT treatment or among cells not susceptible to DT in recipient mice (Figure 7.1E). Therefore, Foxp3 induction among Foxp3^{-} precursors and proliferation of pre-existing Foxp3^{+} cells both contribute to the accumulation of fetal-specific Tregs during pregnancy.

To determine the significance of Tregs with specificity for pre-existing fetal antigen that persist postpartum, we measured their response during secondary pregnancy. Postpartum mice impregnated 14 days after delivery a second time with 2W1S-expressing males up-regulated Foxp3 expression with accelerated kinetics compared with primary pregnancy (Figure 7.4A). This acceleration in fetal-specific Treg expansion in separate groups of mice was recapitulated within the same mouse by measuring 2W1S^{+} Treg accumulation among donor cells from postpartum mice (secondary expansion) adoptively transferred prior to mating, compared with cells in virgin recipient mice (primary expansion) (Figure 7.4B). By substituting cells from postpartum Foxp3$^{DTR/DTR}$ mice for adoptive transfer and using DT to eliminate donor Tregs prior to mating, we also evaluated whether this more vigorous secondary expansion of fetal-specific Tregs reflects Foxp3 induction in Foxp3^{-}

cells or proliferation of pre-existing Foxp3$^+$ cells. In sharp contrast to primary pregnancy, the ablation of adoptively transferred Tregs from postpartum Foxp3$^{DTR/DTR}$ mice almost uniformly eliminated the subsequent expansion of Foxp3$^+$ Tregs (Figures 7.4C and 7.1E). Thus, recurrent pregnancy primes the accelerated accumulation of fetal-specific Foxp3$^+$ cells that expand from pre-existing Tregs retained after primary pregnancy.

Expanding this model, that allows the identification of endogenous maternal T cells with fetal specificity, functional anergy among fetal 2W1S-specific cells was investigated. 2W1S$^+$ cells recovered from mice midgestation or postpartum each compared with non-pregnant controls did not produce appreciable IFN-γ after in vitro stimulation, and these results are consistent with the hypo-responsiveness for fetal-specific T cells in Treg-sufficient mice (Figure 7.5) (Erlebacher et al., 2007; Rowe et al., 2011). Therefore, to more fully evaluate the degree of functional anergy for fetal 2W1S$^+$ cells, we measured their response to infection with recombinant *Listeria monocytogenes* engineered to express 2W1S$_{52-68}$ peptide (Lm-2W1S) that potently stimulates CD4 Th1-differentiation and IFN-γ production (Ertelt et al., 2009; Pepper et al., 2010). We found 2W1S$^+$ cells expanded in an antigen specific fashion after Lm-2W1S infection even in mice impregnated by 2W1S-expressing males (Figure 7.6). However, 2W1S$^+$ cells in pregnant mice where 2W1S represents a fetal antigen remained anergic and produced only background levels of IFN-γ (Figure 7.7A). By contrast, >15% of 2W1S$^+$ cells in Lm-2W1S infected virgin female mice were IFN-γ$^+$ (Figure 7.7A). This hypo-responsiveness was unique to CD4 cells recognizing fetal antigen because mice impregnated with non-

2W1S-expressing males contained similar elevated levels of $2W1S^{+}IFN\text{-}\gamma^{+}$ cells (Figure 7.7A). Given the sustained enrichment of fetal-specific Tregs after delivery (Figure 7.1A,B), these experiments were extended to investigate whether diminished IFN-γ production among fetal-specific CD4 cells was similarly sustained. Remarkably, IFN-γ production remained anemic in mice previously exposed to 2W1S as a fetal antigen, while postpartum mice without prior fetal 2W1S exposure produced IFN-γ comparable to non-pregnant controls (Figure 7.7B). Therefore, pregnancy imprints functional anergy for fetal-specific CD4 cells that is sustained postpartum.

To dissociate whether pregnancy induced anergy for fetal-specific CD4 cells was cell-intrinsic or imposed by features associated with the postpartum environment, IFN-γ production among adoptively transferred CD4 cells from postpartum mice in virgin recipient mice was evaluated (Figure 7.7C). We found $2W1S^{+}$ cells among adoptively transferred postpartum and naïve recipient mice each produced similar elevated levels of IFN-γ that were sharply increased compared with $2W1S^{+}$ cells in unmanipulated postpartum mice (Figure 7.7B,C). Thus, anergy among CD4 cells with specificity to pre-existing fetal antigen is not cell-intrinsic, but maintained by the postpartum environment.

Lastly, to investigate the importance of $Foxp3^{+}$ Tregs in sustaining anergy to CD4 cells with specificity to pre-existing fetal antigen, we interrogated how replacing the entire Treg compartment in postpartum mice previously exposed to 2W1S as a fetal antigen with naïve $Foxp3^{+}$ cells from virgin mice would impact $2W1S^{+}$ cell cytokine production.

Consistent with our prior studies using adoptively transferred CD4 cells to refill the empty cellular compartment in Foxp3$^{DTR/DTR}$ mice sustained on low-dose DT (Rowe et al., 2011), Tregs from naïve mice efficiently reconstituted Treg-ablated Foxp3$^{DTR/DTR}$ postpartum mice within 7 days (Figure 7.8A). Using this approach, we found replacing Foxp3^{+} cells in postpartum mice with Tregs from naïve mice restored IFN-γ production for 2W1S^{+} cells following Lm-2W1S infection (Figure 7.8B). Furthermore, while only rare Foxp3^{+}Helios^{hi} among 2W1S^{+} cells were found among postpartum mice reconstituted with naïve Tregs, a significant proportion (~20%) of 2W1S^{+} cells that expanded in response to Lm-2W1S infection in intact post partum mice remained Foxp3^{+} and Helios^{lo} (Figure 7.8B). Thus, the muted expansion of Foxp3^{+} CD4 cells after Lm infection in naïve mice (Ertelt et al., 2009), and among naïve Tregs is restored by pregnancy-induced Treg activation.

DISCUSSION

These findings establish a model whereby fetal-specific Foxp3$^+$ Tregs selectively expand during pregnancy (Figure 7.9). In this regard while maternal Tregs have been reported to expand 1-2-fold when examined in a non-antigen specific manner (Prins et al., 2009; Rowe et al., 2011; Santner-Nanan et al., 2009; Sasaki et al., 2004), our results enumerating fetal specific Foxp3$^+$ cells demonstrate those responsive to fetal antigen expand 50-100-fold through parturition when the mass of fetal cells is greatest (Figure 7.1A). After delivery, fetal-specific Tregs are sustained at enriched levels, and are functionally distinct from "naïve" Tregs as they re-accumulate with accelerated kinetics and outcompete naïve Tregs during secondary pregnancy. Similar to the distinct characteristics of naïve and memory effector T cells (Wakim and Bevan, 2010), these findings reveal the exciting possibility of exploiting memory Tregs in an antigen-specific fashion to dissociate detrimental from beneficial immune responses. Applied to human pregnancy, these results reinforce the need to discriminate the specificity of maternal Tregs especially in rare cases of preeclampsia where their expansion appears normal (Paeschke et al., 2005). Finally, although these studies were extended for 30 days postpartum that represents a significant duration relative to the estrous cycle and gestational period in mice, longer-term studies are needed to establish when pregnancy induced regulatory memory wanes. Given the increased risks of preeclampsia in recurrent human pregnancy when the interpregnancy interval is significantly extended, an eventual

decay back to baseline levels similar to other CD4 subsets is expected (Conde-Agudelo and Belizan, 2000; Pepper et al., 2010).

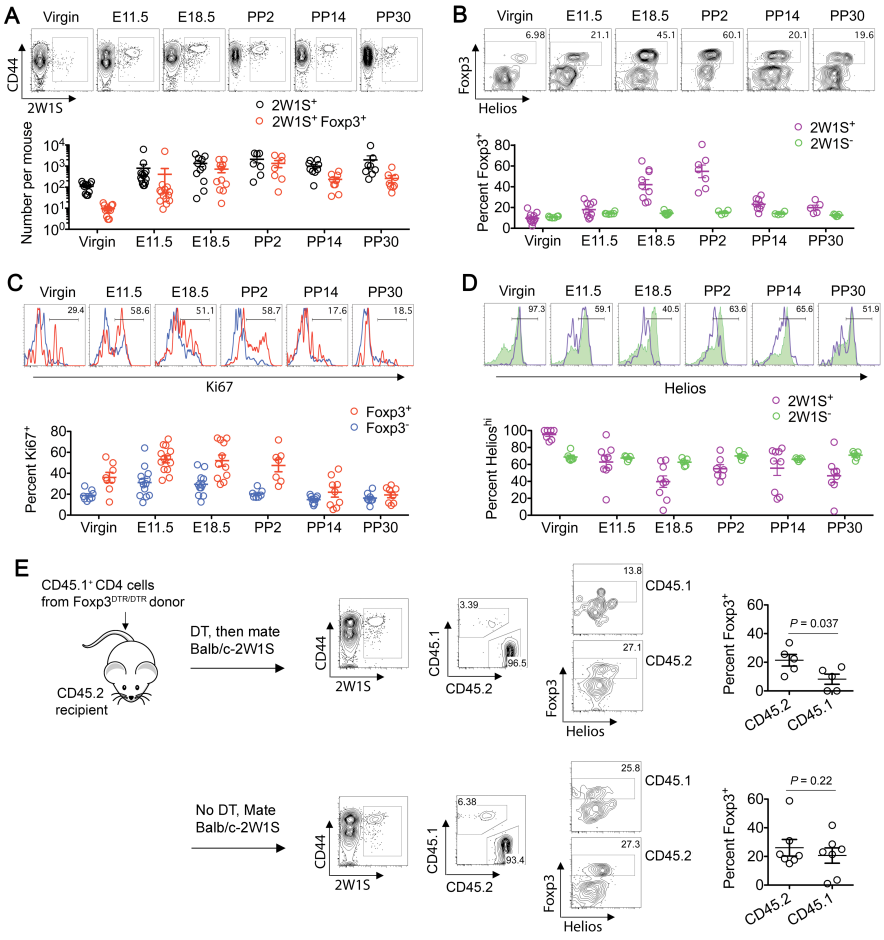

Figure 7.1 - Expansion and proliferation of fetal-specific CD4 and Foxp3$^+$ Tregs throughout pregnancy. A. Number of total 2W1S$^+$ or 2W1S$^+$Foxp3$^+$ CD4 cells per mouse at the indicated pregnancy time point after mating 2W1S-expressing Balb/c males with WT C57Bl/6 females. B. Percentage Foxp3$^+$ among 2W1S$^+$ or 2W1S$^-$ CD4 cells. C. Percentage Ki67$^+$ among 2W1S$^+$Foxp3$^+$ and 2W1S$^+$Foxp3$^-$ CD4 cells. D. Percentage Helios^{hi} among 2W1S$^+$Foxp3$^+$or 2W1S$^-$Foxp3$^+$ CD4 cells. E. Percentage Foxp3$^+$ among Treg-ablated (top) or Treg-sufficient (bottom) donor CD45.1$^+$ or recipient CD45.2$^+$ 2W1S$^+$ CD4 cells midgestation (E11.5). Purified CD4 cells (40 x 10^6) from CD45.1$^+$ Foxp3$^{DTR/DTR}$ mice were transferred into CD45.2$^+$ females that were either treated with DT (two 0.5 μg/dose) (top) or no DT controls (bottom), and mated with 2W1S-expressing males four days thereafter. All data are representative of three or more experiments each with similar results. * $P < 0.05$.

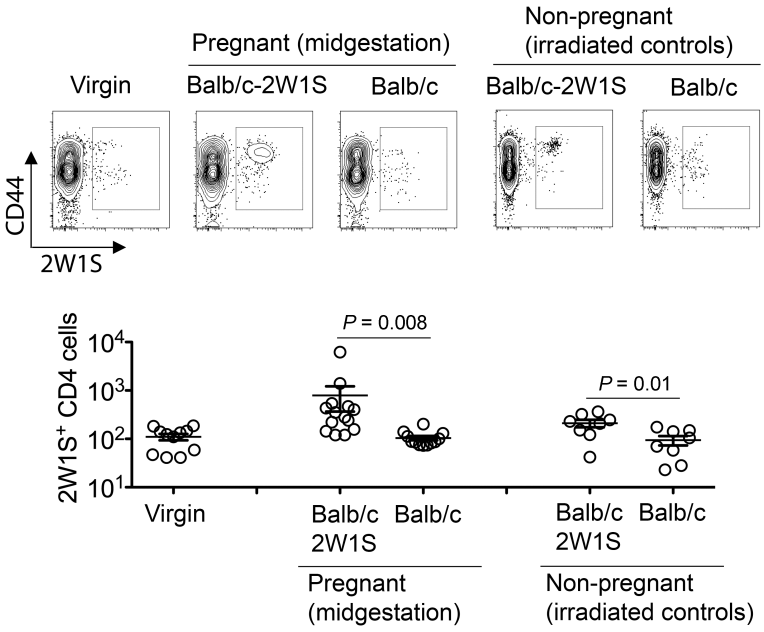

Figure 7.2 - Expansion of fetal 2W1S$^+$ CD4 cells is specific to mating with 2W1S-expressing males. Representative FACS plots (top) and cumulative data illustrating the accumulation of 2W1S tetramer positive CD4 cells in virgin, non-irradiated C57Bl/6 females midgestation (E11.5) after mating with 2W1S-expressing Balb/c or control Balb/c males, or irradiated (100 rads) non-pregnant C57Bl/6 females after continuous mating with 2W1S-expressing Balb/c or control Balb/c males. These data are representative of two independent experiments each with similar results.

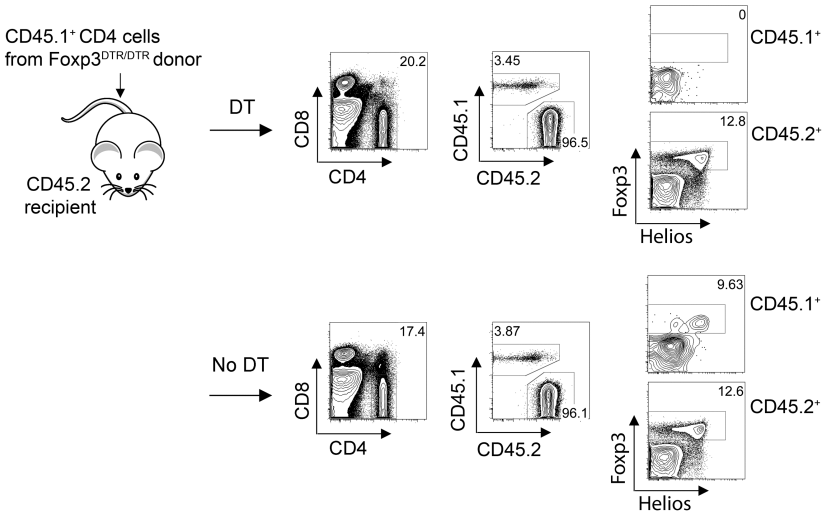

Figure 7.3 - Adoptively transferred Tregs are efficiently eliminated among Foxp3$^{DTR/DTR}$ donor cells. Representative FACS plots illustrating the percent Foxp3^{+} Tregs among each subset of CD4 cells twelve days after adoptive transfer. Purified CD4 cells from CD45.1^{+} Foxp3$^{DTR/DTR}$ mice were adoptively transferred into naïve CD45.2^{+} recipients that were administered diphtheria toxin (two 0.5 μg doses 8 hours apart) (top) or no DT controls (bottom).

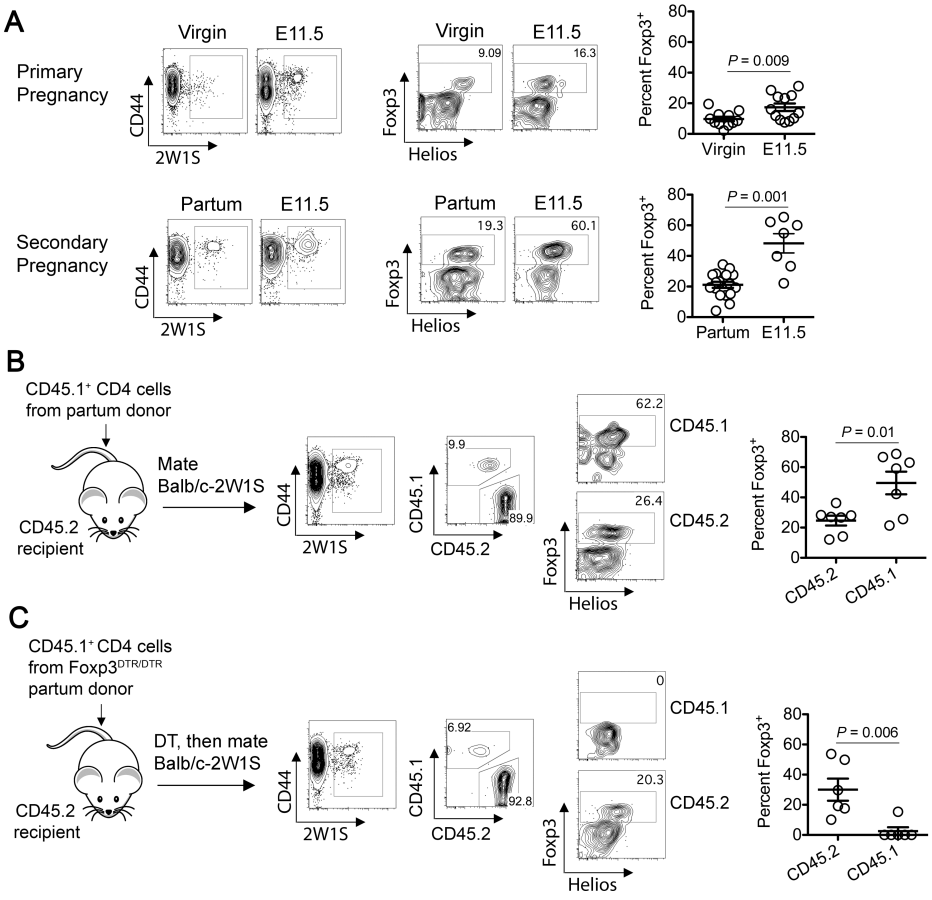

Figure 7.4 - Accelerated expansion of Treg with fetal-specificity during secondary pregnancy from pre-existing Foxp3$^+$ cells retained after primary pregnancy. A. Percentage Foxp3$^+$ among virgin (top, primary pregnancy) or day 14 postpartum (bottom, secondary pregnancy) females before mating or midgestation (E11.5) after mating with 2W1S-expressing males. B. Percentage Foxp3$^+$ among postpartum donor (CD45.1) or recipient (CD45.2) 2W1S$^+$ CD4 cells midgestation (E11.5). Purified CD4 cells (40 x 10^6) from postpartum CD45.1$^+$ Foxp3$^{WT/WT}$ mice were transferred into virgin CD45.2$^+$ females, and mated with 2W1S-expressing males. C. Percentage Foxp3$^+$ among postpartum Foxp3$^{DTR/DTR}$ donor (CD45.1) or naïve recipient (CD45.2) 2W1S$^+$ CD4 cells midgestation (E11.5). Purified CD4 cells (40 x 10^6) from CD45.1$^+$ day 30 post-partum Foxp3$^{DTR/DTR}$ mice were transferred into virgin CD45.2$^+$ females that were treated with DT (two doses 8 hours apart, 0.5 μg/dose), and mated with 2W1S-expressing males four days thereafter. All data are representative of three or more experiments.

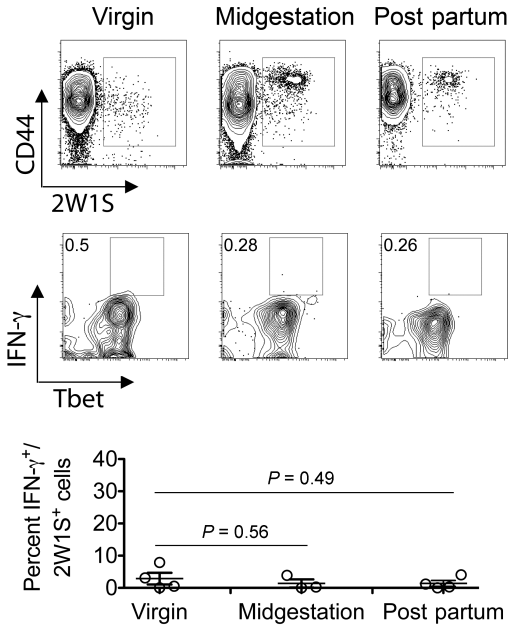

Figure 7.5 - 2W1S$^+$ CD4 cells in naïve, pregnant, and postpartum mice do not produce IFN-γ after stimulation directly ex vivo. Representative FACS plots (top) and cumulative data illustrating percentage of IFN-γ producing 2W1S tetramer positive CD4 cells after stimulation with PMA/Ionomycin for five hours in culture supplemented with brefeldin A.

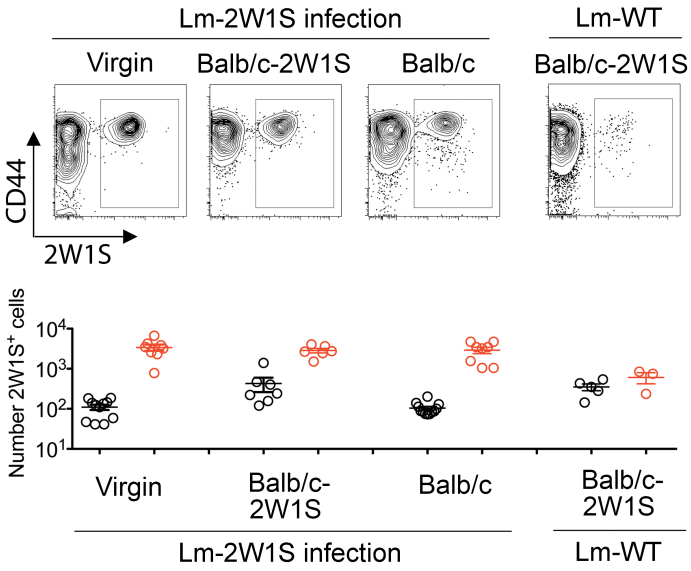

Figure 7.6 - Lm-2W1S primes the selective expansion of 2W1S$^+$ CD4 cells to a similar degree in naïve virgin, or pregnant mice mated with 2W1S-expressing or control Balb/c males. Representative FACS plots (top) and cumulative data illustrating number of 2W1S$^+$ CD4 cells before infection (black circles) or five days after Lm infection (red circles) for each group of mice.

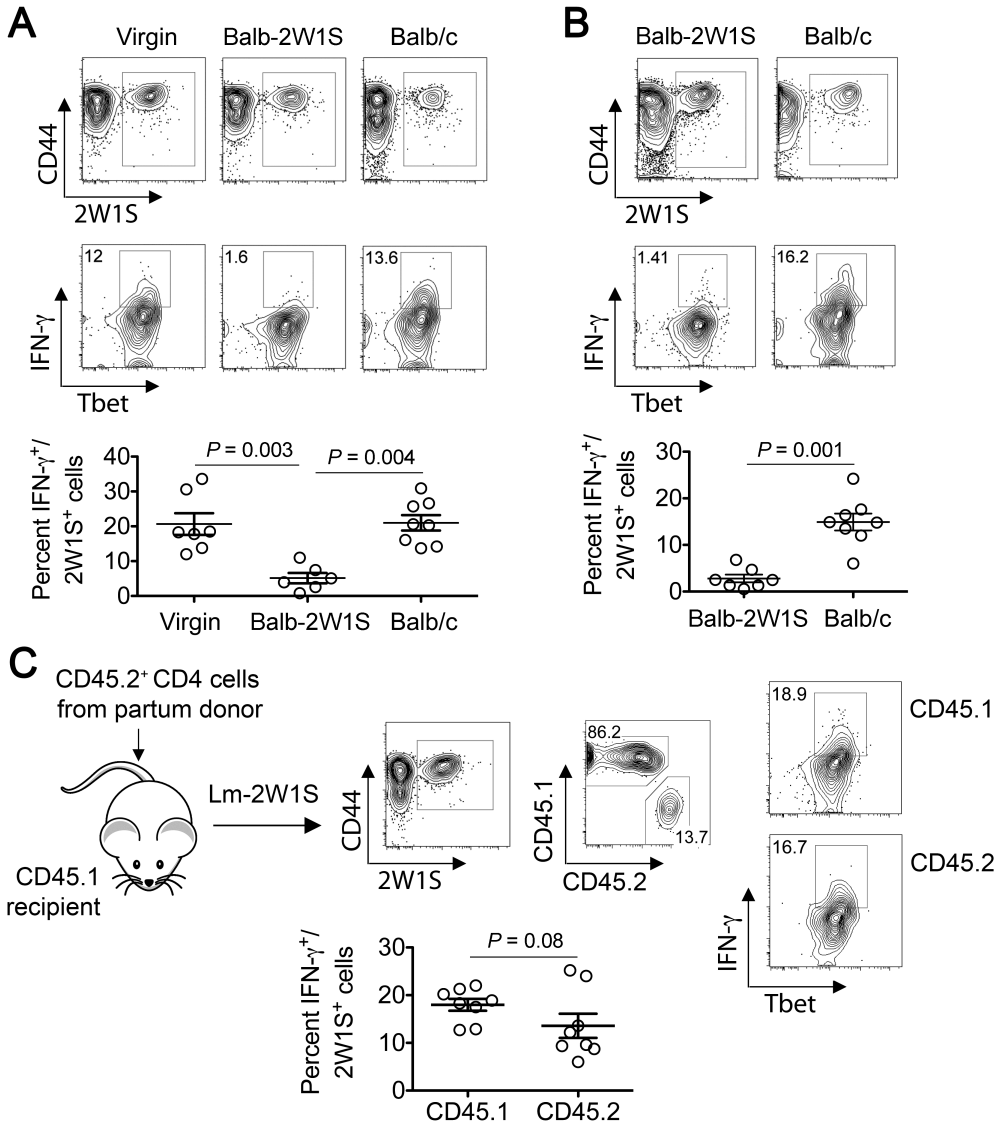

Figure 7.7 - CD4 cells with specificity to fetal or pre-existing fetal antigen are anergic. A. Percentage IFN-γ producing 2W1S$^+$ CD4 cells five days after Lm-2W1S infection in virgin, or pregnant mice midgestation after mating with 2W1S-expressing (Balb/c-2W1S) or control (Balb/c) males. B. Percentage IFN-γ producing 2W1S$^+$ CD4 cells five days after Lm-2W1S infection in postpartum mice impregnated by 2W1S-expressing (Balb/c-2W1S) or control (Balb/c) males. C. Percentage IFN-γ producing postpartum donor (CD45.2) or virgin recipient (CD45.1) CD4 cells five days after Lm-2W1S infection. Cytokine production was assayed after stimulation with PMA/Ionomycin for 5 hours. All data are representative of two or more experiments.

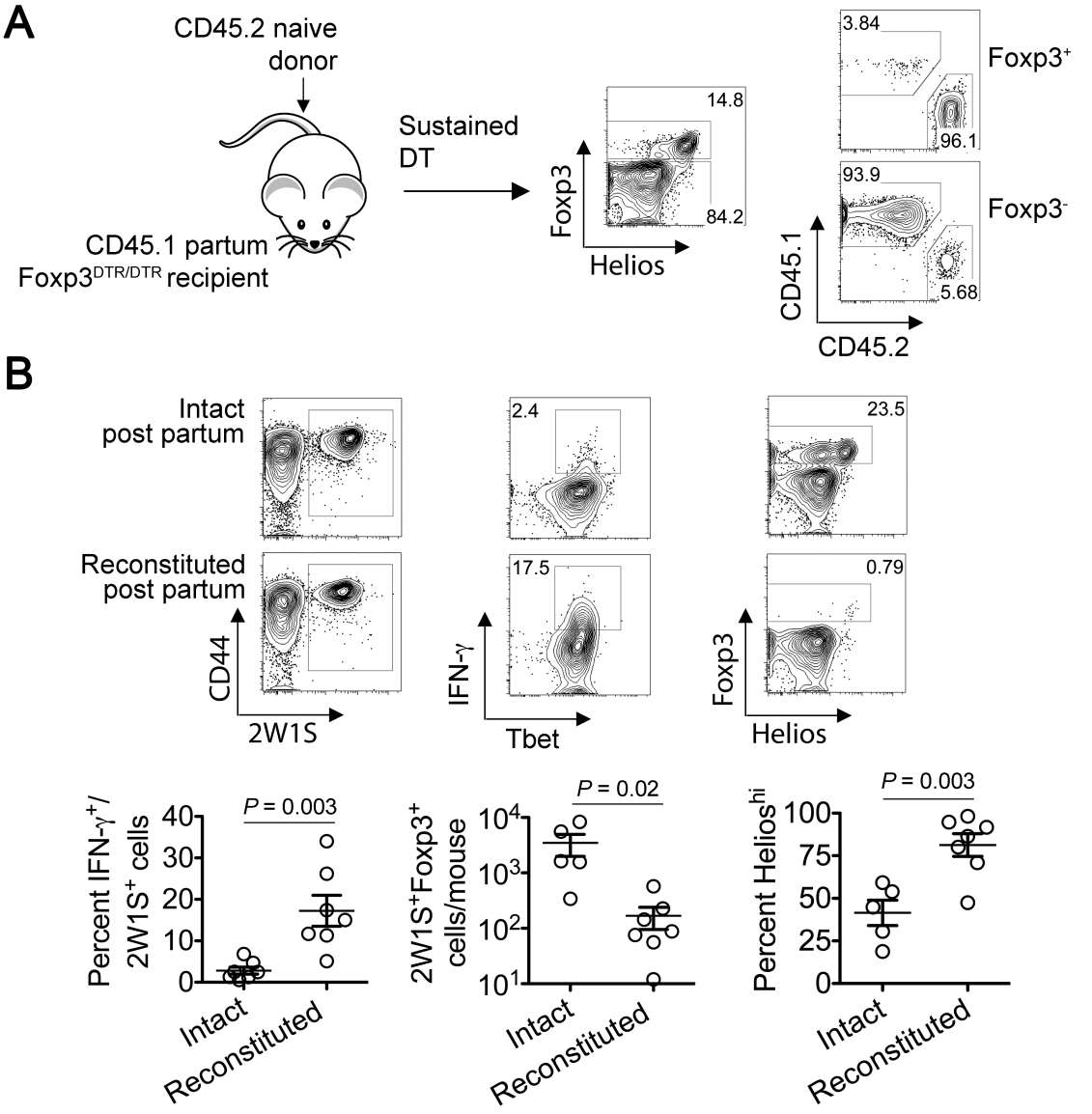

Figure 7.8 - Treg reconstitution reverses anergy among fetal-specific CD4 cells. A. FACS plots illustrating the majority (>95%) of Foxp3$^+$ cells are derived from adoptively transferred donor CD4 cells that repopulate the empty Treg compartment in Foxp3$^{DTR/DTR}$ mice. B. Percentage IFN-γ producing 2W1S$^+$ cells, accumulation of 2W1S$^+$Foxp3$^+$ cells, and Helios expression among 2W1S$^+$Foxp3$^+$ Tregs for each group of mice five days after Lm-2W1S infection. Cytokine production was assayed for cells after stimulation with PMA/Ionomycin for 5 hours. All data are representative of two or more experiments each with similar results.

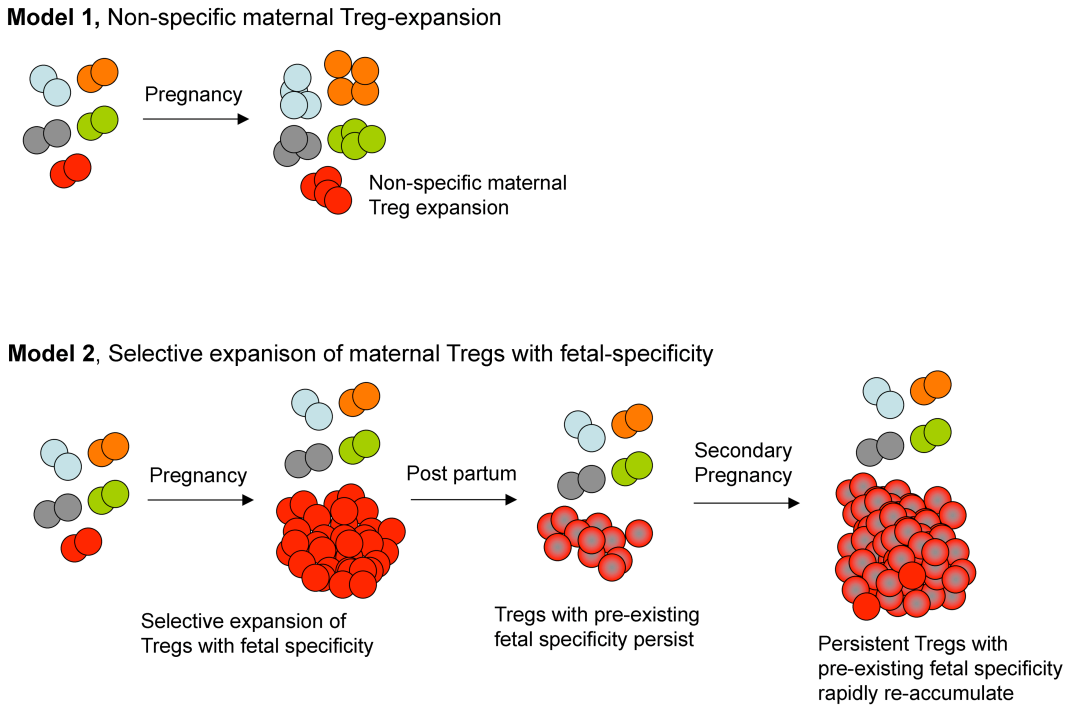

Figure 7.9 - Models comparing maternal Treg expansion. In model 1, pregnancy primes the non-specific expansion of maternal Foxp3^{+} Tregs that is supported by data before investigation using antigen-specific tools. In model 2, maternal Tregs with specificity for fetal antigen selectively expand and accumulate during pregnancy. This model is supported by data presented in this paper where maternal Tregs of a single specificity are found to expand 50 to 100-fold while Foxp3-expression among bulk maternal CD4 cells are only elevated 1 to 2-fold . Furthermore, we show pregnany-activated maternal Tregs are phenotypically distinct, persist after delivery, and rapidly re-expand during secondary pregnancy.

Chapter 8

Discussion[‡‡]

[‡‡] Reprinted in part from Immunology. Jared H. Rowe, James M. Ertelt, and Sing Sing Way. "Foxp3$^+$ regulatory T cells, immune-stimulation and host defense against infection." **© Wiley-Blackwell Publishing. Originally published in *Immunology*. 2012. 136(1):1-10.**

Foxp3^{+} Regulatory T Cell Impacts on Host Defense

Foxp3^{+} Tregs have consistently been shown to impede host defense following infection with bacterial pathogens. This is best illustrated in the context of pregnancy-associated infection susceptibility where the physiological expansion of maternal Tregs required for sustaining tolerance to paternally derived allo-antigens expressed by the developing fetus occurs (Aluvihare et al., 2004; Kahn and Baltimore). In particular following allogeneic mating using defined strains of inbred mice that more closely recapitulates the magnitude of maternal Treg expansion found in human pregnancy, mice with expanded maternal Tregs are markedly more susceptible to infection with intracellular bacterial pathogens like *Listeria monocytogenes* and *Salmonella enterica* each with a natural predisposition for prenatal infection (Gellin et al., 1991; Mylonakis et al., 2002; Pejcic-Karapetrovic et al., 2007; Rowe et al.). Reciprocally, pregnancy associated susceptibility to these pathogens was eliminated with maternal Foxp3^{+} cell ablation when allogeneic pregnancies were established in Foxp3^{DTR} female mice followed by the initiation of DT treatment beginning mid-gestation (Rowe et al.). However, given the necessity for sustained fetal tolerance maintained by expanded maternal Tregs, the ablation of these cells while beneficial for host defense also triggers fetal resorption and pregnancy loss (Aluvihare et al., 2004; Kahn and Baltimore, ; Rowe et al.). In a similar fashion, the expansion of Foxp3^{+} Tregs within the first three days after intranasal *Francisella tularensis* infection has been described to blunt early innate host defense that may represent a unique immune evasion strategy for this pathogen (Periasamy et al., 2011).

The significance of expanded immune suppressive Foxp3$^+$ Tregs in compromising host defense against prenatal infection is further supported by increased susceptibility to *Listeria* for non-pregnant mice with expanded Foxp3$^+$ Tregs that express a constitutively active isoform of STAT5b, and reduced susceptibility with the ablation of expanded Tregs in these and control mice with baseline levels of Tregs (Burchill et al., 2003; Rowe et al.). Therefore, while intracellular bacterial pathogens like *Listeria* and *Salmonella* are capable of *in utero* fetal invasion (Bakardjiev et al., 2006; Le Monnier et al., 2007; Pejcic-Karapetrovic et al., 2007), infection susceptibility during pregnancy is not simply due to the presence of fetal tissue that is susceptible to direct invasion, and instead more likely reflects systemic defects in host defense dictated by expanded maternal Tregs. These findings with experimental *Listeria* infection in mice are also consistent with the epidemiological features of this infection in humans where a significant portion of disseminated maternal infection cases occur without evidence of fetal direct invasion (Mylonakis et al., 2002). Thus, the physiological expansion of maternal Foxp3$^+$ Tregs during pregnancy compromises host defense, and these immune defects are exploited by pathogens like *Listeria* and *Salmonella* with a predisposition for prenatal infection. Importantly, since the expansion of maternal Tregs is blunted during syngeneic pregnancy where the only potential source of antigen heterogeneity between maternal and fetal antigens are those encoded on the Y chromosome, the importance of expanded maternal Tregs in host defense for other prenatal pathogens may have been overlooked in prior studies, and deserve re-investigation using allogeneic pregnancy.

The impacts on host defense dictated by the physiological expansion of immune suppressive Tregs also have broader implications beyond this instance of prenatal infection susceptibility. For example, the progressive expansion of Tregs among peripheral $CD4^+$ T cells occurs with aging throughout the lifespan of humans and mice (Gottenberg et al., 2005; Gregg et al., 2005; Lages et al., 2008; Sharma et al., 2006). In particular, individuals over 60 years have a 3-fold increased proportion of Tregs compared with individuals less than 40 years (Gottenberg et al., 2005; Gregg et al., 2005). In turn when pregnancy associated cases are excluded, individuals over 60 years are also markedly more susceptible to disseminated *Listeria* infection compared with those less than 60 years (Gillespie et al., 2006). Reciprocally following natural West Nile virus infection, symptomatic infection is more common in younger compared with older individuals, and these findings are consistent with the protective role provided by Tregs in this infection (Brown et al., 2007; Lanteri et al., 2009). However, the expansion of Tregs with aging alone does not explain other epidemiological data for this infection where individuals over 70 years compared with those aged 20-69 years have 5-fold increased mortality with West Nile virus infection (O'Leary et al., 2004). Together, these findings suggest other physiological changes with aging play more significant roles in infection-induced mortality despite the expansion of protective Tregs. Nevertheless, these results clearly illustrate how physiological shifts in Tregs likely dictate naturally-occurring variations in susceptibility to specific pathogens among individuals.

Although these results may suggest susceptibility to some infections, and bacterial pathogens in particular, are unavoidable consequences of pregnancy and aging, the increasingly established heterogeneity and functional specialization among Foxp3$^+$ cells also opens up the exciting possibility of therapeutically dissociating the Treg-mediated detrimental impacts on infection susceptibility against some pathogens from their protective roles in other types of infections and their beneficial roles in maintaining immune tolerance (Sakaguchi et al., 2009; Shevach, 2009; Vignali et al., 2008; Wing and Sakaguchi). For example, Tregs are enriched for CTLA-4 expression, and the sustained ablation of CTLA-4 exclusively in Foxp3$^+$ cells throughout development results in non-specific T cell activation and systemic autoimmunity (Friedline et al., 2009; Wing et al., 2008). Importantly, while CTLA-4 ablation in Foxp3$^+$ cells reproduces some features of Treg deficiency, it does not recapitulate the more rapid onset of fatal systemic autoimmunity in mice with naturally occurring or targeted defects in all Tregs due to defects in Foxp3 (Fontenot et al., 2003; Khattri et al., 2003). Comparatively, sustained ablation of IL-10 in Foxp3$^+$ cells throughout development results in minimal systemic autoimmunity, but instead causes inflammation limited to sites with contact to the external environment such as the skin, lung, and intestine (Rubtsov et al., 2008). This discordance in phenotype with sustained ablation of defined molecules in Foxp3$^+$ cells illustrates non-overlapping and specialized context specific roles for individual Treg intrinsic molecules in immune tolerance. However, the ablation of each Treg-intrinsic molecule throughout development using this approach preclude the investigation for how each molecule impacts host defense against infection that ideally requires the

synchronized and coordinated ablation of each molecule in all Foxp3$^+$ cells in adult mice. Using adoptively transferred Tregs containing targeted defects in individual Treg-intrinsic molecules to reconstitute Foxp3$^+$ cell ablated mice overcomes this technical barrier for systemically interrogating the importance of each Treg-intrinsic molecule in host defense against acute infection. Our initial studies using this approach illustrate Treg intrinsic IL-10, but not CTLA-4, participates in compromising host defense against *Listeria monocytogenes* (Rowe et al.). Therefore, establishing the Foxp3$^+$ cell intrinsic molecules that compromise or augment host defense, and dissociating these from the Treg intrinsic molecules required for sustaining immune tolerance represent pivotally important next steps in this exciting area with enormous translational implications.

The co-expression of many effector CD4$^+$ T cell lineage promoting transcription factors by Foxp3$^+$ Tregs that allows functionally distinct Treg subsets to expand in parallel with effector T cells has been recently established. For example, Tregs that express the Th1 lineage defining transcription factor T-bet expand with Th1 effector CD4$^+$ T cells following Th1 stimulation conditions, while the ablation of T-bet specifically in Foxp3$^+$ cells results in uncontrolled Th1 inflammation and autoimmunity (Koch et al., 2009). Similarly, Foxp3$^+$ cell expression of the transcription factors STAT-3, IRF-4, BCL-6, and GATA-3 have each been described to suppress other specialized effector CD4$^+$ T cell subsets that would otherwise cause un-checked self-reactive inflammation (Chaudhry et al., 2009; Chung et al., 2011; Linterman et al., 2011; Wohlfert et al., ; Zheng et al., 2009). Importantly, the specialization and dynamic regulation among these various Treg subsets

also play important roles in coordinating and fine-tuning immune responses after infection. For example, under Th1 inflammatory conditions triggered by *M. tuberculosis*, T-bet expressing Tregs and effector T cells both expand and are recruited into the sites of infection creating a balanced response that facilitates pathogen control, but not eradication (Koch et al., 2009). On the other hand under Th2 inflammatory conditions triggered by pulmonary thymic stromal lymphopoietin or intestinal *Heligmosomoides polygyrus* infection, T-bet$^+$ Tregs fail to accumulate and are instead replaced by Tregs enriched for the Th2 promoting transcription factor GATA-3 (Koch et al., 2009; Wohlfert et al.). Interestingly, although the ablation of Foxp3$^+$ Tregs early after *Heligmosomoides polygyrus* infection augments parasite-specific effector Th2 responses and intestinal inflammation, no significant impacts of pathogen burden or fitness were identified (Rausch et al., 2009).

Specialization among Tregs during persistent infection is not limited only to expression of CD4$^+$ T cell lineage defining transcription factors, but also extends to individual cell-intrinsic molecules that likely mediate immune suppression. Foxp3$^+$ Tregs recovered from the pulmonary lymph node and lung selectively up-regulate ICOS and PD-1 expression at relatively early and late time points, respectively, after aerosol *M. tuberculosis* infection while these shifts do not occur for Tregs in lymph nodes that do not drain the site of infection (Scott-Browne et al., 2007). Similarly when the impacts of Treg-ablation are progressively reduced from early to late time points after systemic *Salmonella* infection, Foxp3$^+$ Tregs in the spleen progressively loose CTLA-4 expression

that is replaced by increased GITR expression (Johanns et al., 2010b). Thus, functionally distinct Treg subsets that express unique combinations of cell intrinsic molecules accumulate and shift throughout the course of persistent infection. Establishing the role of individual Treg-intrinsic molecules in dictating the progression of persistent infection, and investigating how cell intrinsic shifts in expression of each effector $CD4^+$ T cell lineage defining transcription factors controls Treg suppression represent important next steps in further unraveling the dynamic interplay between Tregs and effector T cells in host defense during persistent infection.

In addition to the paralleled accumulation of lineage-specific Tregs and effector T cells, the co-expansion of $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells exhibiting the same specificity for pathogen associated antigens also occurs during some persistent infections. For example, Tregs and effector T cells with specificity to the same pathogen-expressed antigen expand in parallel following intradermal *Leishmania*, pulmonary *M. tuberculosis*, systemic *Salmonella*, or intracerebral coronavirus infections (Johanns et al., 2010b; Shafiani et al., ; Suffia et al., 2006; Zhao et al., 2011). By contrast, for other infections including those caused by *Listeria monocytogenes* in immune competent mice and persistent Friend retrovirus in B and $CD8^+$ T cell deficient mice, only the selective expansion of pathogen-specific $Foxp3^-$ effector $CD4^+$ T cells occur (Antunes et al., 2008; Ertelt et al., 2009). However for persistent infections that prime the expansion of pathogen-specific Tregs, these cells are likely to play pivotally important roles in pathogen persistence because augmenting the absolute numbers of these cells in *M.*

tuberculosis infected mice results in dose dependent increased pathogen burden and delayed expansion of pathogen-specific effector T cells (Shafiani et al.). Similarly, $Foxp3^+$ Tregs with specificity to defined species of enteric commensal bacteria are found in intestinal tissues, and these cells selectively avert intestinal inflammation in colonized mice (Lathrop et al.). Thus, with the identification of more microbe-specific MHC class II peptide antigens and the development of enrichment tools to track very small populations of antigen-specific $CD4^+$ T cells (Moon et al., 2009), microbe-specific $Foxp3^+$ Tregs will undoubtedly prove to play more significant roles in regulating both host defense and immune homeostasis. In this regard, interrogating the differentiation stability for pathogen-specific Tregs, and investigating if the functional plasticity described for Tregs with specificity for self-antigen are applicable for infection induced Tregs represent important areas for further investigation (Zhao et al., 2011; Zhou et al., 2009a; Zhou et al., 2009b).

Plasticity in Treg Suppression

Given the active immune suppression by Tregs that occurs *in vivo*, counter-regulatory mechanisms that override Treg suppression must be engaged when immune activation occurs naturally during infection or immunization. In this regard, several infection response pathways have been shown to bypass the impacts of Treg suppression. For example, antigen presenting cell (APCs) stimulation with highly conserved microbial ligands (e.g. LPS or CpG DNA) through Toll-like receptors (TLRs) drives effector T cell proliferation despite the presence of Tregs (Pasare and Medzhitov, 2003). This is in large part mediated by IL-6 production by activated APCs because effector T cell proliferation is reduced when IL-6 deficient APCs or this cytokine is neutralized in co-culture (Pasare and Medzhitov, 2003). Similarly, other purified TLR agonists and inflammatory cytokines that induce the maturation of dendritic cells and augment expression cell surface molecules that promote T cell stimulation (e.g. CD80, CD86, and MHC) have also been reported to override Treg suppression through IL-6 independent pathways (Kubo et al., 2004; Takahashi et al., 1998; Thornton and Shevach, 1998). Furthermore, even in the absence of APCs, cell-intrinsic stimulation through defined TLRs can also trigger shifts in Treg suppression. For example, purified TLR2 agonists stimulate reductions in suppressive potency for mouse Tregs, and TLR8 agonists trigger similar reductions in potency for human Tregs (Liu et al., 2006; Peng et al., 2005; Sutmuller et al., 2006). On the other hand, microbial ligands can also augment Treg suppressive potency. Mouse $CD25^+$ Tregs selectively express TLR4, and LPS stimulation augments

their suppressive potency (Caramalho et al., 2003); while flagellin stimulation via TLR5 augments the suppressive potency of human Tregs (Crellin et al., 2005). Taken together, these *in vitro* studies illustrate the enormous potential whereby microbes and the response to infection can influence immune activation though shifts in Treg suppression.

The cumulative impacts whereby pathogens that express multiple TLR ligands and the ensuing immune response on shifts in Treg suppressive potency have also been characterized for GFP^+ cells recovered from $Foxp3^{GFP}$ reporter mice directly *ex vivo* following infection (Fontenot et al., 2005b). For example at relatively early time points during persistent *Salmonella* infection when the activation of effector T cells is blunted and the pathogen burden is progressively increasing, the suppressive potency for GFP^+ Tregs is augmented (Johanns et al., 2010b). Conversely at later infection time points when effector T cells are highly activated and progressive reductions in pathogen burden occur, the suppressive potency for $Foxp3^+$ cells is reduced. Together with the waning impacts of $Foxp3^+$ cell ablation with infection progression, these results illustrate how shifts in Treg-suppression can dictate the tempo of persistent infection (Johanns et al., 2010b). Similarly following acute *Listeria* infection, reductions in suppressive potency are found for GFP^+ Tregs that immediately precede the expansion of pathogen-specific effector T cells (Ertelt et al.). The expansion of circulating Tregs with increased suppressive potency is associated with increased parasite burdens for patients with severe malaria infection (Minigo et al., 2009). However, no significant changes in suppressive potency were found for $Foxp3^+$ Tregs isolated directly *ex-vivo* after *Plasmodium berghei*

infection in mice (Steeg et al., 2009). Nevertheless, these findings illustrate how infection-induced shifts in Foxp3$^+$ Treg suppressive potency may play important and increasingly appreciated roles in infection outcomes.

Importantly, the increasing established plasticity in Treg suppression can also be exploited for boosting the immune response of vaccines against pathogen-specific or tumor-associated antigens (van Duin et al., 2006; Wang et al., 2006; Zhou et al., 2009a). In this regard, reduced Treg suppression after stimulation with various purified microbial ligands suggest classical vaccine adjuvants derived from crude microbial preparations may simulate immune activation by overriding Treg-mediated immune suppression. Indeed, the transient ablation of Foxp3$^+$ cells alone during stimulation with purified peptide is sufficient to trigger the robust activation, expansion, and formation of memory CD8$^+$ T cells that confers protection against subsequent *Listeria* infection in an antigen-specific fashion (Ertelt et al.). Similarly, Foxp3$^+$ cell ablation augments the expansion and activation of antigen-specific CD8$^+$ T cell primed by the live attenuated viral vector *modified vaccinia virus Ankara* (Kastenmuller et al.). These findings are consistent with the enhanced vaccine induced immunogenicity that occurs with Treg-ablation using anti-CD25 antibody treatment, and the sustained priming of protective CD8$^+$ T cells by attenuated *Listeria* even in mice lacking all known signal 3 inflammatory cytokines (Curtsinger and Mescher, 2010; Ertelt et al., 2010; Haring et al., 2006; Kursar et al., 2002; Moore et al., 2005; Toka et al., 2004). Thus, overriding immune suppression by Tregs likely plays pivotally important roles in stimulating protective T cell responses *in*

vivo. However, while immune adjuvants and vaccine vectors have traditionally been evaluated for their ability to activate T cells indirectly through stimulation of professional APCs that in turn elaborate defined stimulation signals (T cell receptor [signal 1], co-stimulation [signal 2], and inflammatory cytokines [signal 3]) (Curtsinger and Mescher, 2010; Haring et al., 2006; Kastenmuller et al.), overriding active suppression by Tregs represents a more fundamental prerequisite "signal zero" essential for stimulating effector T cell activation *in vivo*. Although this term has recently been used to describe the activation of innate immunity or chemokine gradients that each also participate in T cell activation (Bousso and Albert, ; Mays and Wilson), we propose this more generalizable descriptor is more appropriate for overriding the impacts of suppression mediated by Tregs and other immune suppressive cells that actively restrains T cell activation (Figure 8.1).

Concluding Remarks

Since the identification of regulatory T cells as a separate and defined lineage of $CD4^+$ T cells, there has been an explosion of studies describing the role these cells play in almost every aspect of the immune response. With the establishment of Foxp3 expression as the lineage specific marker for regulatory T cells and the development of transgenic mouse tools for manipulating $Foxp3^+$ cells *in vivo*, newfound protective roles for these cells in host defense against some infections have been recently uncovered (Table 8.1). In turn for other infections, the detrimental roles $Foxp3^+$ cells play in host defense have been reinforced (Table 8.1). Along the way, an amazing degree of heterogeneity and functional specialization for $Foxp3^+$ regulatory T cells in terms of antigen-specificity, paralleled differentiation along effector $CD4^+$ T cell lineages, and usage of individual cell intrinsic molecules to mediate context specific immune suppression have each been established. Coupled with increasing refined approaches for expanding human regulatory T cells or manipulating the suppressive potency of these cells using purified adjuvants (Brunstein et al., ; Hippen et al., ; van Duin et al., 2006; Wang et al., 2006), these multiple layers of heterogeneity in regulatory T cells reveal many exciting opportunities for therapeutically dissociating the detrimental and beneficial impacts these cells play in host defense against infection and immune homeostasis.

Importantly, the physiologic expansion of Tregs, which occurs naturally during pregnancy, is essential for the active maintenance of maternal-fetal tolerance. However,

these beneficial impacts of Foxp3$^+$ Tregs on fetal tolerance occur at the expense of maternal susceptibility to prenatal pathogens. In particular, pathogens like *Listeria* with a unique predilection to cause serious disseminated infections in pregnant women, have evolved the ability to exploit this temporary defect in host defense caused by Treg expansion. Interestingly, we find that through the functional dissociation of Treg suppressive mechanisms, selective inhibition of IL-10 presented a novel strategy to bolster maternal host defense to prenatal *Listeria* infections. Moreover, the ability for the intracellular invasion of this pathogen to fracture maternal-fetal tolerance and trigger sterile abortion, likely represents the dynamic interplay between Treg functional plasticity and could explain the spontaneous miscarriages demonstrated by many prenatal pathogens. Lastly, the fetal-specific expansion of Tregs driven by pregnancy and subsequent "regulatory" memory following parturition, represents the first demonstration of a physiologic system for priming long-lived memory Foxp3$^+$ Tregs. Together the findings presented in our studies have begun to unravel some of these fundamental questions about the biology of Tregs, in dictating the outcome of infections. Therefore, the natural extension of this work will be to begin dissociating the functional diversity of Tregs using strategies, which manipulate this cell type based on antigen specificity.

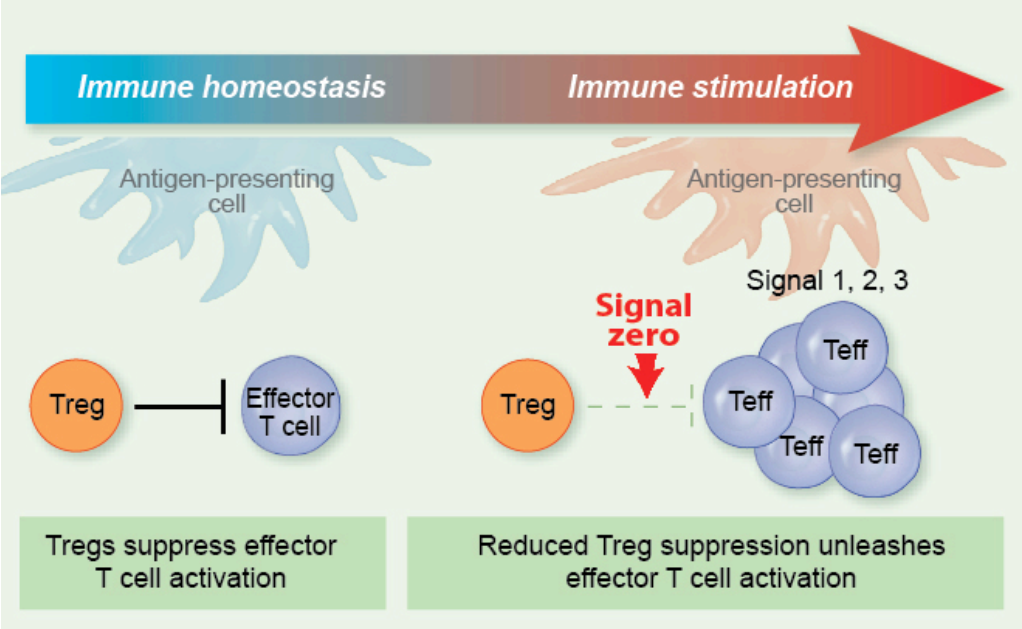

Figure 8.1 - Model whereby overriding Foxp3^{+} Treg-mediated immune suppression represents a prerequisite "signal zero" for effector T cell activation *in vivo*. During immune homeostasis, Tregs actively suppress effector cell activation. Following immune stimulation, Treg-suppression is blunted allowing effector T cell activation through previously described cell intrinsic stimulation signals (T cell receptor [signal 1], co-stimulation [signal 2], inflammatory cytokines [signal 3]).

Class	Pathogen	Impacts of Foxp3$^+$ cell manipulation on host defense		Reference
Virus	Herpes simplex virus-2	Protective	Foxp3$^+$ cell ablation accelerates mortality and increases viral load	*Lund JM* 2008
	Lymphocytic choriomeningitis virus	Protective	Foxp3$^+$ cell ablation increases viral load	*Lund JM* 2008
	West Nile virus	Protective	Foxp3$^+$ cell ablation increases viral load and mortality	*Lanteri MC* 2009
Parasite	*Plasmodium berghei*	Protective	Foxp3$^+$ cell expansion protects against severe disease and reduces pathogen burden that are each reversed by Foxp3$^+$ cell ablation	*Haque A* 2008
	Plasmodium berghei	No effect	Foxp3$^+$ cell ablation from baseline levels has no impact on survival or pathogen burden	*Haque A* 2008, *Steeg C* 2009
	Toxoplasma gondii	Protective	Natural collapse of Foxp3$^+$ Tregs following infection results in fatal infection that is reversed by Treg stabilization using IL-2 cytokine/antibody complexes	*Oldenhove G* 2009
	Heligmosomoides polygyrus	No effect	No changes pathogen burden with Foxp3$^+$ cell ablation	*Rausch S* 2009
Fungi	*Candida albicans*	Protective	Tregs co-transferred with effector CD4$^+$ T cells enhance fungal clearance	*Pandiyan P* 2011
Bacteria	*Listeria monocytogenes*	Detrimental	Foxp3$^+$ cell expansion results in increased pathogen burden that is reversed by Foxp3$^+$ cell ablation	*Rowe JH* 2011
	Salmonella enterica	Detrimental	Foxp3$^+$ cell expansion during pregnancy results in increased pathogen burden that is reversed by Foxp3$^+$ cell ablation	*Rowe JH* 2011
	Salmonella enterica	Detrimental	Foxp3$^+$ cell ablation accelerates bacterial clearance and effector T cell activation	*Johanns TM* 2010
Myco-bacteria	*Mycobacterium tuberculosis*	Detrimental	Selective depletion of Foxp3$^+$ cells in mixed chimera mice reduces pathogen burden	*Scott-Browne JP* 2007
	Mycobacterium tuberculosis	Detrimental	Adoptive transfer of pathogen-specific Foxp3$^+$ cells blunts effector cell expansion and increased pathogen burden	*Shafiani S* 2010

Table 8.1 - Impacts of Foxp3$^+$ cell manipulation on host defense

Bibliography

1. Aluvihare, V.R., Kallikourdis, M., and Betz, A.G. (2004). Regulatory T cells mediate maternal tolerance to the fetus. Nature immunology *5*, 266-271.
2. Anderson, K.M., Czinn, S.J., Redline, R.W., and Blanchard, T.G. (2006). Induction of CTLA-4-mediated anergy contributes to persistent colonization in the murine model of gastric Helicobacter pylori infection. J Immunol *176*, 5306-5313.
3. Antunes, I., Tolaini, M., Kissenpfennig, A., Iwashiro, M., Kuribayashi, K., Malissen, B., Hasenkrug, K., and Kassiotis, G. (2008). Retrovirus-specificity of regulatory T cells is neither present nor required in preventing retrovirus-induced bone marrow immune pathology. Immunity *29*, 782-794.
4. Asseman, C., Mauze, S., Leach, M.W., Coffman, R.L., and Powrie, F. (1999). An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. The Journal of experimental medicine *190*, 995-1004.
5. Auerbuch, V., Brockstedt, D.G., Meyer-Morse, N., O'Riordan, M., and Portnoy, D.A. (2004). Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes. The Journal of experimental medicine *200*, 527-533.
6. Bahjat, K.S., Liu, W., Lemmens, E.E., Schoenberger, S.P., Portnoy, D.A., Dubensky, T.W., Jr., and Brockstedt, D.G. (2006). Cytosolic entry controls CD8+-T-cell potency during bacterial infection. Infection and immunity *74*, 6387-6397.
7. Bakardjiev, A.I., Theriot, J.A., and Portnoy, D.A. (2006). Listeria monocytogenes traffics from maternal organs to the placenta and back. PLoS pathogens *2*, e66.
8. Barber, D.L., Mayer-Barber, K.D., Feng, C.G., Sharpe, A.H., and Sher, A. (2011). CD4 T cells promote rather than control tuberculosis in the absence of PD-1-mediated inhibition. J Immunol *186*, 1598-1607.
9. Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J., and Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. Nature *439*, 682-687.

10. Barber, E.M., Fazzari, M., and Pollard, J.W. (2005). Th1 cytokines are essential for placental immunity to Listeria monocytogenes. Infection and immunity *73*, 6322-6331.
11. Beckerman, K.P., Rogers, H.W., Corbett, J.A., Schreiber, R.D., McDaniel, M.L., and Unanue, E.R. (1993). Release of nitric oxide during the T cell-independent pathway of macrophage activation. Its role in resistance to Listeria monocytogenes. J Immunol *150*, 888-895.
12. Belkaid, Y. (2007). Regulatory T cells and infection: a dangerous necessity. Nature reviews *7*, 875-888.
13. Belkaid, Y., Piccirillo, C.A., Mendez, S., Shevach, E.M., and Sacks, D.L. (2002). CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature *420*, 502-507.
14. Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F., and Ochs, H.D. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nature genetics *27*, 20-21.
15. Bhadra, R., Gigley, J.P., Weiss, L.M., and Khan, I.A. (2011). Control of Toxoplasma reactivation by rescue of dysfunctional CD8+ T-cell response via PD-1-PDL-1 blockade. Proceedings of the National Academy of Sciences of the United States of America *108*, 9196-9201.
16. Boasso, A., Hardy, A.W., Landay, A.L., Martinson, J.L., Anderson, S.A., Dolan, M.J., Clerici, M., and Shearer, G.M. (2008). PDL-1 upregulation on monocytes and T cells by HIV via type I interferon: restricted expression of type I interferon receptor by CCR5-expressing leukocytes. Clinical immunology (Orlando, Fla *129*, 132-144.
17. Boockvar, K.S., Granger, D.L., Poston, R.M., Maybodi, M., Washington, M.K., Hibbs, J.B., Jr., and Kurlander, R.L. (1994). Nitric oxide produced during murine listeriosis is protective. Infection and immunity *62*, 1089-1100.
18. Bousso, P., and Albert, M.L. (2010). Signal 0 for guided priming of CTLs: NKT cells do it too. Nature immunology *11*, 284-286.

19. Boyman, O., Kovar, M., Rubinstein, M.P., Surh, C.D., and Sprent, J. (2006). Selective stimulation of T cell subsets with antibody-cytokine immune complexes. Science (New York, N.Y *311*, 1924-1927.

20. Brombacher, F., Dorfmuller, A., Magram, J., Dai, W.J., Kohler, G., Wunderlin, A., Palmer-Lehmann, K., Gately, M.K., and Alber, G. (1999). IL-12 is dispensable for innate and adaptive immunity against low doses of Listeria monocytogenes. International immunology *11*, 325-332.

21. Brooks, D.G., Trifilo, M.J., Edelmann, K.H., Teyton, L., McGavern, D.B., and Oldstone, M.B. (2006). Interleukin-10 determines viral clearance or persistence in vivo. Nature medicine *12*, 1301-1309.

22. Brown, J.A., Factor, D.L., Tkachenko, N., Templeton, S.M., Crall, N.D., Pape, W.J., Bauer, M.J., Ambruso, D.R., Dickey, W.C., and Marfin, A.A. (2007). West Nile viremic blood donors and risk factors for subsequent West Nile fever. Vector Borne Zoonotic Dis *7*, 479-488.

23. Brown, K.E., Freeman, G.J., Wherry, E.J., and Sharpe, A.H. (2010). Role of PD-1 in regulating acute infections. Current opinion in immunology *22*, 397-401.

24. Brundage, R.A., Smith, G.A., Camilli, A., Theriot, J.A., and Portnoy, D.A. (1993). Expression and phosphorylation of the Listeria monocytogenes ActA protein in mammalian cells. Proc Natl Acad Sci U S A *90*, 11890-11894.

25. Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nature genetics *27*, 68-73.

26. Brunstein, C.G., Miller, J.S., Cao, Q., McKenna, D.H., Hippen, K.L., Curtsinger, J., Defor, T., Levine, B.L., June, C.H., Rubinstein, P.*, et al.* (2011). Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. Blood *117*, 1061-1070.

27. Brzoza, K.L., Rockel, A.B., and Hiltbold, E.M. (2004). Cytoplasmic entry of Listeria monocytogenes enhances dendritic cell maturation and T cell differentiation and function. J Immunol *173*, 2641-2651.

28. Buchel, E., Van Steenbergen, W., Nevens, F., and Fevery, J. (2002). Improvement of autoimmune hepatitis during pregnancy followed by flare-up after delivery. The American journal of gastroenterology *97*, 3160-3165.
29. Burchill, M.A., Goetz, C.A., Prlic, M., O'Neil, J.J., Harmon, I.R., Bensinger, S.J., Turka, L.A., Brennan, P., Jameson, S.C., and Farrar, M.A. (2003). Distinct effects of STAT5 activation on CD4+ and CD8+ T cell homeostasis: development of CD4+CD25+ regulatory T cells versus CD8+ memory T cells. J Immunol *171*, 5853-5864.
30. Caramalho, I., Lopes-Carvalho, T., Ostler, D., Zelenay, S., Haury, M., and Demengeot, J. (2003). Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. The Journal of experimental medicine *197*, 403-411.
31. Carrero, J.A., Calderon, B., and Unanue, E.R. (2004). Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection. The Journal of experimental medicine *200*, 535-540.
32. Carrero, J.A., Calderon, B., and Unanue, E.R. (2006). Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes. The Journal of experimental medicine *203*, 933-940.
33. Cecchinato, V., Tryniszewska, E., Ma, Z.M., Vaccari, M., Boasso, A., Tsai, W.P., Petrovas, C., Fuchs, D., Heraud, J.M., Venzon, D.*, et al.* (2008). Immune activation driven by CTLA-4 blockade augments viral replication at mucosal sites in simian immunodeficiency virus infection. J Immunol *180*, 5439-5447.
34. Chaudhry, A., Rudra, D., Treuting, P., Samstein, R.M., Liang, Y., Kas, A., and Rudensky, A.Y. (2009). CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science (New York, N.Y *326*, 986-991.
35. Chung, Y., Tanaka, S., Chu, F., Nurieva, R.I., Martinez, G.J., Rawal, S., Wang, Y.H., Lim, H., Reynolds, J.M., Zhou, X.H.*, et al.* (2011). Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nature medicine *17*, 983-988.

36. Conde-Agudelo, A., and Belizan, J.M. (2000). Maternal morbidity and mortality associated with interpregnancy interval: cross sectional study. BMJ (Clinical research ed *321*, 1255-1259.

37. Cossart, P. (2011). Illuminating the landscape of host-pathogen interactions with the bacterium Listeria monocytogenes. Proceedings of the National Academy of Sciences of the United States of America *108*, 19484-19491.

38. Cousens, L.P., Peterson, R., Hsu, S., Dorner, A., Altman, J.D., Ahmed, R., and Biron, C.A. (1999). Two roads diverged: interferon alpha/beta- and interleukin 12-mediated pathways in promoting T cell interferon gamma responses during viral infection. The Journal of experimental medicine *189*, 1315-1328.

39. Crellin, N.K., Garcia, R.V., Hadisfar, O., Allan, S.E., Steiner, T.S., and Levings, M.K. (2005). Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. J Immunol *175*, 8051-8059.

40. Curtis, M.M., Rowell, E., Shafiani, S., Negash, A., Urdahl, K.B., Wilson, C.B., and Way, S.S. (2010). Fidelity of pathogen-specific CD4+ T cells to the Th1 lineage is controlled by exogenous cytokines, interferon-gamma expression, and pathogen lifestyle. Cell Host Microbe *8*, 163-173.

41. Curtis, M.M., and Way, S.S. (2009). Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. Immunology *126*, 177-185.

42. Curtsinger, J.M., and Mescher, M.F. (2010). Inflammatory cytokines as a third signal for T cell activation. Current opinion in immunology *22*, 333-340.

43. Curtsinger, J.M., Valenzuela, J.O., Agarwal, P., Lins, D., and Mescher, M.F. (2005). Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J Immunol *174*, 4465-4469.

44. Dai, W.J., Kohler, G., and Brombacher, F. (1997). Both innate and acquired immunity to Listeria monocytogenes infection are increased in IL-10-deficient mice. J Immunol *158*, 2259-2267.

45. Day, C.L., Kaufmann, D.E., Kiepiela, P., Brown, J.A., Moodley, E.S., Reddy, S., Mackey, E.W., Miller, J.D., Leslie, A.J., DePierres, C*., et al.* (2006). PD-1

expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature *443*, 350-354.

46. de Fougerolles, A.R., and Baines, M.G. (1987). Modulation of the natural killer cell activity in pregnant mice alters the spontaneous abortion rate. Journal of reproductive immunology *11*, 147-153.
47. Doherty, T.M., Chougnet, C., Schito, M., Patterson, B.K., Fox, C., Shearer, G.M., Englund, G., and Sher, A. (1999). Infection of HIV-1 transgenic mice with Mycobacterium avium induces the expression of infectious virus selectively from a Mac-1-positive host cell population. J Immunol *163*, 1506-1515.
48. Dong, H., Zhu, G., Tamada, K., and Chen, L. (1999). B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med *5*, 1365-1369.
49. Duraiswamy, J., Ibegbu, C.C., Masopust, D., Miller, J.D., Araki, K., Doho, G.H., Tata, P., Gupta, S., Zilliox, M.J., Nakaya, H.I.*, et al.* (2011). Phenotype, function, and gene expression profiles of programmed death-1(hi) CD8 T cells in healthy human adults. J Immunol *186*, 4200-4212.
50. Edelson, B.T., and Unanue, E.R. (2002). MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity. J Immunol *169*, 3869-3875.
51. Ehst, B.D., Ingulli, E., and Jenkins, M.K. (2003). Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. Am J Transplant *3*, 1355-1362.
52. Ejrnaes, M., Filippi, C.M., Martinic, M.M., Ling, E.M., Togher, L.M., Crotty, S., and von Herrath, M.G. (2006). Resolution of a chronic viral infection after interleukin-10 receptor blockade. The Journal of experimental medicine *203*, 2461-2472.
53. Erlebacher, A., Vencato, D., Price, K.A., Zhang, D., and Glimcher, L.H. (2007). Constraints in antigen presentation severely restrict T cell recognition of the allogeneic fetus. The Journal of clinical investigation *117*, 1399-1411.

54. Ertelt, J.M., Johanns, T.M., Rowe, J.H., and Way, S.S. (2010). Interleukin (IL)-21-independent pathogen-specific CD8+ T-cell expansion, and IL-21-dependent suppression of CD4+ T-cell IL-17 production. Immunology *131*, 183-191.

55. Ertelt, J.M., Rowe, J.H., Johanns, T.M., Lai, J.C., McLachlan, J.B., and Way, S.S. (2009). Selective priming and expansion of antigen-specific Foxp3- CD4+ T cells during Listeria monocytogenes infection. J Immunol *182*, 3032-3038.

56. Ertelt, J.M., Rowe, J.H., Mysz, M.A., Singh, C., Roychowdhury, M., Aguilera, M.N., and Way, S.S. (2011a). Foxp3+ regulatory T cells impede the priming of protective CD8+ T cells. J Immunol *187*, 2569-2577.

57. Ertelt, J.M., Rowe, J.H., Mysz, M.A., Singh, C., Roychowdhury, M., Aguilera, M.N., and Way, S.S. (2011b). Foxp3+ Regulatory T Cells Impede the Priming of Protective CD8+ T Cells. J Immunol *187*, 2569-2577.

58. Fallarino, F., Grohmann, U., Hwang, K.W., Orabona, C., Vacca, C., Bianchi, R., Belladonna, M.L., Fioretti, M.C., Alegre, M.L., and Puccetti, P. (2003). Modulation of tryptophan catabolism by regulatory T cells. Nature immunology *4*, 1206-1212.

59. Flynn, J.L., Chan, J., Triebold, K.J., Dalton, D.K., Stewart, T.A., and Bloom, B.R. (1993). An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. The Journal of experimental medicine *178*, 2249-2254.

60. Fontenot, J.D., Dooley, J.L., Farr, A.G., and Rudensky, A.Y. (2005a). Developmental regulation of Foxp3 expression during ontogeny. The Journal of experimental medicine *202*, 901-906.

61. Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nature immunology *4*, 330-336.

62. Fontenot, J.D., Rasmussen, J.P., Williams, L.M., Dooley, J.L., Farr, A.G., and Rudensky, A.Y. (2005b). Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity *22*, 329-341.

63. Foulds, K.E., Zenewicz, L.A., Shedlock, D.J., Jiang, J., Troy, A.E., and Shen, H. (2002). Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. J Immunol *168*, 1528-1532.

64. Frankel, F.R. (2005). Vaccine wakes from the dead. Nat Med *11*, 833-834.

65. Frankel, F.R., Hegde, S., Lieberman, J., and Paterson, Y. (1995). Induction of cell-mediated immune responses to human immunodeficiency virus type 1 Gag protein by using Listeria monocytogenes as a live vaccine vector. J Immunol *155*, 4775-4782.

66. Friedline, R.H., Brown, D.S., Nguyen, H., Kornfeld, H., Lee, J., Zhang, Y., Appleby, M., Der, S.D., Kang, J., and Chambers, C.A. (2009). CD4+ regulatory T cells require CTLA-4 for the maintenance of systemic tolerance. The Journal of experimental medicine *206*, 421-434.

67. Fulton, R.B., Meyerholz, D.K., and Varga, S.M. (2010). Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. J Immunol *185*, 2382-2392.

68. Furze, R.C., Culley, F.J., and Selkirk, M.E. (2006). Differential roles of the co-stimulatory molecules GITR and CTLA-4 in the immune response to Trichinella spiralis. Microbes Infect *8*, 2803-2810.

69. Gellin, B.G., and Broome, C.V. (1989). Listeriosis. Jama *261*, 1313-1320.

70. Gellin, B.G., Broome, C.V., Bibb, W.F., Weaver, R.E., Gaventa, S., and Mascola, L. (1991). The epidemiology of listeriosis in the United States--1986. Listeriosis Study Group. American journal of epidemiology *133*, 392-401.

71. Gendron, R.L., Nestel, F.P., Lapp, W.S., and Baines, M.G. (1990). Lipopolysaccharide-induced fetal resorption in mice is associated with the intrauterine production of tumour necrosis factor-alpha. Journal of reproduction and fertility *90*, 395-402.

72. Gillespie, I.A., McLauchlin, J., Grant, K.A., Little, C.L., Mithani, V., Penman, C., Lane, C., and Regan, M. (2006). Changing pattern of human listeriosis, England and Wales, 2001-2004. Emerging infectious diseases *12*, 1361-1366.

73. Gottenberg, J.E., Lavie, F., Abbed, K., Gasnault, J., Le Nevot, E., Delfraissy, J.F., Taoufik, Y., and Mariette, X. (2005). CD4 CD25high regulatory T cells are not impaired in patients with primary Sjogren's syndrome. Journal of autoimmunity *24*, 235-242.

74. Gottschalk, R.A., Corse, E., and Allison, J.P. (2012). Expression of Helios in peripherally induced Foxp3+ regulatory T cells. J Immunol *188*, 976-980.

75. Gregg, R., Smith, C.M., Clark, F.J., Dunnion, D., Khan, N., Chakraverty, R., Nayak, L., and Moss, P.A. (2005). The number of human peripheral blood CD4+ CD25high regulatory T cells increases with age. Clinical and experimental immunology *140*, 540-546.

76. Guerin, L.R., Prins, J.R., and Robertson, S.A. (2009). Regulatory T-cells and immune tolerance in pregnancy: a new target for infertility treatment? Human reproduction update *15*, 517-535.

77. Ha, S.J., Mueller, S.N., Wherry, E.J., Barber, D.L., Aubert, R.D., Sharpe, A.H., Freeman, G.J., and Ahmed, R. (2008). Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. The Journal of experimental medicine *205*, 543-555.

78. Hansen, D.S., and Schofield, L. (2010). Natural regulatory T cells in malaria: host or parasite allies? PLoS pathogens *6*, e1000771.

79. Haque, A., Best, S.E., Amante, F.H., Mustafah, S., Desbarrieres, L., de Labastida, F., Sparwasser, T., Hill, G.R., and Engwerda, C.R. (2010). CD4+ natural regulatory T cells prevent experimental cerebral malaria via CTLA-4 when expanded in vivo. PLoS pathogens *6*, e1001221.

80. Haring, J.S., Badovinac, V.P., and Harty, J.T. (2006). Inflaming the CD8+ T cell response. Immunity *25*, 19-29.

81. Harty, J.T., and Bevan, M.J. (1995). Specific immunity to Listeria monocytogenes in the absence of IFN gamma. Immunity *3*, 109-117.

82. Hess, J., Ladel, C., Miko, D., and Kaufmann, S.H. (1996). Salmonella typhimurium aroA- infection in gene-targeted immunodeficient mice: major role of

CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. J Immunol *156*, 3321-3326.

83. Hippen, K.L., Merkel, S.C., Schirm, D.K., Sieben, C.M., Sumstad, D., Kadidlo, D.M., McKenna, D.H., Bromberg, J.S., Levine, B.L., Riley, J.L.*, et al.* (2011). Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity. Sci Transl Med *3*, 83ra41.

84. Hodi, F.S., Butler, M., Oble, D.A., Seiden, M.V., Haluska, F.G., Kruse, A., Macrae, S., Nelson, M., Canning, C., Lowy, I.*, et al.* (2008). Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients. Proc Natl Acad Sci U S A *105*, 3005-3010.

85. Hogquist, K.A., Jameson, S.C., Heath, W.R., Howard, J.L., Bevan, M.J., and Carbone, F.R. (1994). T cell receptor antagonist peptides induce positive selection. Cell *76*, 17-27.

86. Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science (New York, N.Y *299*, 1057-1061.

87. Hsieh, C.S., Lee, H.M., and Lio, C.W. (2012). Selection of regulatory T cells in the thymus. Nature reviews *12*, 157-167.

88. Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R.M., and Aguet, M. (1993). Immune response in mice that lack the interferon-gamma receptor. Science (New York, N.Y *259*, 1742-1745.

89. Jacobs, T., Graefe, S.E., Niknafs, S., Gaworski, I., and Fleischer, B. (2002). Murine malaria is exacerbated by CTLA-4 blockade. J Immunol *169*, 2323-2329.

90. Jiang, S., Rasmussen, R.A., Nolan, K.M., Frankel, F.R., Lieberman, J., McClure, H.M., Williams, K.M., Babu, U.S., Raybourne, R.B., Strobert, E., and Ruprecht, R.M. (2007). Live attenuated Listeria monocytogenes expressing HIV Gag: immunogenicity in rhesus monkeys. Vaccine *25*, 7470-7479.

91. Johanns, T.M., Ertelt, J.M., Rowe, J.H., and Way, S.S. (2010a). Regulatory T cell suppressive potency dictates the balance between bacterial proliferation and clearance during persistent Salmonella infection. PLoS pathogens *6*, e1001043.

92. Johanns, T.M., Ertelt, J.M., Rowe, J.H., and Way, S.S. (2010b). Regulatory T cell suppressive potency dictates the balance between bacterial proliferation and clearance during persistent Salmonella infection. PLoS pathogens, (in press).

93. Josefowicz, S.Z., Lu, L.F., and Rudensky, A.Y. (2012). Regulatory T cells: mechanisms of differentiation and function. Annual review of immunology *30*, 531-564.

94. Jun, H., Seo, S.K., Jeong, H.Y., Seo, H.M., Zhu, G., Chen, L., and Choi, I.H. (2005). B7-H1 (CD274) inhibits the development of herpetic stromal keratitis (HSK). FEBS letters *579*, 6259-6264.

95. Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F.*, et al.* (2002a). In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity *17*, 211-220.

96. Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F.*, et al.* (2002b). In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity *17*, 211-220.

97. Kahn, D.A., and Baltimore, D. (2010). Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. Proceedings of the National Academy of Sciences of the United States of America *107*, 9299-9304.

98. Kamijo, R., Le, J., Shapiro, D., Havell, E.A., Huang, S., Aguet, M., Bosland, M., and Vilcek, J. (1993). Mice that lack the interferon-gamma receptor have profoundly altered responses to infection with Bacillus Calmette-Guerin and subsequent challenge with lipopolysaccharide. The Journal of experimental medicine *178*, 1435-1440.

99. Kamimura, D., and Bevan, M.J. (2007). Naive CD8+ T cells differentiate into protective memory-like cells after IL-2 anti IL-2 complex treatment in vivo. The Journal of experimental medicine *204*, 1803-1812.

100. Kanai, T., Totsuka, T., Uraushihara, K., Makita, S., Nakamura, T., Koganei, K., Fukushima, T., Akiba, H., Yagita, H., Okumura, K.*, et al.* (2003). Blockade of B7-H1 suppresses the development of chronic intestinal inflammation. J Immunol *171*, 4156-4163.

101. Kao, C., Oestreich, K.J., Paley, M.A., Crawford, A., Angelosanto, J.M., Ali, M.A., Intlekofer, A.M., Boss, J.M., Reiner, S.L., Weinmann, A.S., and Wherry, E.J. (2011). Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8(+) T cell responses during chronic infection. Nature immunology *12*, 663-671.

102. Kaparakis, M., Laurie, K.L., Wijburg, O., Pedersen, J., Pearse, M., van Driel, I.R., Gleeson, P.A., and Strugnell, R.A. (2006). CD4+ CD25+ regulatory T cells modulate the T-cell and antibody responses in helicobacter-infected BALB/c mice. Infection and immunity *74*, 3519-3529.

103. Kastenmuller, K., Wille-Reece, U., Lindsay, R.W., Trager, L.R., Darrah, P.A., Flynn, B.J., Becker, M.R., Udey, M.C., Clausen, B.E., Igyarto, B.Z.*, et al.* (2011a). Protective T cell immunity in mice following protein-TLR7/8 agonist-conjugate immunization requires aggregation, type I IFN, and multiple DC subsets. The Journal of clinical investigation *121*, 1782-1796.

104. Kastenmuller, W., Gasteiger, G., Subramanian, N., Sparwasser, T., Busch, D.H., Belkaid, Y., Drexler, I., and Germain, R.N. (2011b). Regulatory T Cells Selectively Control CD8+ T Cell Effector Pool Size via IL-2 Restriction. J Immunol *187*, 3186-3197.

105. Kaufmann, D.E., Kavanagh, D.G., Pereyra, F., Zaunders, J.J., Mackey, E.W., Miura, T., Palmer, S., Brockman, M., Rathod, A., Piechocka-Trocha, A.*, et al.* (2007). Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. Nat Immunol *8*, 1246-1254.

106. Kearney, E.R., Walunas, T.L., Karr, R.W., Morton, P.A., Loh, D.Y., Bluestone, J.A., and Jenkins, M.K. (1995). Antigen-dependent clonal expansion of a trace population of antigen-specific CD4+ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. J Immunol *155*, 1032-1036.

107. Keir, M.E., Francisco, L.M., and Sharpe, A.H. (2007). PD-1 and its ligands in T-cell immunity. Current opinion in immunology *19*, 309-314.

108. Khattri, R., Cox, T., Yasayko, S.A., and Ramsdell, F. (2003). An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nature immunology *4*, 337-342.

109. Kim, J.M., Rasmussen, J.P., and Rudensky, A.Y. (2007). Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nature immunology *8*, 191-197.

110. Kirman, J., McCoy, K., Hook, S., Prout, M., Delahunt, B., Orme, I., Frank, A., and Le Gros, G. (1999). CTLA-4 blockade enhances the immune response induced by mycobacterial infection but does not lead to increased protection. Infect Immun *67*, 3786-3792.

111. Koch, M.A., Tucker-Heard, G., Perdue, N.R., Killebrew, J.R., Urdahl, K.B., and Campbell, D.J. (2009). The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nature immunology *10*, 595-602.

112. Kolar, P., Knieke, K., Hegel, J.K., Quandt, D., Burmester, G.R., Hoff, H., and Brunner-Weinzierl, M.C. (2009). CTLA-4 (CD152) controls homeostasis and suppressive capacity of regulatory T cells in mice. Arthritis and rheumatism *60*, 123-132.

113. Krishnan, L., Guilbert, L.J., Russell, A.S., Wegmann, T.G., Mosmann, T.R., and Belosevic, M. (1996). Pregnancy impairs resistance of C57BL/6 mice to Leishmania major infection and causes decreased antigen-specific IFN-gamma response and increased production of T helper 2 cytokines. J Immunol *156*, 644-652.

114. Krishnan, L., Pejcic-Karapetrovic, B., Gurnani, K., Zafer, A., and Sad, S. (2010). Pregnancy does not deter the development of a potent maternal protective CD8+

T-cell acquired immune response against Listeria monocytogenes despite preferential placental colonization. Am J Reprod Immunol *63*, 54-65.

115. Kubo, T., Hatton, R.D., Oliver, J., Liu, X., Elson, C.O., and Weaver, C.T. (2004). Regulatory T cell suppression and anergy are differentially regulated by proinflammatory cytokines produced by TLR-activated dendritic cells. J Immunol *173*, 7249-7258.
116. Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell *75*, 263-274.
117. Kursar, M., Bonhagen, K., Fensterle, J., Kohler, A., Hurwitz, R., Kamradt, T., Kaufmann, S.H., and Mittrucker, H.W. (2002). Regulatory CD4+CD25+ T cells restrict memory CD8+ T cell responses. The Journal of experimental medicine *196*, 1585-1592.
118. Kursar, M., Koch, M., Mittrucker, H.W., Nouailles, G., Bonhagen, K., Kamradt, T., and Kaufmann, S.H. (2007). Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. J Immunol *178*, 2661-2665.
119. Lafon, M., Megret, F., Meuth, S.G., Simon, O., Velandia Romero, M.L., Lafage, M., Chen, L., Alexopoulou, L., Flavell, R.A., Prehaud, C., and Wiendl, H. (2008). Detrimental contribution of the immuno-inhibitor B7-H1 to rabies virus encephalitis. J Immunol *180*, 7506-7515.
120. Lages, C.S., Suffia, I., Velilla, P.A., Huang, B., Warshaw, G., Hildeman, D.A., Belkaid, Y., and Chougnet, C. (2008). Functional regulatory T cells accumulate in aged hosts and promote chronic infectious disease reactivation. J Immunol *181*, 1835-1848.
121. Lanteri, M.C., O'Brien, K.M., Purtha, W.E., Cameron, M.J., Lund, J.M., Owen, R.E., Heitman, J.W., Custer, B., Hirschkorn, D.F., Tobler, L.H.*, et al.* (2009). Tregs control the development of symptomatic West Nile virus infection in humans and mice. The Journal of clinical investigation *119*, 3266-3277.
122. Latchman, Y.E., Liang, S.C., Wu, Y., Chernova, T., Sobel, R.A., Klemm, M., Kuchroo, V.K., Freeman, G.J., and Sharpe, A.H. (2004). PD-L1-deficient mice

show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. Proc Natl Acad Sci U S A *101*, 10691-10696.

123. Lathrop, S.K., Bloom, S.M., Rao, S.M., Nutsch, K., Lio, C.W., Santacruz, N., Peterson, D.A., Stappenbeck, T.S., and Hsieh, C.S. (2011). Peripheral education of the immune system by colonic commensal microbiota. Nature *478*, 250-254.

124. Lazar-Molnar, E., Chen, B., Sweeney, K.A., Wang, E.J., Liu, W., Lin, J., Porcelli, S.A., Almo, S.C., Nathenson, S.G., and Jacobs, W.R., Jr. (2010). Programmed death-1 (PD-1)-deficient mice are extraordinarily sensitive to tuberculosis. Proceedings of the National Academy of Sciences of the United States of America *107*, 13402-13407.

125. Le Monnier, A., Autret, N., Join-Lambert, O.F., Jaubert, F., Charbit, A., Berche, P., and Kayal, S. (2007). ActA is required for crossing of the fetoplacental barrier by Listeria monocytogenes. Infection and immunity *75*, 950-957.

126. Lee, S.J., O'Donnell, H., and McSorley, S.J. (2010). B7-H1 (programmed cell death ligand 1) is required for the development of multifunctional Th1 cells and immunity to primary, but not secondary, Salmonella infection. J Immunol *185*, 2442-2449.

127. Lepenies, B., Gaworski, I., Tartz, S., Langhorne, J., Fleischer, B., and Jacobs, T. (2007). CTLA-4 blockade differentially influences the outcome of non-lethal and lethal Plasmodium yoelii infections. Microbes Infect *9*, 687-694.

128. Lieberman, L.A., and Hunter, C.A. (2002). Regulatory pathways involved in the infection-induced production of IFN-gamma by NK cells. Microbes and infection / Institut Pasteur *4*, 1531-1538.

129. Linterman, M.A., Pierson, W., Lee, S.K., Kallies, A., Kawamoto, S., Rayner, T.F., Srivastava, M., Divekar, D.P., Beaton, L., Hogan, J.J., *et al.* (2011). Foxp3+ follicular regulatory T cells control the germinal center response. Nature medicine *17*, 975-982.

130. Littman, D.R., and Rudensky, A.Y. (2010). Th17 and regulatory T cells in mediating and restraining inflammation. Cell *140*, 845-858.

131. Liu, H., Komai-Koma, M., Xu, D., and Liew, F.Y. (2006). Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. Proceedings of the National Academy of Sciences of the United States of America *103*, 7048-7053.

132. Luhn, K., Simmons, C.P., Moran, E., Dung, N.T., Chau, T.N., Quyen, N.T., Thao le, T.T., Van Ngoc, T., Dung, N.M., Wills, B.*, et al.* (2007). Increased frequencies of CD4+ CD25(high) regulatory T cells in acute dengue infection. The Journal of experimental medicine *204*, 979-985.

133. Lund, J.M., Hsing, L., Pham, T.T., and Rudensky, A.Y. (2008). Coordination of early protective immunity to viral infection by regulatory T cells. Science (New York, N.Y *320*, 1220-1224.

134. Ma, C.J., Ni, L., Zhang, Y., Zhang, C.L., Wu, X.Y., Atia, A.N., Thayer, P., Moorman, J.P., and Yao, Z.Q. (2011). PD-1 negatively regulates interleukin-12 expression by limiting STAT-1 phosphorylation in monocytes/macrophages during chronic hepatitis C virus infection. Immunology *132*, 421-431.

135. Magram, J., Connaughton, S.E., Warrier, R.R., Carvajal, D.M., Wu, C.Y., Ferrante, J., Stewart, C., Sarmiento, U., Faherty, D.A., and Gately, M.K. (1996). IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. Immunity *4*, 471-481.

136. Maier, H., Isogawa, M., Freeman, G.J., and Chisari, F.V. (2007). PD-1:PD-L1 interactions contribute to the functional suppression of virus-specific CD8+ T lymphocytes in the liver. J Immunol *178*, 2714-2720.

137. Martins, G.A., Tadokoro, C.E., Silva, R.B., Silva, J.S., and Rizzo, L.V. (2004). CTLA-4 blockage increases resistance to infection with the intracellular protozoan Trypanosoma cruzi. J Immunol *172*, 4893-4901.

138. Mays, L.E., and Wilson, J.M. (2011). The complex and evolving story of T cell activation to AAV vector-encoded transgene products. Mol Ther *19*, 16-27.

139. McCoy, K., Camberis, M., and Gros, G.L. (1997). Protective immunity to nematode infection is induced by CTLA-4 blockade. J Exp Med *186*, 183-187.

140. Mendez, S., Reckling, S.K., Piccirillo, C.A., Sacks, D., and Belkaid, Y. (2004). Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity. The Journal of experimental medicine *200*, 201-210.

141. Mercado, R., Vijh, S., Allen, S.E., Kerksiek, K., Pilip, I.M., and Pamer, E.G. (2000). Early programming of T cell populations responding to bacterial infection. J Immunol *165*, 6833-6839.

142. Minigo, G., Woodberry, T., Piera, K.A., Salwati, E., Tjitra, E., Kenangalem, E., Price, R.N., Engwerda, C.R., Anstey, N.M., and Plebanski, M. (2009). Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. PLoS pathogens *5*, e1000402.

143. Miwa, N., Hayakawa, S., Miyazaki, S., Myojo, S., Sasaki, Y., Sakai, M., Takikawa, O., and Saito, S. (2005). IDO expression on decidual and peripheral blood dendritic cells and monocytes/macrophages after treatment with CTLA-4 or interferon-gamma increase in normal pregnancy but decrease in spontaneous abortion. Molecular human reproduction *11*, 865-870.

144. Moon, J.J., Chu, H.H., Hataye, J., Pagan, A.J., Pepper, M., McLachlan, J.B., Zell, T., and Jenkins, M.K. (2009). Tracking epitope-specific T cells. Nature protocols *4*, 565-581.

145. Moon, J.J., Chu, H.H., Pepper, M., McSorley, S.J., Jameson, S.C., Kedl, R.M., and Jenkins, M.K. (2007). Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity *27*, 203-213.

146. Moon, J.J., Dash, P., Oguin, T.H., 3rd, McClaren, J.L., Chu, H.H., Thomas, P.G., and Jenkins, M.K. (2011). Quantitative impact of thymic selection on Foxp3+ and Foxp3- subsets of self-peptide/MHC class II-specific CD4+ T cells. Proceedings of the National Academy of Sciences of the United States of America *108*, 14602-14607.

147. Moore, A.C., Gallimore, A., Draper, S.J., Watkins, K.R., Gilbert, S.C., and Hill, A.V. (2005). Anti-CD25 antibody enhancement of vaccine-induced

immunogenicity: increased durable cellular immunity with reduced immunodominance. J Immunol *175*, 7264-7273.

148. Muller, U., Steinhoff, U., Reis, L.F., Hemmi, S., Pavlovic, J., Zinkernagel, R.M., and Aguet, M. (1994). Functional role of type I and type II interferons in antiviral defense. Science (New York, N.Y *264*, 1918-1921.
149. Munn, D.H., Zhou, M., Attwood, J.T., Bondarev, I., Conway, S.J., Marshall, B., Brown, C., and Mellor, A.L. (1998). Prevention of allogeneic fetal rejection by tryptophan catabolism. Science (New York, N.Y *281*, 1191-1193.
150. Munoz-Suano, A., Hamilton, A.B., and Betz, A.G. (2011). Gimme shelter: the immune system during pregnancy. Immunological reviews *241*, 20-38.
151. Mylonakis, E., Paliou, M., Hohmann, E.L., Calderwood, S.B., and Wing, E.J. (2002). Listeriosis during pregnancy: a case series and review of 222 cases. Medicine *81*, 260-269.
152. Netea, M.G., Sutmuller, R., Hermann, C., Van der Graaf, C.A., Van der Meer, J.W., van Krieken, J.H., Hartung, T., Adema, G., and Kullberg, B.J. (2004). Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells. J Immunol *172*, 3712-3718.
153. Nishimura, H., Minato, N., Nakano, T., and Honjo, T. (1998). Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. International immunology *10*, 1563-1572.
154. Nomura, T., Kawamura, I., Tsuchiya, K., Kohda, C., Baba, H., Ito, Y., Kimoto, T., Watanabe, I., and Mitsuyama, M. (2002). Essential role of interleukin-12 (IL-12) and IL-18 for gamma interferon production induced by listeriolysin O in mouse spleen cells. Infection and immunity *70*, 1049-1055.
155. O'Connell, R.M., Saha, S.K., Vaidya, S.A., Bruhn, K.W., Miranda, G.A., Zarnegar, B., Perry, A.K., Nguyen, B.O., Lane, T.F., Taniguchi, T.*, et al.* (2004). Type I interferon production enhances susceptibility to Listeria monocytogenes infection. The Journal of experimental medicine *200*, 437-445.
156. O'Leary, D.R., Marfin, A.A., Montgomery, S.P., Kipp, A.M., Lehman, J.A., Biggerstaff, B.J., Elko, V.L., Collins, P.D., Jones, J.E., and Campbell, G.L. (2004).

The epidemic of West Nile virus in the United States, 2002. Vector Borne Zoonotic Dis *4*, 61-70.

157. O'Riordan, M., Yi, C.H., Gonzales, R., Lee, K.D., and Portnoy, D.A. (2002). Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. Proceedings of the National Academy of Sciences of the United States of America *99*, 13861-13866.
158. Ochs, H.D., Gambineri, E., and Torgerson, T.R. (2007). IPEX, FOXP3 and regulatory T-cells: a model for autoimmunity. Immunol Res *38*, 112-121.
159. Odorizzi, P.M., and Wherry, E.J. (2012). Inhibitory receptors on lymphocytes: insights from infections. J Immunol *188*, 2957-2965.
160. Oldenhove, G., Bouladoux, N., Wohlfert, E.A., Hall, J.A., Chou, D., Dos Santos, L., O'Brien, S., Blank, R., Lamb, E., Natarajan, S.*, et al.* (2009). Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. Immunity *31*, 772-786.
161. Orgun, N.N., Mathis, M.A., Wilson, C.B., and Way, S.S. (2008). Deviation from a strong Th1-dominated to a modest Th17-dominated CD4 T cell response in the absence of IL-12p40 and type I IFNs sustains protective CD8 T cells. J Immunol *180*, 4109-4115.
162. Orgun, N.N., and Way, S.S. (2008). A critical role for phospholipase C in protective immunity conferred by listeriolysin O-deficient Listeria monocytogenes. Microbial pathogenesis *44*, 159-163.
163. Orr, M.T., Orgun, N.N., Wilson, C.B., and Way, S.S. (2007). Cutting Edge: Recombinant Listeria monocytogenes expressing a single immune-dominant peptide confers protective immunity to herpes simplex virus-1 infection. J Immunol *178*, 4731-4735.
164. Ostensen, M., and Villiger, P.M. (2007). The remission of rheumatoid arthritis during pregnancy. Seminars in immunopathology *29*, 185-191.
165. Ou, R., Zhou, S., Huang, L., and Moskophidis, D. (2001). Critical role for alpha/beta and gamma interferons in persistence of lymphocytic choriomeningitis virus by clonal exhaustion of cytotoxic T cells. Journal of virology *75*, 8407-8423.

166. Paeschke, S., Chen, F., Horn, N., Fotopoulou, C., Zambon-Bertoja, A., Sollwedel, A., Zenclussen, M.L., Casalis, P.A., Dudenhausen, J.W., Volk, H.D., and Zenclussen, A.C. (2005). Pre-eclampsia is not associated with changes in the levels of regulatory T cells in peripheral blood. Am J Reprod Immunol *54*, 384-389.

167. Pamer, E.G. (2004). Immune responses to Listeria monocytogenes. Nat Rev Immunol *4*, 812-823.

168. Pandiyan, P., Conti, H.R., Zheng, L., Peterson, A.C., Mathern, D.R., Hernandez-Santos, N., Edgerton, M., Gaffen, S.L., and Lenardo, M.J. (2011). CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse Candida albicans Th17 cell infection model. Immunity *34*, 422-434.

169. Pasare, C., and Medzhitov, R. (2003). Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science (New York, N.Y *299*, 1033-1036.

170. Paterson, Y., and Johnson, R.S. (2004). Progress towards the use of Listeria monocytogenes as a live bacterial vaccine vector for the delivery of HIV antigens. Expert Rev Vaccines *3*, S119-134.

171. Pearce, E.L., and Shen, H. (2007). Generation of CD8 T cell memory is regulated by IL-12. J Immunol *179*, 2074-2081.

172. Pejcic-Karapetrovic, B., Gurnani, K., Russell, M.S., Finlay, B.B., Sad, S., and Krishnan, L. (2007). Pregnancy impairs the innate immune resistance to Salmonella typhimurium leading to rapid fatal infection. J Immunol *179*, 6088-6096.

173. Peng, G., Guo, Z., Kiniwa, Y., Voo, K.S., Peng, W., Fu, T., Wang, D.Y., Li, Y., Wang, H.Y., and Wang, R.F. (2005). Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. Science (New York, N.Y *309*, 1380-1384.

174. Pepper, M., Linehan, J.L., Pagan, A.J., Zell, T., Dileepan, T., Cleary, P.P., and Jenkins, M.K. (2010). Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. Nature immunology *11*, 83-89.

175. Periasamy, S., Singh, A., Sahay, B., Rahman, T., Feustel, P.J., Pham, G.H., Gosselin, E.J., and Sellati, T.J. (2011). Development of tolerogenic dendritic cells and regulatory T cells favors exponential bacterial growth and survival during early respiratory tularemia. Journal of leukocyte biology *90*, 493-507.

176. Petrovas, C., Casazza, J.P., Brenchley, J.M., Price, D.A., Gostick, E., Adams, W.C., Precopio, M.L., Schacker, T., Roederer, M., Douek, D.C., and Koup, R.A. (2006). PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. The Journal of experimental medicine *203*, 2281-2292.

177. Piccinni, M.P., Beloni, L., Livi, C., Maggi, E., Scarselli, G., and Romagnani, S. (1998). Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. Nature medicine *4*, 1020-1024.

178. Portnoy, D.A., Auerbuch, V., and Glomski, I.J. (2002). The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity. The Journal of cell biology *158*, 409-414.

179. Portnoy, D.A., Jacks, P.S., and Hinrichs, D.J. (1988). Role of hemolysin for the intracellular growth of Listeria monocytogenes. The Journal of experimental medicine *167*, 1459-1471.

180. Poulsen, K.P., Faith, N.G., Steinberg, H., and Czuprynski, C.J. (2011). Pregnancy reduces the genetic resistance of C57BL/6 mice to Listeria monocytogenes infection by intragastric inoculation. Microbial pathogenesis *50*, 360-366.

181. Prins, J.R., Boelens, H.M., Heimweg, J., Van der Heide, S., Dubois, A.E., Van Oosterhout, A.J., and Erwich, J.J. (2009). Preeclampsia is associated with lower percentages of regulatory T cells in maternal blood. Hypertens Pregnancy *28*, 300-311.

182. Pulko, V., Harris, K.J., Liu, X., Gibbons, R.M., Harrington, S.M., Krco, C.J., Kwon, E.D., and Dong, H. (2011). B7-h1 expressed by activated CD8 T cells is essential for their survival. J Immunol *187*, 5606-5614.

183. Raue, H.P., and Slifka, M.K. (2007). Pivotal advance: CTLA-4+ T cells exhibit normal antiviral functions during acute viral infection. J Leukoc Biol *81*, 1165-1175.

184. Rausch, S., Huehn, J., Loddenkemper, C., Hepworth, M.R., Klotz, C., Sparwasser, T., Hamann, A., Lucius, R., and Hartmann, S. (2009). Establishment of nematode infection despite increased Th2 responses and immunopathology after selective depletion of Foxp3+ cells. European journal of immunology *39*, 3066-3077.

185. Read, S., Greenwald, R., Izcue, A., Robinson, N., Mandelbrot, D., Francisco, L., Sharpe, A.H., and Powrie, F. (2006). Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. J Immunol *177*, 4376-4383.

186. Read, S., Malmstrom, V., and Powrie, F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med *192*, 295-302.

187. Reiley, W.W., Shafiani, S., Wittmer, S.T., Tucker-Heard, G., Moon, J.J., Jenkins, M.K., Urdahl, K.B., Winslow, G.M., and Woodland, D.L. (2010). Distinct functions of antigen-specific CD4 T cells during murine Mycobacterium tuberculosis infection. Proceedings of the National Academy of Sciences of the United States of America *107*, 19408-19413.

188. Robertson, S.A. (2010). Immune regulation of conception and embryo implantation-all about quality control? Journal of reproductive immunology *85*, 51-57.

189. Robertson, S.A., Care, A.S., and Skinner, R.J. (2007). Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in mice. Biology of reproduction *76*, 738-748.

190. Rosenblum, M.D., Gratz, I.K., Paw, J.S., Lee, K., Marshak-Rothstein, A., and Abbas, A.K. (2011). Response to self antigen imprints regulatory memory in tissues. Nature *480*, 538-542.

191. Rowe, J.H., Ertelt, J.M., Aguilera, M.N., Farrar, M.A., and Way, S.S. (2011). Foxp3(+) Regulatory T Cell Expansion Required for Sustaining Pregnancy

Compromises Host Defense against Prenatal Bacterial Pathogens. Cell Host Microbe *10*, 54-64.

192. Rowe, J.H., Ertelt, J.M., and Way, S.S. (2012). Foxp3(+) regulatory T cells, immune stimulation and host defence against infection. Immunology *136*, 1-10.

193. Rowe, J.H., Johanns, T.M., Ertelt, J.M., and Way, S.S. (2008). PDL-1 blockade impedes T cell expansion and protective immunity primed by attenuated Listeria monocytogenes. J Immunol *180*, 7553-7557.

194. Rubinstein, M.P., Kovar, M., Purton, J.F., Cho, J.H., Boyman, O., Surh, C.D., and Sprent, J. (2006). Converting IL-15 to a superagonist by binding to soluble IL-15R{alpha}. Proceedings of the National Academy of Sciences of the United States of America *103*, 9166-9171.

195. Rubtsov, Y.P., Rasmussen, J.P., Chi, E.Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W.R., Jr*., et al.* (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity *28*, 546-558.

196. Saito, S., Nakashima, A., Shima, T., and Ito, M. (2010). Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. Am J Reprod Immunol *63*, 601-610.

197. Sakaguchi, S. (2003). Control of immune responses by naturally arising CD4+ regulatory T cells that express toll-like receptors. The Journal of experimental medicine *197*, 397-401.

198. Sakaguchi, S. (2004). Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annual review of immunology *22*, 531-562.

199. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol *155*, 1151-1164.

200. Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P., and Yamaguchi, T. (2009). Regulatory T cells: how do they suppress immune responses? International immunology *21*, 1105-1111.

201. Sakai, S., Kawamura, I., Okazaki, T., Tsuchiya, K., Uchiyama, R., and Mitsuyama, M. (2010). PD-1-PD-L1 pathway impairs T(h)1 immune response in the late stage of infection with Mycobacterium bovis bacillus Calmette-Guerin. International immunology *22*, 915-925.

202. Santner-Nanan, B., Peek, M.J., Khanam, R., Richarts, L., Zhu, E., Fazekas de St Groth, B., and Nanan, R. (2009). Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. J Immunol *183*, 7023-7030.

203. Sasaki, Y., Darmochwal-Kolarz, D., Suzuki, D., Sakai, M., Ito, M., Shima, T., Shiozaki, A., Rolinski, J., and Saito, S. (2007). Proportion of peripheral blood and decidual CD4(+) CD25(bright) regulatory T cells in pre-eclampsia. Clinical and experimental immunology *149*, 139-145.

204. Sasaki, Y., Sakai, M., Miyazaki, S., Higuma, S., Shiozaki, A., and Saito, S. (2004). Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. Molecular human reproduction *10*, 347-353.

205. Scanga, C.A., Bafica, A., and Sher, A. (2007). Viral gene expression in HIV transgenic mice is activated by Mycobacterium tuberculosis and suppressed after antimycobacterial chemotherapy. J Infect Dis *195*, 246-254.

206. Schreiner, B., Mitsdoerffer, M., Kieseier, B.C., Chen, L., Hartung, H.P., Weller, M., and Wiendl, H. (2004). Interferon-beta enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. Journal of neuroimmunology *155*, 172-182.

207. Schuchat, A., Swaminathan, B., and Broome, C.V. (1991). Epidemiology of human listeriosis. Clinical microbiology reviews *4*, 169-183.

208. Scott-Browne, J.P., Shafiani, S., Tucker-Heard, G., Ishida-Tsubota, K., Fontenot, J.D., Rudensky, A.Y., Bevan, M.J., and Urdahl, K.B. (2007). Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. The Journal of experimental medicine *204*, 2159-2169.

209. Seo, S.K., Jeong, H.Y., Park, S.G., Lee, S.W., Choi, I.W., Chen, L., and Choi, I. (2007). Blockade of endogenous B7-H1 suppresses antibacterial protection after primary Listeria monocytogenes infection. Immunology.

210. Seo, S.K., Jeong, H.Y., Park, S.G., Lee, S.W., Choi, I.W., Chen, L., and Choi, I. (2008). Blockade of endogenous B7-H1 suppresses antibacterial protection after primary Listeria monocytogenes infection. Immunology *123*, 90-99.

211. Sercan, O., Hammerling, G.J., Arnold, B., and Schuler, T. (2006). Innate immune cells contribute to the IFN-gamma-dependent regulation of antigen-specific CD8+ T cell homeostasis. J Immunol *176*, 735-739.

212. Sercan, O., Stoycheva, D., Hammerling, G.J., Arnold, B., and Schuler, T. (2010). IFN-gamma receptor signaling regulates memory CD8+ T cell differentiation. J Immunol *184*, 2855-2862.

213. Shafiani, S., Tucker-Heard, G., Kariyone, A., Takatsu, K., and Urdahl, K.B. (2010). Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. The Journal of experimental medicine *207*, 1409-1420.

214. Sharma, S., Dominguez, A.L., and Lustgarten, J. (2006). High accumulation of T regulatory cells prevents the activation of immune responses in aged animals. J Immunol *177*, 8348-8355.

215. Sharpe, A.H., Wherry, E.J., Ahmed, R., and Freeman, G.J. (2007). The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol *8*, 239-245.

216. Shevach, E.M. (2009). Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity *30*, 636-645.

217. Shrikant, P., Khoruts, A., and Mescher, M.F. (1999). CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell- and IL-2-dependent mechanism. Immunity *11*, 483-493.

218. Somerset, D.A., Zheng, Y., Kilby, M.D., Sansom, D.M., and Drayson, M.T. (2004). Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. Immunology *112*, 38-43.

219. Steeg, C., Adler, G., Sparwasser, T., Fleischer, B., and Jacobs, T. (2009). Limited role of CD4+Foxp3+ regulatory T cells in the control of experimental cerebral malaria. J Immunol *183*, 7014-7022.

220. Subudhi, S.K., Zhou, P., Yerian, L.M., Chin, R.K., Lo, J.C., Anders, R.A., Sun, Y., Chen, L., Wang, Y., Alegre, M.L., and Fu, Y.X. (2004). Local expression of B7-H1 promotes organ-specific autoimmunity and transplant rejection. J Clin Invest *113*, 694-700.

221. Suffia, I.J., Reckling, S.K., Piccirillo, C.A., Goldszmid, R.S., and Belkaid, Y. (2006). Infected site-restricted Foxp3+ natural regulatory T cells are specific for microbial antigens. The Journal of experimental medicine *203*, 777-788.

222. Suri-Payer, E., Amar, A.Z., Thornton, A.M., and Shevach, E.M. (1998). CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. J Immunol *160*, 1212-1218.

223. Sutmuller, R.P., den Brok, M.H., Kramer, M., Bennink, E.J., Toonen, L.W., Kullberg, B.J., Joosten, L.A., Akira, S., Netea, M.G., and Adema, G.J. (2006). Toll-like receptor 2 controls expansion and function of regulatory T cells. The Journal of clinical investigation *116*, 485-494.

224. Suvas, S., Azkur, A.K., Kim, B.S., Kumaraguru, U., and Rouse, B.T. (2004). CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. J Immunol *172*, 4123-4132.

225. Suvas, S., Kumaraguru, U., Pack, C.D., Lee, S., and Rouse, B.T. (2003). CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. The Journal of experimental medicine *198*, 889-901.

226. Suvas, S., and Rouse, B.T. (2006). Treg control of antimicrobial T cell responses. Current opinion in immunology *18*, 344-348.

227. Suzuki, Y., Orellana, M.A., Schreiber, R.D., and Remington, J.S. (1988). Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. Science (New York, N.Y *240*, 516-518.

228. Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J., and Sakaguchi, S. (1998). Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. International immunology *10*, 1969-1980.

229. Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T.W., and Sakaguchi, S. (2000). Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med *192*, 303-310.

230. Tang, A.L., Teijaro, J.R., Njau, M.N., Chandran, S.S., Azimzadeh, A., Nadler, S.G., Rothstein, D.M., and Farber, D.L. (2008). CTLA4 expression is an indicator and regulator of steady-state CD4+ FoxP3+ T cell homeostasis. J Immunol *181*, 1806-1813.

231. Taylor, J.J., Mohrs, M., and Pearce, E.J. (2006). Regulatory T cell responses develop in parallel to Th responses and control the magnitude and phenotype of the Th effector population. J Immunol *176*, 5839-5847.

232. Taylor, M.D., Harris, A., Babayan, S.A., Bain, O., Culshaw, A., Allen, J.E., and Maizels, R.M. (2007). CTLA-4 and CD4+ CD25+ regulatory T cells inhibit protective immunity to filarial parasites in vivo. J Immunol *179*, 4626-4634.

233. Taylor, M.W., and Feng, G.S. (1991). Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism. Faseb J *5*, 2516-2522.

234. Thornton, A.M., Korty, P.E., Tran, D.Q., Wohlfert, E.A., Murray, P.E., Belkaid, Y., and Shevach, E.M. (2010). Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol *184*, 3433-3441.

235. Thornton, A.M., and Shevach, E.M. (1998). CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. The Journal of experimental medicine *188*, 287-296.

236. Tivol, E.A., Borriello, F., Schweitzer, A.N., Lynch, W.P., Bluestone, J.A., and Sharpe, A.H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and

fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity *3*, 541-547.

237. Toka, F.N., Suvas, S., and Rouse, B.T. (2004). CD4+ CD25+ T cells regulate vaccine-generated primary and memory CD8+ T-cell responses against herpes simplex virus type 1. Journal of virology *78*, 13082-13089.

238. Torcia, M.G., Santarlasci, V., Cosmi, L., Clemente, A., Maggi, L., Mangano, V.D., Verra, F., Bancone, G., Nebie, I., Sirima, B.S.*, et al.* (2008). Functional deficit of T regulatory cells in Fulani, an ethnic group with low susceptibility to Plasmodium falciparum malaria. Proceedings of the National Academy of Sciences of the United States of America *105*, 646-651.

239. Trautmann, L., Janbazian, L., Chomont, N., Said, E.A., Gimmig, S., Bessette, B., Boulassel, M.R., Delwart, E., Sepulveda, H., Balderas, R.S.*, et al.* (2006). Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. Nature medicine *12*, 1198-1202.

240. Trupin, L.S., Simon, L.P., and Eskenazi, B. (1996). Change in paternity: a risk factor for preeclampsia in multiparas. Epidemiology (Cambridge, Mass *7*, 240-244.

241. Urbani, S., Amadei, B., Tola, D., Massari, M., Schivazappa, S., Missale, G., and Ferrari, C. (2006). PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. Journal of virology *80*, 11398-11403.

242. Uzonna, J.E., Wei, G., Yurkowski, D., and Bretscher, P. (2001). Immune elimination of Leishmania major in mice: implications for immune memory, vaccination, and reactivation disease. J Immunol *167*, 6967-6974.

243. van Duin, D., Medzhitov, R., and Shaw, A.C. (2006). Triggering TLR signaling in vaccination. Trends in immunology *27*, 49-55.

244. Vignali, D.A., Collison, L.W., and Workman, C.J. (2008). How regulatory T cells work. Nature reviews *8*, 523-532.

245. Wakim, L.M., and Bevan, M.J. (2010). From the thymus to longevity in the periphery. Current opinion in immunology *22*, 274-278.

246. Walsh, C.M., Smith, P., and Fallon, P.G. (2007). Role for CTLA-4 but not CD25+ T cells during Schistosoma mansoni infection of mice. Parasite Immunol *29*, 293-308.

247. Walther, M., Jeffries, D., Finney, O.C., Njie, M., Ebonyi, A., Deininger, S., Lawrence, E., Ngwa-Amambua, A., Jayasooriya, S., Cheeseman, I.H.*, et al.* (2009). Distinct roles for FOXP3 and FOXP3 CD4 T cells in regulating cellular immunity to uncomplicated and severe Plasmodium falciparum malaria. PLoS pathogens *5*, e1000364.

248. Walther, M., Tongren, J.E., Andrews, L., Korbel, D., King, E., Fletcher, H., Andersen, R.F., Bejon, P., Thompson, F., Dunachie, S.J.*, et al.* (2005). Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. Immunity *23*, 287-296.

249. Wang, R.F., Peng, G., and Wang, H.Y. (2006). Regulatory T cells and Toll-like receptors in tumor immunity. Seminars in immunology *18*, 136-142.

250. Waterhouse, P., Penninger, J.M., Timms, E., Wakeham, A., Shahinian, A., Lee, K.P., Thompson, C.B., Griesser, H., and Mak, T.W. (1995). Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science (New York, N.Y *270*, 985-988.

251. Way, S.S., Havenar-Daughton, C., Kolumam, G.A., Orgun, N.N., and Murali-Krishna, K. (2007). IL-12 and type-I IFN synergize for IFN-gamma production by CD4 T cells, whereas neither are required for IFN-gamma production by CD8 T cells after Listeria monocytogenes infection. J Immunol *178*, 4498-4505.

252. Way, S.S., Kollmann, T.R., Hajjar, A.M., and Wilson, C.B. (2003). Cutting edge: protective cell-mediated immunity to Listeria monocytogenes in the absence of myeloid differentiation factor 88. J Immunol *171*, 533-537.

253. Weber, J. (2007). Review: anti-CTLA-4 antibody ipilimumab: case studies of clinical response and immune-related adverse events. Oncologist *12*, 864-872.

254. Wegmann, T.G., Lin, H., Guilbert, L., and Mosmann, T.R. (1993). Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunology today *14*, 353-356.

255. White, C.A., Johansson, M., Roberts, C.T., Ramsay, A.J., and Robertson, S.A. (2004). Effect of interleukin-10 null mutation on maternal immune response and reproductive outcome in mice. Biology of reproduction *70*, 123-131.

256. Whitmire, J.K., Benning, N., and Whitton, J.L. (2005a). Cutting edge: early IFN-gamma signaling directly enhances primary antiviral CD4+ T cell responses. J Immunol *175*, 5624-5628.

257. Whitmire, J.K., Tan, J.T., and Whitton, J.L. (2005b). Interferon-gamma acts directly on CD8+ T cells to increase their abundance during virus infection. The Journal of experimental medicine *201*, 1053-1059.

258. Wildin, R.S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J.L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L.*, et al.* (2001). X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nature genetics *27*, 18-20.

259. Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. Science (New York, N.Y *322*, 271-275.

260. Wing, K., and Sakaguchi, S. (2010). Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nature immunology *11*, 7-13.

261. Wohlfert, E.A., Grainger, J.R., Bouladoux, N., Konkel, J.E., Oldenhove, G., Ribeiro, C.H., Hall, J.A., Yagi, R., Naik, S., Bhairavabhotla, R.*, et al.* (2011). GATA3 controls Foxp3+ regulatory T cell fate during inflammation in mice. The Journal of clinical investigation *121*, 4503-4515.

262. Wu, C., Ferrante, J., Gately, M.K., and Magram, J. (1997). Characterization of IL-12 receptor beta1 chain (IL-12Rbeta1)-deficient mice: IL-12Rbeta1 is an essential component of the functional mouse IL-12 receptor. J Immunol *159*, 1658-1665.

263. Xiao, Z., Casey, K.A., Jameson, S.C., Curtsinger, J.M., and Mescher, M.F. (2009). Programming for CD8 T cell memory development requires IL-12 or type I IFN. J Immunol *182*, 2786-2794.

264. Yamazaki, T., Akiba, H., Koyanagi, A., Azuma, M., Yagita, H., and Okumura, K. (2005). Blockade of B7-H1 on macrophages suppresses CD4+ T cell proliferation by augmenting IFN-gamma-induced nitric oxide production. J Immunol *175*, 1586-1592.

265. Yang, H., Qiu, L., Chen, G., Ye, Z., Lu, C., and Lin, Q. (2008). Proportional change of CD4+CD25+ regulatory T cells in decidua and peripheral blood in unexplained recurrent spontaneous abortion patients. Fertility and sterility *89*, 656-661.

266. Zelinskyy, G., Kraft, A.R., Schimmer, S., Arndt, T., and Dittmer, U. (2006). Kinetics of CD8+ effector T cell responses and induced CD4+ regulatory T cell responses during Friend retrovirus infection. European journal of immunology *36*, 2658-2670.

267. Zhang, Y., Ma, C.J., Ni, L., Zhang, C.L., Wu, X.Y., Kumaraguru, U., Li, C.F., Moorman, J.P., and Yao, Z.Q. (2011). Cross-talk between programmed death-1 and suppressor of cytokine signaling-1 in inhibition of IL-12 production by monocytes/macrophages in hepatitis C virus infection. J Immunol *186*, 3093-3103.

268. Zhao, J., Zhao, J., Fett, C., Trandem, K., Fleming, E., and Perlman, S. (2011). IFN-gamma- and IL-10-expressing virus epitope-specific Foxp3(+) T reg cells in the central nervous system during encephalomyelitis. The Journal of experimental medicine *208*, 1571-1577.

269. Zhao, X., Zhang, M., Li, Z., and Frankel, F.R. (2006). Vaginal protection and immunity after oral immunization of mice with a novel vaccine strain of Listeria monocytogenes expressing human immunodeficiency virus type 1 gag. J Virol *80*, 8880-8890.

270. Zheng, Y., Chaudhry, A., Kas, A., deRoos, P., Kim, J.M., Chu, T.T., Corcoran, L., Treuting, P., Klein, U., and Rudensky, A.Y. (2009). Regulatory T-cell suppressor

program co-opts transcription factor IRF4 to control T(H)2 responses. Nature *458*, 351-356.

271. Zhou, X., Bailey-Bucktrout, S., Jeker, L.T., and Bluestone, J.A. (2009a). Plasticity of CD4(+) FoxP3(+) T cells. Current opinion in immunology *21*, 281-285.

272. Zhou, X., Bailey-Bucktrout, S.L., Jeker, L.T., Penaranda, C., Martinez-Llordella, M., Ashby, M., Nakayama, M., Rosenthal, W., and Bluestone, J.A. (2009b). Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. Nature immunology *10*, 1000-1007.