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I am submitting herewith a dissertation written by Sharvan Sehrawat entitled "Control of Herpetic Stromal Keratitis: Role of Regulatory T cells." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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REGULATION OF HERPETIC STROMAL KERATITIS: ROLE OF REGULATORY T CELLS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
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Sharvan Sehrawat
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This dissertation is dedicated to my mother, who taught me the ‘mantra’ of performing assigned task with perseverance and passion and the art of being versatile. She was always besides me from the beginning.

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ABSTRACT

Herpetic stromal keratitis (HSK) is an immunoinflammatory corneal lesion caused by herpes simplex virus (HSV) infection. One of the dire consequences of which is blindness resulting from tissue destructive immunopathological reaction in corneal stroma. The lesion is considered to be orchestrated mainly by $CD4^+$ T cells of Th1 phenotype. Studies on two animal models viz. immunocompetent and immunodeficient, shed light on the issues on specificity of the cells which at least in immunocomprised TCR transgenic animals were shown to be activated in a bystander manner. However, initial infiltration by innate immune cells in response to replicating virus set the stage for the chronic inflammation in the corneal stroma. Paradoxically, these cells are also critical in the control of virus in the cornea.

The first part (Part I) of this dissertation focuses on the understanding of HSV-1 induced immunoinflammatory processes in the cornea and trigeminal ganglia including the secondary lymphoid tissues and the involvement of regulatory mechanisms. The next three parts (Part II-IV) focus on the control of the inflammatory lesion and anti-inflammatory mechanisms that are activated following virus infection in the lymphoid organs and cornea. Results in Part II evaluate the immunotherapeutic potential of regulatory T cells in controlling the progression of the inflammatory lesions after ocular HSV infection. Results of the third section show that sequestration of T effector cells in the lymphoid organs and limited access to site of inflammation using a drug FTY720 after HSV infection resulted in diminished severity of SK and expansion of antigen-specific regulatory T cells that could contribute to the diminution of lesion severity. The fourth section describes the role of a previously unexplored inhibitory interaction between a Th1 specific cell surface marker, TIM-3 and its ligand galectin-9 in the causation of the viral induced

corneal immunopathology. The administration of galectin-9 seemed to be an effective approach to terminate Th1 responses and promote regulatory cells activity thereby controlling the severity of lesions.

In this study, experiments were designed to control the progression of the ongoing inflammatory reaction in the cornea in order to evaluate some of the therapeutic strategies for HSK.

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ABBREVIATIONS

APC	Antigen presenting cell
CCL2, 5, 20	Chemokine (C-C motif) ligand 2, 5, 20
CD	Cluster of differentiation
COX-2	Cyclooxygenase-2
CTL	Cytotoxic T lymphocyte
CXCL3, 8	CXC chemokine ligand 3, 8
CXCR2	CXC chemokines receptor 2
FGF	Fibroblast growth factor
FTY-720	2-amino-(2-(2-(4-octophenyl) ethyl)-1,3-propanediol hydrochloride
Gal-3,9	Galectin-3,9
HSK	Herpetic Stromal Keratitis
HSV-1	Herpes simplex virus 1
ICAM-1	Intercellular adhesion molecule 1
IFN-α, γ	Interferon alpha, gamma
IL-1, 2, 6, 10, 12, 17, 18, 23	Interleukin 1, 2, 6, 12, 17, 18, 23
IP-10	Interferon inducible protein 10
iTregs	induced Regulatory T cells
MCP-1	Monocyte chemoattractant protein 1
MIP-1, 2	Macrophage inflammatory protein 1, 2

MMP-9	Matrix metalloproteinase-9
NFκB	Nuclear factor- kappa B
PAMP	Pathogen Associated Molecular Patterns
PECAM	Platelet endothelial cell adhesion molecule
PFU	Plaque forming unit
PGE2	Prostaglandin E2
PMN	Polymorphonuclear leukocyte
SIP1,2,3,4,5	Sphingosine 1 Phophate receptors1,2,3,4,5
siRNA	small interfering RNA
SK	Stromal Keratitis
TGF-β	Transforming growth factor beta
TIM-3	T cell immunoglobulin and mucin protein
TLR 2, 4, 9	Toll like receptor 2, 4, 9
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
VLA4	Very late antigen 4

PART-I

BACKGROUND AND OVERVIEW

Etiology and epidemiology of SK

Herpetic Stromal Keratitis is an immunoblinding ocular lesion in normally transparent cornea and is caused by herpes simplex virus (HSV) infection. It is the most common cause of infectious blindness in the developed world. As per recent studies, the incidence of the disease lies between 4.1 -20.7 /100,000 of the population per year in the western world (1). Recent changes in the life style and behavior among the population presents a typical incidence pattern of herpes viral infections with more people being tested seropositive for HSV-2 than for HSV-1 (2). The incidence of HSK in the younger population, where it tends to be more severe, is on the rise in developing countries and is further complicated by factors such as malnutrition, co-infections and lack of accessibility to available treatments (3). Ocular lesions in human are mostly caused by recurrent activation of HSV 1 from the trigeminal ganglion (TG) and reflects the interaction between viral and host factors (2). The incidence of HSK by primary infection is three times lower than by recurrent infection. Because of unknown reasons, frequent cases of HSV 2 induced HSK occur in neonates but such are rare in adult population.

HSV can establish both productive and latent infection depending on the cell type infected. The non-neuronal cells allow the replication of viral genome while in sensory neuronal cells replication is limited and the virus tries to establish latency that can be life long. Classically, the life cycle of HSV in the host is divided into four stages: entry, spread, establishment of latency and reactivation. After the primary infection of the skin or mucosal surfaces with HSV, it spreads to the neuronal cell bodies where latency is established (4). Recent studies have established that maintenance of latency is dominantly achieved by both viral encoded factors such as miRNAs (5) or the host immune mechanism involving $CD8^+$ T cells responses (6). Depending upon the psychological, physical or the immune status of host, virus gets periodically

activated from latency in TG and released to the periphery. After reactivation, virus has a tendency to migrate to the initial site of infection and causes different types of painful inflammatory reactions depending on the involvement of tissue. But if the site of predilection happens to be eye, it causes either a sub-clinical or clinical inflammation in the epithelium that could be mitigated by anti-virals or a more severe chronic immunoinflammatory syndrome especially in the stroma where the reaction is called herpetic stromal keratitis or HSK that impairs vision and may lead to blindness if left unattended (3). HSV infections could also potentially result in viral retinitis and/or encephalitis in both immunosuppressive and immunocompetent individuals (3). Major symptoms of HSK lesions includes but not limited to corneal necrosis, ulceration and scaring, stromal edema and neovascularization that may necessitate corneal transplantation (7). These lesions are primarily orchestrated by $CD4^+$ T cells, the antigen-specificity of which remains poorly defined.

Animal models and pathogenesis of SK

Most of the studies directed to elucidate the pathogenesis of HSK were performed on animal models for human HSK. While for primary infection, mouse is the most studied animal model, the rabbit is the preferred animal model for recurring infection. However similar type of lesions occurs after primary or the recurrent infection (8). Understanding progressing events that finally lead to corneal inflammation has mainly come from primary ocular infection in mice. Several strains of mice including both immunocompromised and immunocompetent animals such as BALB/c, C57BL6, CAL-20, 129/SVEV have been described for studying HSK (9). In immunocompetent animals SK lesions are evident within 6-7 days after ocular infection with HSV 1 that peak in severity between 15-21 days. The lesions are primarily contributed by $CD4^+$

T cells that are detected in abundance at day 7-8 pi and most of which are likely to be HSV reactive. Another model is a TCR transgenic mice on a $RAG^{-/-}$ background, which were shown to develop SK upon ocular infection with HSV, even though their $CD4^+$ T cells were almost all reactive with $OVA_{323-339}$ peptide and not detectably cross-reactive with HSV antigens (10). The $CD4^+$ T cells in the ocular lesions of such animals were shown to react with the $KJ_{1.26}$ mAb noted by others to react with the TCR of *H-2*^{d} $CD4^+$ T cells that recognize the $OVA_{323-339}$ peptide (11). Since this KJ^+ TCR had no demonstrable reactivity with HSV, it was thought that the activation of KJ^+ $CD4^+$ T cells was not TCR mediated but involved activation by one or more cytokines (12). This model was referred to as a bystander model of SK (13). Yet another model that has been characterized is SK induced in SCID animals after reconstitution with $CD4^+$ but not $CD8^+$ T cells isolated from either HSV immune or naïve animals (14-16). All these animal models present typical SK lesions characterized by corneal haze, edema, necrosis, ulceration and neovascularization.

Ocular infection with HSV 1 in immunocompetent animals is followed by initial replication of virus especially in the corneal epithelium for up to 5-6 days. Live viral particles and the transcribed mRNA copies of viral genes could be detected from the corneal swabs during this time but not beyond 7 days post infection using conventional viral titration and RT-PCR assays respectively (1). However, viral DNA could be detected in the cornea even up to 21 days post infection (pi) the time when the disease is at its peak and spontaneous healing may start in a minority of animals. The nature and pathophysiological significance of the persisting viral genomic DNA species in terms of their transcriptional and translational efficiency to make viral proteins is not yet elucidated. The studies focused on these aspects would shed light on some of the previously unknown players in the causation of SK. After the initial phase of viral infection

and replication in the cornea, there is a prominent infiltration of inflammatory cells near the corneal epithelium that mainly consists of neutrophils (PMN) (17). These cells could potentially exert anti-viral defense by producing nitric oxides, reactive oxygen species, TNF-α, IFN-γ or perhaps just by engulfing viral particles. Furthermore, the kinetics of their infiltration correlated with the clearance of replicating virus from the cornea as shown in Fig.1.1 (All figures are supplied in appendix following the main part). Other studies where neutrophils were depleted prior to ocular HSV infection showed a delay in viral clearance from the cornea (18). In addition to their role in viral clearance, they may also provide conducive conditions for the ensuing inflammatory response by releasing mediators such as IL-1β, IL-8 (MIP-1α) and IL-12 and TNF-α. Neutrophil secreted NO could unmask corneal antigens that can be continuous source for the influx of reactive T cells. The matrix metelloproteinases such as MMP-9 breaks stromal matrix and along with neutrophil or perhaps stromal cell secreted VEGF-A contribute to the neovascularisation of usually avascular cornea. VEGF-A protein expression is evident in the cornea within 24 hrs and is mainly produced by epithelial cells or the stromal cells after ocular HSV infection. IL-1 and IL-6 produced by infiltrating inflammatory cells are the subsequent inducers of VEGF. Inhibition of angiogenesis by targeting MMP-9 and VEGF by siRNA approach were shown to reduce the extent of neovascularization (19, 20). Some additional angiokines such as bFGF, E-L-R motif containing chemokines (MIP-2) are also upregulated in cornea after HSV infection (21). In addition to PMNs, other cells such as DCs, NK cells, γδ-T cells, macrophages etc. could contribute both towards viral clearance and the subsequent inflammation by secreting type I IFNs, and other cytokines as well as chemokines such as IL-6, IL-1β, IL-12, MIP-2, TNF-α, IFN-γ, IL-23, IL-17 etc (1). Once the vascular bed is formed, there is continuous infiltration of cells because of leakage of newly formed vessels. IL-1 and IL-6

were shown to be the critical cytokines to initiate the subsequent inflammatory events and could be produced by epithelial cells initially after viral infection. HSV DNA and perhaps some of its other components expressing PAMPs can activate PRR such as toll like receptors (TLRs)-2,4 and 9 on the innate cells which provide stimulation for the activation of the NFκB pathway (22) . CpG motifs derived from viral DNA in the cornea could stimulate TLR 9 and induce IL-1 and IL-6 that contribute to the immunopathological lesions along with an efficient induction of adaptive immune response (23). IL-6 could be produced by un-infected cells by IL-1 stimulation in a paracrine manner which in turn trigger MIP-2 (also known as CXCL8) production that is involved in the attraction of PMNs. COX-2, is another important mediator of inflammation that could be induced by IL-1 in the cornea and acts through production of PGE2 (24). Recent studies have shown the important role of TNF-α in the causation of SK with animals lacking in this cytokine were unable to control the virus in cornea and thus exhibited enhanced lesion severity (25).

IL-12 produced by PMNs, macrophages and Langerhans DCs was shown to be another important candidate cytokine involved in the pathogenesis of SK (26, 27). It is involved in the downstream production of IFN-γ by macrophages, NK cells, neutrophils and $CD4^+$ T cells. Both proinflammatory and anti-inflammatory activities have been attributed to IFN-γ. It aids in the PMN influx by upregulating PECAM-1 and ICAM-1 on corneal epithelial cells (28, 29), endothelium cells and keratocytes needed for the exit of inflammatory cells. It also helps to prime the $CD4^+$ T cell responses by up regulating MHC II on antigen presenting cells. The most important anti-inflammatory activity of IFN-γ is attributed to its potential of inducing anti-angiogenesis factors including, but probably not limited to, IP-10 (30). Its role in promoting $Foxp3^+$ regulatory T cells induction was also described recently (31-33). IL-23/IL-17 axis is

another recently identified pathway involved in pathogenesis of various types of autoimmune inflammatory lesions. IL-23 produced by innate cells such as DCs is responsible for the stabilization of cells with the Th17 phenotype. IL-17 produced by inflammatory Th17 or perhaps fibroblast or neutrophils help recruit more PMNs. The role of IFN-γ producing Th1 cells is well studied in SK pathogenesis but a precise role of Th17 cells has not yet been described. In mouse SK lesion $CD4^+$ T cell outnumbers $CD8^+$ T cells but the reasons for their preferential accumulation remains unclear (34). The inflammatory reaction in the trigeminal ganglion has a preponderance of $CD8^+$ T cells in addition to $CD4^+$ T cells. The antigen-specificity of $CD4^+$ T cells that infiltrate cornea remains largely unknown because of a lack of specific $CD4^+$ T cells epitope derived from HSV or the tetramer. It is anticipated that $CD4^+$ T cells that infiltrate into cornea initially constitute a population enriched in HSV reactivity. Later on lesions would be dominated by bystander cell populations. Because of less stringent requirements of antigenic stimulation of Th17 cells (35) , it is possible that the role of these cells in the pathogenesis of SK predominates in the later stages of inflammation where viral antigen availability is limited. Thus, one valid hypothesis could be that the acute phase is mainly dominated by Th1 cells while the chronicity of lesion is maintained by Th17 cells. Some of our initial observations support this hypothesis (unpublished observations).

Current Treatment options

Anti-virals and anti-inflammatory agents

SK is a sight-threatening and difficult to control inflammatory reaction in the cornea. Currently, there is a dearth of agents which are approved for treating keratitis. Major classes of

drugs in use are either anti-virals or/and anti-inflammatories which include corticosteroids as well as non-steroidal anti-inflammatory drugs such as Cyclooxygenase-2 (COX-2) blockers (9, 36, 37). However, neither is beyond adverse effects. Viral species develop resistance to the anti-virals especially in immunocompromised individuals if used for prolonged periods of time (38, 39). Similarly use of corticosteroids might result in numerous adverse effects (40, 41). In some patients corticosteroid may enhance HSV-1 replication thereby exacerbating HSK lesions (42).

Future therapies

Targets for future therapies have mainly come from animal studies. Some of the studies in the mouse model of HSK have demonstrated that cytokine and chemokine blockers such as the IL-1 receptor antagonist are effective in controlling the severity of lesions (43). Inhibiting the transcription of genes for angiogenesis factor VEGF-A using siRNA approach was shown to be effective in reducing HSK lesions in the mouse (20). However, the mechanism by which it worked remains poorly understood. A recent report suggested a non-specific inhibition of angiogenesis by all ds RNA segments which stimulate TLR-3 and thereby induce production of IFN-γ (44). IFN-γ has been shown to have anti-angiogenesis activities. Other strategies that targeted the angiogenesis response included use of a recombinant *Salmonella typhimurium* containing a plasmid that encoded VEGFR-2 (45). This strategy induced anti-VEGFR-2 $CD8^+$ T cells that targeted pathological endothelial cells and inhibited neovascularization. However, the adverse effects because of non-specific killing of endothelial cells are expected. Several other approaches that made use of some components of HSV genome such as gB, gD (46-49) or the live mutant of vhs-/ICP8- HSV strain (50) have been shown to reduce the incidence of HSK. Various vaccine strategies along with the use of modifiers such as CpG, heat shock proteins

(hsps) etc have been described that enhanced the efficacy of HSV specific immune response (51, 52). The importance of vaccines has been envisaged because none of the anti-virals available is able to prevent the establishment of latency. Therefore, there is an increasing need of designing newer therapeutic vaccines and study their efficacy in terms of neurosurveillance and preventing reactivation of virus from the TG. A therapeutic agent BAY 57-1293, a helicase-primase inhibitor when used orally on daily basis showed some effects on reducing the reactivation of virus from latent TG and a subsequent dampening effects on virus replication in cornea that reduced the lesion severity.

Other recently identified novel immunotherapeutic molecules are being studied to add to the options for treating SK. One of them is apoprotein E (apopE) mimetic dimer peptide (apoEdp) and was shown to possess anti-viral and anti-inflammatory activities against ocular HSV infection when topical treatment begun 24 hr post infection and continued until day 10 (53). Another agent include topical treatment with >0.1% of cyclosporine that had some level of efficacy in diminishing the corneal haze in mice (54).

Regulatory T cells as an immunotherapeutic

Another subset of T lymphocytes that infiltrate the cornea is $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells (Tregs). In addition to other mechanisms such as the cytokine IL-10 (15, 55) Treg are shown to be involved in controlling the ongoing inflammatory processes in the cornea (15). In addition, these cells might help in the resolution of the clinical lesions. Studies suggest that depletion of Treg from the mice prior to infection results in more severe SK lesions (15). Sakaguchi et al.'s seminal observations in the mid-1990s (56) reawakened interest in Tregs and opened up the prospect of using these cells immunotherapeutically. Most of the $Foxp3^{+}$ Tregs are considered to be thymus-derived and are largely reactive to a range of self Ags (57). These cells

are involved in preventing and constraining autoimmunity, however the cells of the same phenotype may also participate in responses to foreign antigens and tumors. Indeed, regulatory cells may function beneficially to control tissue damage caused by auto reactivity, allergies and allotransplants, as well as responses to many tissue damaging pathogens (55). For therapeutic purposes, it would be preferable to use Tregs of known Ag specificity so as to increase potency and avoid potential side effects of inhibiting desirable immune responses. Some have expanded specific self-reactive Tregs in vitro and demonstrated in vivo efficacy using adoptive transfer approaches. This has been a "hot topic" recently in the Treg field since it is particularly relevant in terms of devising useful ways of manipulating the response therapeutically.

Strategies to cause conversion of conventional cells into Tregs

That the conversion of conventional $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ Tregs can be accomplished was appreciated some time ago by the Horwitz and Wahl groups who showed that TGF-β stimulation was a key event for the conversion process. Thus, the addition of TGF-β and IL-2 in TCR stimulated naïve $CD4^+$ T cells either from human or mice can result in $Foxp3^+$ Tregs induction (58, 59). When such converted cells were administered in vivo, they could suppress the proliferation of naïve $CD4^+$ T cells and prevented dust-mite induced allergic pathogenesis in lungs (59). The feasibility of in vivo conversion of $Foxp3^+$ regulatory T cells from conventional $CD4^+$T cells in periphery was shown in a TCR tg mouse model in which minute doses of a peptide were directed to certain types of dendritic cells (DCs) which were minimally activated (60, 61). Addition of TGF-β and IL-2 enhanced the conversion efficiency. These cells could further be expanded by immunization with the cognate antigen and were highly suppressive. A recent study suggested that exosomes like particles (ELPs), which are also a source of TGF-β, released from the thymus could play an important role in the peripheral

generation of Tregs (62). Foxp3 could be induced in Foxp3^{-}CD4^{+}T cells by administration a copolymer-1 (COP-1) in patients suffering from multiple sclerosis. The administration of COP-1 expanded regulatory T cells and these cells exhibited potent suppressive activity. The mechanism by which the induction of Foxp3 could be achieved was found to be dependent on the production of IFN-γ and TGF-β (63). A paradoxical situation where pro-inflammatory cytokines could induce anti-inflammatory regulatory T cells put forward a paradigm that the body has an inherent mechanism of dealing with excessive inflammation. The role of IFN-γ in induction of Foxp3 was also confirmed in other studies (31-33). The induction and enhancement of suppressive activity of Tregs by IFN-γ was found to be dependent upon STAT-1 signaling and production of NO (32, 64). NO is also produced by inflammatory innate cells such as neutrophils and myeloid suppressor cells (65, 66). It would be interesting to investigate if these cells have some role in the generation, maintenance or the expansion of Tregs. A role of another proinflammatory cytokine, TNF-α, in promotion of Treg responses was described recently (67). Tregs have been shown to have higher expression of TNFR-2 and the signal transduced by TNF-α in Tregs enhanced their proliferation and suppressive activity by up-regulating the expression of IL-2 receptor alpha chain (CD25) and Foxp3 (67, 68).

In addition to TGF-β, other anti-inflammatory molecules such as Thrombospondin-1 (TSP) when interacts with its CD47 receptor promotes the generation of human regulatory T cells (69). TSP is predominantly secreted by platelets and APC, and plays a major role in inhibiting angiogenesis, tumor cell growth and promoting apoptosis (70). Various domains of TSP exert both stimulatory and inhibitory effects on T cells via two TSP receptors, i.e., α4β1 and CD47, respectively. However, intact TSP is predominantly an inhibitor of TCR signal transduction (71) and IL-12 responsiveness of naive and adult T cells (72). TSP has also been

shown to be the main activator of TGF-β (28). Ligation of Thrombospodin to CD47 expressed on activated $CD4^+$ T cells rendered them anergic and imparted a regulatory phenotype that was accompanied by upregulation of CTLA-4, Foxp3, GITR, CD25 and OX40. The suppressive mechanism by these cells was contact dependent but TGF-β independent (69).

Intravenous immunoglobulin (IVIG) therapy is practiced as tolerance inducing strategy but recent studies have shown that one of the mechanism by which these therapeutic regimen could achieve tolerance is by inducing $Foxp3^+$ regulatory T cells (73). The presence of Treg epitopes also called as 'Tregitopes', in the constant region of Ig provided some explanation as to why Tregs were expanded in these patients. Tregitopes could stimulate the proliferation of Tregs (74). Administration of tryptophan catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO) expressing leukemia cells in the spleens could expand Treg population thereby inducing a state of tolerance (75).

An essential role of Tregs in maintaining maternal tolerance to the fetus during pregnancy has been explored recently (76, 77). Estrogen is one of the hormones which is highly expressed during pregnancy and plays an important role in establishment of pregnancy by activating blastocyst and initial implantation (77). Conventional $CD4^+CD25^-$ cells expressed the receptor for estrogen (ER). When estrogen is bound to ER on these conventional T cells, it induced $Foxp3^+$ expression and the cells attained the ability to suppress proliferation of TCR stimulated responder CD^+4 T cells in *in vitro* assays by producing TGF-β or IL-10. The conversion process was inhibited when a specific inhibitor (ICI 182780) of estrogen receptor was added in the cultures (76-78). These studies put Tregs on the center stage for maintaining the maternal tolerance to fetus.

Another immunomodulating neuropeptide, α–melatonin stimulating hormone (α-MSH) was shown to promote the generation of regulatory T cells in synergy with TGF-β (79, 80). The cells generated by this protocol produced TGF-β and exhibited suppressive activity in an antigen specific manner when transferred in vivo and prevented progression of experimental autoimmune uvoretinitis (EAU).

Role of antigen presenting cells and co-stimulatory molecules in the conversion process

Almost all of the conversion studies have made use of APCs or the micro beads coated with antibodies to co-stimulatory molecules in the cultures of TCR stimulated $CD4^+$ T cells, however, the requirement of individual costimulatory or inhibitory interaction pair between stimulated T cells and APCs remained largely unexplored until recently. Studies in recent years have investigated the role of some of the costimulatory or inhibitory interaction pair of molecules in the peripheral generation of Tregs. The first molecule that was shown to have a pivotal role in the conversion process was B7. Thus, conversion of transferred conventional $CD4^+$ T cells into $CD4^+CD25^+$ Tregs could be achieved in wild type congenic animals but not in B7-/- animals (81). Another inhibitory molecule Cytotoxic T lymphocyte antigen-4 (CTLA-4), a member of CD28-family of molecules and is expressed on TCR and anti-CD28 stimulated $CD4^+$ T cells. The major function of this molecule is to suppress the functioning of $CD4^+$ T cells by competing with CD28 for binding to B7.1 and B7.2 molecules (82) and decreasing the contact time of APCs with $CD4^+$ T cells (83). CTLA-4 Ig fusion protein has been used as an immunosuppressive agent in some autoimmunities such as rheumatoid arthritis and transplant rejection. Recently, it has become evident the addition of CTLA-4xIg in TCR stimulated cultures could convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ Tregs having potent suppressive activity. Furthermore, Tregs population expanded *in vivo* when CTLA-4Ig was administered (84). The

conversion and the expansion process were dependent on the presence of APCs and inhibited by the addition of anti-B7.2 blocking antibody. This study further attested to the involvement of B7 molecules in the conversion process. PD-1 and PDL-1 inhibitory signal was also found to play important role in the conversion of conventional $CD4^+$ T cells into Tregs ((85),our unpublished observation). Thus, it was shown that $CD8a^+$ DCs were more efficient than $CD8a^-$ DCs in converting $CD4^+$T cells into Tregs. It was further shown that DCs taken out from PDL-1 knockout animals were less efficient in converting TCR stimulated $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ regulatory T cells in presence of TGF-β. Furthermore, administration of anti-PDL1 but not anti-PDL-2 antibody significantly inhibited the induction of Foxp3 *ex vivo* (85). The overall numbers of $Foxp3^+$ Tregs were not found to be significantly different in either PD-1 or PDL-1 knockout animals as compared to wild type counterparts ((86), our unpublished observations). This observation could be explained in terms of the operation of compensatory mechanisms, which could have enhanced the Treg numbers, in the absence of signaling through PD-1. Some specialized subsets of $CD103^+$DCs isolated from the gut express enzymes such as retinal dehydrogenase that permit the conversion of vitamin A into retinoic acid (87-89). Furthermore, retinoic acid along with TGF-β act as a factor that drives the conversion of TCR stimulated $Foxp3^-$ conventional T cells into $Foxp3^+$ regulatory cells. Retinoic acid may also drive the conversion of human cells although in this instance the co-addition of TBF-β is not required (90). Apart from promoting the conversion of Tregs, antigen presenting cells through ligation of some co stimulatory pair of molecule inhibit the process of conversion. Thus, OX40/OX40L interaction pair inhibited TGF-β dependent conversion of TCR stimulated $CD4^+$ T cells into Tregs. At the same time it promoted the proliferation of effector T cells (91). Another molecule, galectin-9, a natural product of many cell types has been found to have a Treg generation

promoting activity. Thus, animals lacking galectin-9 had reduced numbers of Tregs and addition of galectin-9 promoted TGF-β induced conversion of conventional T cells to become $Foxp3^+$ (92). In part IV of this dissertation, we have shown that galectin-9 could promote Treg generation even in the presence of inflammatory milieu.

Until recently, neurons were thought to have an immuno-regulatory functions but their expression of B7 and TGF-β provided a mechanistic explanation of their being highly immuno-regulatory. Thus neurons expressing B7 and TGF-β could confer a regulatory phenotype to interacting $CD4^+$ T cells by inducing Foxp3 in them (93).

Involvement of T cell intrinsic mechanisms in the conversion process

TGF-β signaling in naïve cells stimulated through their TCR is transduced by either of SMAD proteins (SMAD2, 3 or 4) or through p38 MAP kinase pathway. Signalling through SMAD proteins led to the upregulation of all TGF-β dependent genes and thus imparted T cells a regulatory phenotype (94). At the same time signaling through TGF-β also activated the p38 MAP kinase pathway and inhibition of this pathway by specific inhibitor, SB203580, prevented the conversion of CD^+CD25^- T cells into $Foxp3^+$ Tregs (94). However, in already differentiated Tregs, this treatment did not affect the suppressive activity suggesting that signalling through p38 is important in the initial stages of differentiation. Another group of proteins that were found to have an important role in promoting expansion of regulatory T cells are members of GTP binding proteins family. These include protein product encoded by Ras genes (N-Ras, K-Ras, and H-Ras) and have diverse intracellular signaling functions including the control of cell differentiation, proliferation, growth, and apoptosis (95). These proteins are considered as switch-signals and act by cycling between inactive-GDP or active-GTP conformations (96). The final signaling event through these proteins is activation of ERK/MAP kinase pathway that in

turn activates T cells. The inhibition of N-Ras and K-Ras genes by shRNA and small molecule inhibitors such as farnesylthiosalycylic acid, led to increased expression of Foxp3 in $CD4^+$ T cells that was accompanied by enhanced suppressive activity (97). An importance of another signaling molecule STAT 3 in the conversion of Tregs and maintenance of their activity was realized recently where ablation of STAT-3 using shRNA or neutralizing antibody led to decreased conversion of $CD4^+CD25^-$ T cells into Tregs and animals failed to prevent occurrence of acute graft-vs-host disease (98). A dominant role of CTLA-4 in maintaining the suppressive activity of $CD4^+CD25^+Foxp3^+$Tregs have been demonstrated recently (99). Thus selective depletion of CTLA-4 gene in Tregs resulted in fatal autoimmune disease in animals (99). GRAIL (gene related to anergy in lymphocytes), a ubiquitin-protein necessary for the induction of anergy in $CD4^+$ T cells was found to be highly up regulated in $Foxp3^+$ Tregs and the forced expression of this molecule in T cell line could impart them a suppressive phenotype in the absence of Foxp3 expression (100)

Signalling through IL-2 has been shown to be a critical event in the generation of Tregs (101). In the absence of IL-2 signalling other cytokines such as IL-7, and IL-15 can have some compensatory role in the promotion of Treg generation and expansion (102).

Epigenetic control of Foxp3 locus in regulatory T cells

The role of the microenvironment constituted by cytokines such as TGF-β, IL-2, IFN-γ, TNF-α etc. in the induction of Foxp3 in a cell type that is committed to become regulatory is well known from the studies over many years (59, 60). But little is known about the stability of phenotype attained by these cells as compared to thymus derived natural Tregs. The epigenetic mechanism that include histone modifications and selective demethylation in CpG motifs have been shown to play pivotal role in stable cellular lineages of one type or the other. These events

impart heritability fixed expression patterns of distinct genes in the cell (103-105). The involvement of these mechanisms in the commitment of regulatory T cells is not clearly known at this time and is a current area of active research in the biology of Tregs. Some studies have attested to the importance of DNA demethylation, acetylation of histone proteins such as H3, H4 and trimethylation of H3 in the conserved regions in *foxp3* locus in Tregs but not in conventional $CD4^{+}CD25^{-}$ cells (106). These modifications were also shown by human $CD4^{+}CD25^{hi}$ Tregs suggesting the evolutionary importance of these events that allow persistent expression of Foxp3 and thus a regulatory phenotype. TGF-β induced Tregs show a weakly demethylated CpG motifs within conserved region of *foxp3* locus that might not impart them a stable phenotype. Futher support for these studies came from recent observation where the use of agents that either inhibited DNA methylation of CpG motifs such as azacytidine or deacetylation of certain histone protein such as Trichostatin A (TSA) or valproic acid (VPA). This resulted in promotion of Foxp3 expression even in absence the of TGF-β but also enhanced their suppressive activity (107, 108). The appropriately modified foxp3 gene further regulates Treg specific characteristics such as anergy and suppressive functions by suppressing transcriptional and promotor binding activity of protein such as activator protein 1 (AP-1) by interacting with phosphorylated c-Jun (109). In adition, the functional Foxp3 protein in a cell actively repress transcription of genes by recruiting distinct histone acetyl transferases and histone deacetylases (110). In Tregs, foxp3 physically interacts with transcription factors such as acute myeloid leukemia 1 (AML-1) and Runt-related transcription factor 1 (Runx1) which are required for the transcription of IL-2 and IFN-γ and thus regulate the production of these cytokines (111).

Resistance of Tregs to T cell depleting regimens

Many studies have advocated the differential susceptibility of Tregs to depletion and apoptosis inducing strategies. Thus, it was shown that TCR stimulated Tregs were less susceptible to Fas-Fas-L induced apoptosis than TCR stimulated conventional $CD4^{+}CD25^{-}$ T cells. However, the susceptibility followed a reverse trend for naïve cells (112). The efficacy of anti-thymocyte globulin therapy for depleting peripheral lymphocytes in order to maintain tolerance in the patients suffering from auto immunities or those who received transplants has been demonstrated. This therapy depleted T effector cells but the the population of Tregs was spared (113). Furthermore, ex vivo treatment with anti-thymocyte globulin induced and expanded Treg populations (114). Nur77, a protein of the nuclear receptor family, has been shown to promote apoptosis of thymocytes during negative selection of autoreactive thymocytes. However, Tregs were found to be resistant to Nur77 mediated apoptosis (115). Another reagent that has been studied for inducing tolerance in autoimmiunities such as EAE and type 1 diabetes, is anti-CD3 monoclonal antibody. Its administration in patients reduced the streptozocin induced diabetes and Th1 type of immune response at the same time it increased the expression of latency associated peptide on $CD4^{+}$ T cells (116). The use of this antibody not only prevented the development of diabetes but also reversed the disease (117). A very recent study on lupus, an auto-antibody mediated autoimmune disease, reported the efficacy of nasal administration of anti-CD3 antibody in inhibiting the progression of the lesions development and at the same time inducing IL-10 secreting $CD4^{+}CD25^{-}LAP^{+}$ Regulatory T Cell (118).

Strategies to inhibit the suppressive activity and the conversion process of Tregs

Regulatory T cells are one of the important players in the maintenance of peripheral tolerance and preventing excessive tissue damage as a result of an inflammatory reaction.

However, there are situations where their activity needs to be regulated. Enhancing tumor immunity, anti-microbial immune response, vaccine efficacy etc. are some of the examples where excessive Treg response might hinder in the generation of effective immune response. Therefore, under such circumstances, the activity of Tregs needs to be blunted. Some of the factor that have been shown to negatively regulate Tregs are cytokines IL-6, IL-12, IL-21 etc (119, 120). Curiously, adding inflammatory cytokines such as IL-6 in cultures thought to be suitable for generation of Tregs resulted in the induction of T cells that produced IL-17 and were found to be responsible for excessive CNS inflammation (121). Additionally, it was shown that IL-21 could initiate an alternate pathway of induction of Th17 cells in the absence of IL-6 and also inhibited the activity of Tregs (122). Signaling through some TLRs such as TLR-9 and TLR4 abrogated the activity of Tregs (119, 123). Accordingly, it was shown that gut micro floral CpG DNA by activating TLR-9 inhibit the process of Foxp3 generation and enhanced the immune response to oral infection (123). AKT was shown to be a critical pathway that inhibited *de novo* differentiation of regulatory T cells by changing the transcription signature molecules associated with the Tregs phenotype in undifferentiated $CD4^+$ T cells. It did not affect the Foxp3 expression in already differentiated cells (124). The forced expression of AKT or the agents which could increase its expression could be used to reduce the numbers of Tregs. Another pathway that was shown to inhibit Treg function and differentiation is OX40/OX40L pair (125). Thus it was shown that $CD4^+$ T cells isolated from OX40L Tg mice were highly resistant to conversion into $Foxp3^+$ Tregs and these animals have large numbers of memory T cells ($CD44^{hi}CD62L^-$) which were not only resistant to conversion into Tregs but actively inhibited the conversion of naïve $CD4^+$ T cells to become $Foxp3^+$ by inducing producing IFN-γ and inducing T-bet. In addition, some studies showed that function of Tregs could be abrogated by

inducing signaling through OX40L (125). Accordingly, it was shown that administration of anti-OX40 agonist antibody into tumors, which is enriched in $OX40L^+$ $Foxp3^+$ Tregs, led to rejection of tumors provided sufficient numbers of $CD8^+$ T cells were present (126). Signalling transduced through some of the T cell surface receptors could modulate the conversion process and the activity of Tregs. It was shown that an agonist antibody against TIM-1, a T cell specific surface marker deprogrammed the Tregs, inhibited the conversion of $CD4^+CD25^-$ cells into Tregs and enhanced the function of effector T cells thereby preventing allogenic transplant tolerance (127). In a recent study in renal transplant patients, use of a pharmacological agent, calcineurin inhibitors (CNI), reduced the numbers of Tregs in peripheral blood while rapamycin treatment preserved their numbers (128).

The agents described in this section could be used to turn the activity of Tregs off in situations where they have undesirable effects.

Conclusions

SK is a chronic immunoinflammatory lesion in cornea with a complex pathogenesis. Discoveries of novel pathways and molecular participants in the pathogenesis would provide new targets for designing therapeutics. Based on the previous knowledge of the key player and events involved in the development of SK, strategies that target one or more of the key events need to be investigated. Some of these approaches include inhibition of excessive immune response generation, limiting the access of T effectors to the inflammatory sites in the cornea by preventing their exit from lymphoid organs, or delivering an inhibitory or lethal signal to the cells at the site of inflammation to treat the disease.

The work described in this dissertation evaluated the effects of some of these strategies on modulating SK lesion. Tregs specific to a well know OVA antigen were generated and characterized. The functional polyspecificity of in vitro generated Tregs was evaluated in HSV induced immunopathology in three separate models of SK. In a second approach cells were sequestered in the lymphoid organs using a small molecule, FTY720, that acts as an agonist to sphingosine receptors and temporarily down regulates their expression so that cells are sequestered in lymph nodes and do not migrate to inflammatory site in the cornea. The prolonged use of this drug generated antigen-specific Tregs that were also shown to regulate the severity of SK lesion previously (15) . In the last approach cells were induced to undergo apoptosis in vivo at the site of inflammation using a recently discovered molecule, galectin-9, that delivers a lethal signal to Th1 effector cells through a surface molecule TIM-3 and a positive signal to regulatory T cells. These approaches could be used individually or in combination with other existing therapies ro resolve SK with minimal unwanted side effects.

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APPENDIX

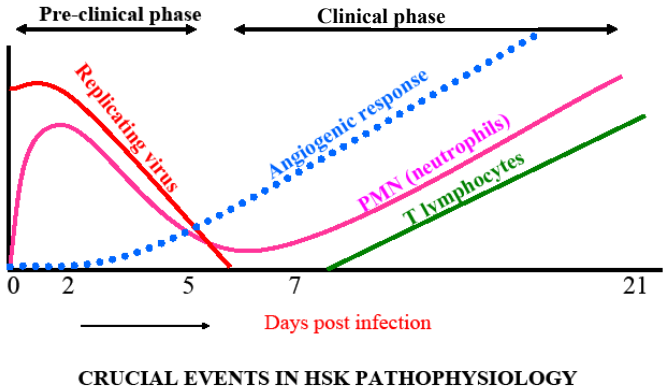

Figure 1. 1 Principal events in herpetic SK pathogenesis

Following ocular HSV infection replicating virus could be detected in the cornea till 5-7 days p.i. Early inflammatory response in the cornea is dominated by the polymorphonuclear leukocytes (PMN). Infiltration of PMNs into the cornea is be characterized by a typical biphasic influx. Angiogenesis or the process of new blood vessel development from the existing limbal vessels starts at 24h p.i. and peaks around 15 days p.i. Influx of pathogenic CD4+ T lymphocytes occurs in the clinical phase around 7-9 days p.i.

PART-II

IN-VITRO GENERATED ANTIGEN-SPECIFIC $CD4^+CD25^+FOXP3^+$ REGULATORY T CELLS CONTROL THE SEVERITY OF HERPES SIMPLEX VIRUS INDUCED OCULAR IMMUNOINFLAMMATORY LESIONS

Research described in this chapter is a slightly modified version of an article that in accepted for publication in *Journal of Virology* by Sharvan Sehrawat, Susmit Suvas, Pranita P Sarangi, Amol Suryawanshi and Barry T Rouse

Sehrawat S, Suvas S, Sarangi PP, Suryawanshi A and Rouse BT. *In vitro* generated antigen-specific $CD4^+CD25^+Foxp3^+$ regulatory T cells control the severity of HSV-induced ocular immunoinflammatory lesions J Virol: 2008 : 82(14); 6838-6851. *Copyright © 2008, American Society for Microbiology.*

In this chapter "our" and "we" refers to me and co-authors. My contribution in the paper includes (1) Selection of the topic (2) Compiling and interpretation of the literature (3) Designing experiments (4) understanding the literature and interpretation of the results (5) providing comprehensible structure to the paper (6) Preparation of graphs and figures (7) Writing and editing

Abstract

Generating and using regulatory T cells to modulate inflammatory disease represents a valuable approach to therapy but not yet been applied as a means to control viral induced immunopathological reactions. In this report we developed a simplified technique that used unfractionated splenocytes as a precursor population and showed that stimulation under optimized conditions for 5 days with solid phase anti-CD3 mAb in the presence of TGF-β and IL-2 could induce up to 90% of $CD4^+$ T cells to become $Foxp3^+$ and able to mediate suppression *in vitro*. $CD11c^+$ DCs were intricately involved in the conversion process and once modified in

the presence of TGF-β could convert $Foxp3^{-}CD4^{+}$ cells into $Foxp3^{+}CD4^{+}$cells by producing TGF-β. The converted cells had undergone cell division and the majority of them expressed activation markers along with surface molecules that would facilitate their migration into tissue sites. The primary reason for our study was to determine if such *in vitro* converted Tregs could be used *in vivo* to influence the outcome of a virus induced immunoinflamatory lesion in the eye caused by HSV infection. We could show in three separate models of herpetic stromal keratitis (SK) that adoptive transfers of *in vitro* converted Treg effectively diminished lesion severity especially when given in the initial phases of infection. The suppression effect *in vivo* appeared to be polyspecific. The protocol we have developed could provide a useful additional approach to control virus-induced inflammatory disease.

Introduction

Numerous types of regulatory T cells were reported to control the immune responses to both self and foreign antigens (1). Impediments in regulatory T cell function can cause disease conditions (2). The best known example is the multiple-organ autoimmune disorders that occur in humans as well as in experimental animals when the gene for Foxp3 transcription factor is mutated (3, 4). Functional inactivation of Foxp3 gene alters the development and immunosuppressive activity of the natural regulatory $CD4^{+}$ T cell population. In recent years, naturally occurring $Foxp3^{+}$ regulatory T cells were shown as the key cell type that maintains peripheral tolerance (5). Additionally, these cells also regulate pathogen and allergen induced inflammatory responses and boosting their functional activity may represent a valuable therapeutic approach to blunt transplant rejection. Previously, it was reported that depletion of nTregs prior to ocular infection with HSV-1, increased the severity of immunoinflammatory

lesions in the cornea (6). However, it would be valuable to know if Tregs can modulate virus induced ongoing immunoinflammatory reactions and influence the disease progression.

In this report, we took advantage of the recent observations that Foxp3$^+$ T cells with regulatory function can be generated from conventional T cells by appropriate *in vitro* activation conditions (7-12). We modified the existing method to develop a simple *in vitro* technique to generate Ova-specific Foxp3$^+$ regulatory CD4$^+$ T cells from CD4$^+$Foxp3$^-$ cells. We then determined the ability of *in vitro* generated Tregs to modulate the severity of an ocular immunoinflammatory reaction caused by infection with Herpes simplex virus (HSV) in conventional and bystander models of HSV-1 induced stromal keratitis. Our results showed that adoptive transfer of *in vitro* generated Ova-specific Foxp3$^+$ Tregs diminished lesion expression in three different models of herpetic stromal keratitis (SK) in both an antigen-specific as well as non-specific manner.

Materials and methods

Mice, Virus, cell lines

Female 6- to 8-wk-old BALB/c DO11.10 RAG2-/- mice were purchased from Taconic, Thy1.2$^+$ BALB/c and CB.17 SCID mice were purchased from Charles River and Thy1.1$^+$ BALB/c mice were a kind gift from Dr. D. Woodland (Trudeau Institute, Saranac Lake, NY). All animals were housed in the animal facilities at the University of Tennessee. BALB/c DO11.10 RAG2-/- and CB.17 SCID mice were kept in our specific pathogen-free facility where food, water, bedding, and instruments were autoclaved and all manipulations were done in a laminar flow hood. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. HSV-I RE

Hendricks and HSV-I KOS was propagated and titrated on Vero cells (ATCC CCL81) using standard protocols. The virus was stored in aliquots at -80°C until use.

Corneal HSV-1 infection and clinical observations

Corneal infections of un-manipulated BALB/c mice and DO11.10RAG2-/- animals and adoptively transferred with iTregs were conducted under deep anesthesia. HSV RE and HSV KOS were used for inducing keratitis lesions in immunocompetent BALB/c and DO11.10RAG2-/- animals, respectively. Sometimes Tregs were adoptively transferred in previously infected animals. Mice were scarified on their corneas with a 27-gauge needle, and a 3-µl drop containing the required viral dose was applied to the eye. The eyes were examined on different days postinfection (p.i.) with a slit-lamp biomicroscope (Kowa, Nagoya, Japan), and the clinical severity of keratitis and angiogenesis of individually scored mice was recorded. The scoring system was as follows: 0, normal eye; +1, mild corneal haze; +2, moderate corneal opacity or but iris visible; +3, severe corneal opacity, iris invisible; +4, opaque cornea, ulcer formation; and +5, necrotizing SK. The angiogenesis was scored as described previously (13).

Antibodies and reagents

CD4-APC (RM4-5), Thy1.1-PerCP (OX-7), DO11.10-PE (KJ1.26), CD25-FITC (7D4), GITR-FITC (DTA-1), Folate Receptor 4-FITC (eBio12A5), CD62L-FITC (MEL-14), CD103-FITC (M290), CD62L-APC (MEL-14) CD49d-PE (MFR4.B), ICAM-1-PE (3E2), ICOS-PE, PDL-1-PE (MIH5), PD-1-FITC (J43), Foxp3-PE(FJK-16s), Foxp3-FITC (FJK-16s), CD69-FITC (H1.2F3), CCR7-PE (4B12), CD11c-PE (HL3), anti-IFN-γ-FITC, anti-IL-10-FITC anti-IL-17-PE were purchased from BD PharMingen (San Diego, CA). Recombinant human TGFβ-1, anti-TGF β1,2,3 antibody (1D11) and anti IL-10 antibodies (AB-417-NA) were obtained from R&D. anti-CD3 (145.2C11), anti-CD28 (37.51) were from BD Bioscience. Anti-PD1 antibody (J43)

and anti-ICOS antibody (7E.17G9) were from ebioscience. Recobinant human IL-2 was obtained from Hemagen. $OVA_{323-339}$ peptide was obtained from Genscript. CFSE was obtained from Molecular Probe and used at a final concentration of 0.5 μM for 15 min. at 37^0C in PBS.

***In vitro* generation of $CD4^+CD25^+Foxp3^+$ regulatory T cells**

Splenocytes isolated from DO11.10Rag2-/- mice were fractionated into $CD4^+$ and $CD4^-$ T cells using $CD4^+$ T cell isolation kit. $1x10^6$ of T depleted SPCs were pulsed with various doses (2μM, 1μM, 0.62μM, 0.31μM and 0.15μM) of OVA peptide for 2 hours at 37^0C and washed three times thereafter. Ova pulsed cells were then cocultured in 1:1 ratio with purified $CD4^+CD25^-Foxp3^-$ cells in the presence of 10ng/ml of rTGF-β, and 25U of IL-2 for five days at 37^0C and thereafter the cells were characterized by Flow cytometry. To discount the possibility of residual ova peptide transfer along with transferred Tregs for subsequent *in vivo* experiments, Ova specific Tregs were also generated with plate-bound anti-CD3 mAb. To this end, 0.125μg/ml of anti-CD3 mAb in a volume of 200 μl was coated overnight in 48 well flat bottomed plate in 0.1M Na_2HPO_4 buffer, pH 9.0. Before establishing culture of splenocytes with the cocktail of various cytokines, plates were washed three times with medium after which splenocytes were cultured as described above but in the absence of ova peptide. Total splenocytes were isolated from DO11.10RAG2-/- animals and red blood cells were lysed using RBC lysing buffer. $2x10^6$/ml of SPCs were cultured with various concentrations of recombinant hTGF-β1 (5ng, 10ng or 15ng), IL-2 (100, 50, 25U) and anti-CD3 antibody in a checker board fashion. The cultures were incubated for varying periods ranging from day 4 to day 7 at 37^0C in a humidified CO_2 incubator to find the optimum dose of all constituents in the cocktail and the incubation periods that yielded a maximum percentage of induced Tregs. In certain experiments,

splenocytes were cultured in 6 well culture plates to generate Tregs in bulk. The generated Tregs were phenotypically characterized by flow cytometry.

Purification of cells

$CD4^+$ and $CD4^-$ T cells were purified from naïve DO11.10 RAG2 -/- mice using $CD4^+$ T cells isolation kits (Miltenyi Biotec). In addition, $CD4^+$ and $CD4^-$ T cells were also purified from *in vitro* cultures for some *in vivo* experiments. $CD11c^+$ DCs were isolated on magnetic beads columns (Miltenyi Biotec) after 48 hours of *in vitro* cultures system either from those that contained the culture medium that resulted in $Foxp3^+$ T cell conversion or medium that lacked TGF-β. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from *in vitro* cultures using $CD4^+$ regulatory T cell isolation kit (Miltenyi Biotec). The depletion of T cells from splenocytes was achieved by using Thy1.2 microbeads (Miltenyi Biotec). All purification procedures were performed as per the manufacturers' instruction.

***In vitro* suppression assay**

In vitro suppression assays were performed to assess and compare the inhibitory activity of iTregs and splenic nTregs against anti-CD3 stimulated $CD4^+CD25^-$ T cells. To assess the suppressive activity of iTregs, $CD4^+CD25^+$ T cells were purified from splenocytes cultured for 5 days in the presence or absence of recombinant TGF-β1. $CD4^+CD25^-$ T cells were isolated from pooled spleens and LNs of DO11.10RAG2-/- and labeled with 0.5μM of CFSE. T depleted splenocytes were isolated from spleens of DO11.10RAG2-/- using Thy1.2 microbeads and irradiated before use in the suppression assays. CFSE labeled $CD4^+CD25^-$ T cells ($5x10^4$) were cultured in the presence of soluble anti-CD3 mAb (1.0μg/ml) and irradiated splenocytes ($1x10^5$) with sequentially diluted $CD4^+CD25^+$ T from either culture in 96-well round-bottomed plates.

After 72 hours, dilution of CFSE was analyzed by flow cytometry. The gate was applied on $CD4^+CFSE^+$ T cells and the intensity of CFSE staining was analyzed.

To compare the suppressive activity of iTregs with splenic nTregs, iTregs were isolated from the *in vitro* culture system and $CD4^+CD25^+$ cells isolated from the pooled spleens of BALB/c animals. $CD4^+CD25^-$ T cells isolated from pooled spleens and LNs of Thy1.1 BALB/c animals and labeled with 0.5μM CFSE. Cultures were set up as described above and the dilution of CFSE in $Thy1.1^+CD4^+$ gated cells was analyzed.

Measurement of *in vivo* activity of iTregs

Three different systems were used for measuring the *in vivo* activity of *in vitro* generated Tregs. In the first series of experiments, **c**ultured splenocytes containing $5x10^5$ of $CD4^+CD25^+Foxp3^+$ T cells were adoptively transferred i.v. in DO11.10 RAG2-/- mice one day prior or six days after ocular infection with $5x10^5$ pfu of HSV I KOS and the lesion were scored every alternate day beginning at day 4. At day 11, mice were sacrificed and cells recovered from cornea, DLN and spleen were analysed by flow cytometry.

In the second set of experiments, $5x10^6$ of isolated $CD4^+CD25^-$ T cells from BALB/c animals were transferred with or without $1x10^6$ of either nTregs or OVA Tregs into SCID animals which were then infected with ocular HSV I ($5x10^5$ pfu). The SK lesion progression and angiogenesis was monitored for 12 days. To look for the proliferation of lesion orchestrating $CD4^+$ T cells, CFSE labeled $CD4^+CD25^-$ T cells from BALB/c animals were transferred alone or with iTregs or nTregs. These animals were infected 24hrs later with $5x10^5$ pfu of HSV I (RE). After 7 days of transfer, the proliferation of $CD4^+CFSE^+$ T cells was analyzed by dilution of CFSE.

Finally, experiments were done to look for the disease modulatory activity of Tregs in immunocompetent BALB/c animals. Five different doses ($2x10^6$, $1x10^6$, $5x10^5$, $2x10^5$ and $5x10^4$) of $Foxp3^+$ T cells were adoptively transferred i.v. in BALB/c animals one day prior to the ocular HSV RE ($5x10^5$ pfu) infection. The disease severity was recorded for 15 days post infection. A minimum dose of Tregs which could inhibit the disease progression in these animals was used for subsequent studies where cells were transferred one day before, three days or 6 days post infection. Animals were sacrificed at different time intervals to collect and analyze lymphoid and non-lymphoid tissues.

Flow cytometric analysis

Cell preparation Single-cell suspensions were prepared from the cornea, draining cervical lymph nodes (DLN), and spleen of mice at different time points p.i. Corneas were excised, pooled group wise and digested with 60 U/ml Liberase (Roche Diagnostics) for 60 min at 37°C in a humidified atmosphere of 5% CO_2 as described earlier (6). After incubation, the corneas were disrupted by grinding with a syringe plunger on a cell strainer and a single-cell suspension was made in complete RPMI 1640 medium.

Staining for flow cytometry The single-cell suspension obtained from LNs, spleen, and corneal samples were stained for different cell surface molecules for FACS. All steps were performed at 4^0C.Briefly, a total of 1×10^6 cells were first blocked with an unconjugated anti-CD32/CD16 mAb for 30 min. in FACS buffer. After washing with FACS buffer, fluorochrome-labeled respective Abs was added for 30 min. Finally, the cells were washed three times and resuspended in 1% paraformaldehyde.

To enumerate the number of IFN-γ producing T cells, intracellular cytokine staining was performed as previously described (26). In brief, 10^6 freshly isolated splenocytes and lymph node

cells were cultured in U bottom 96-well plates. Cells were left untreated, stimulated with 2 MOI of UV inactivated HSV I and incubated overnight at 37°C in 5% CO_2. Brefeldin A (10 µg/ml) was added for the last five hours of the culture period. After this period, cell surface staining was performed, followed by intracellular cytokine staining using a Cytofix/Cytoperm kit (BD PharMingen) in accordance with the manufacturer's recommendations. The Ab used was anti-IFN-γ-FITC and anti-IL-17-PE. The fixed cells were resuspended in 1% paraformaldehyde and acquired with BD FACSCalibur. The data were analyzed using the CellQuestPro 3.1 (BD Biosciences) or Flowjo software.

BrDU incorporation assay

BrDU analysis was performed as described earlier (14). Briefly, mice were divided into four groups: naïve, naïve + iTregs, infected and infected + iTregs. $5x10^5$ iTregs were transferred a day before ocular infection and animals were then fed BrDU in drinking water at 1mg/ml for 10 days after adoptive transfer of iTregs and ocular HSV 1 infection. After 10 days, host $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells that incorporated BrDU were analysed by staining with anti-BrDU antibody using an APC BrDU flow kit from BD Pharmingen as per the manufacturers' instructions.

Statistical analysis

To calculate the statistical significance for disease severity between different groups, unpaired two-tailed Student's *t* test was performed. All other analyses for statistically significant differences were performed with Student's *t* test. $P \leq 0.001$ = *** $P \leq 0.01$ = ** and $P \leq 0.05$ = * were considered significant. Results are expressed as mean ± SD. For some experiments, as mentioned in the fig. legends, one way ANOVA test was applied.

Results

In vitro Generation of Ova-specific Treg (iTregs)

In initial experiments, splenic cells from naive DO11.10 Rag2-/- animals were fractionated on MACS columns into the T cell fraction (greater than 95% KJ^+ $CD4^+$ and hence OVA-specific) as well as the non T cell fraction. These non-T cells were pulsed with various concentrations of the $OVA_{323-339}$ peptide and used to stimulate the T cell fraction in a 1:1 ratio. The culture medium contained IL-2 (25U/ml) as well as human recombinant TGF-β (10ng/ml), conditions expected to be suitable to induce $Foxp3^+$ T cells from conventional precursors (7, 10). Using these conditions, we observed that after a 5 day culture period up to 70% (range 50-70%) of the surviving cells in the culture were $Foxp3^+$. The dose of ova peptide that gave maximum conversion efficiency was 0.31μM.

To simplify matters, we also cultured unfractionated DO11.10 Rag2-/- naive splenocytes in culture plates that had been pre-incubated with various concentrations of rat anti-mouse CD3 antibody. In preliminary experiments using 0.3μg/ml to sensitize plates and the same TGF-β and IL-2 concentrations described above, up to 75% (range 70-75%) of cells were observed to be $Foxp3^+$ after 5 days stimulation (Fig. 2.1A). When peptide (0.31μM) was used for stimulation instead of anti-CD3, up to 45% (range 30-45%) of $CD4^+$ T cells became Foxp3 (data not shown). Subsequently, the system was optimized by using varying concentrations of anti-CD3 and TGF-β, but using the same amount of IL-2. As shown in figure 2.1B and C, a concentration of 0.125μg/ml of anti-CD3 and 10 ng/ml of TGF-β turned out to be optimal. With the optimal conditions, the frequency of $CD4^+$ T cells that were $Foxp3^+$ ranged from 80% to 90% of the $CD4^+$ T cells in different experiments (Fig. 2.1D). These conditions were chosen for subsequent

experiments in which *in vitro* converted cells were used *in vivo* in an attempt to modulate HSV induced ocular inflammatory lesions. During the conversion process, the Foxp3$^+$ T cells underwent division (one to six times) although some converted cells must have died since the overall yield of Foxp3$^+$ cells was usually around two fold the number of input Foxp3$^-$CD4$^+$ T cells (Fig 2.1E).

Phenotypic Characterization of iTregs

Using optimal Foxp3 conversion conditions, cultures were terminated at various times after initiation not only to establish the time when conversion was at its maximum, but also to measure the expression of additional phenotypes on the Foxp3$^+$ T cells that could play a role in their migratory and functional properties *in vivo*. The results are expressed in fig 2.2A. Maximal frequencies of Foxp3 expression were evident after 5 days and these frequencies sometimes exceeded 90% of the surviving CD4$^+$ T cells.

As shown in fig. 2.2B, most of the Foxp3$^+$ T cells at the end of the culture period were CD103$^+$, a molecule important for migration into tissue sites (15). This, as well as some other markers, was present on only a minor population of the CD4$^+$Foxp3$^+$ nTreg population isolated from spleens of naïve immunocompetant mice (Fig 2.2B). Additional phenotypic markers present on the majority of the *in vitro* converted Foxp3$^+$ T cells at the end of the culture period included CD25, GITR, folate receptor 4 (FR4), PD1, ICOS, ICAM-1, CD62L, CD49d, CCR7 and CCR5. Their higher level (mean fluorescence intensity and percent positive) of expression on the *in vitro* converted Tregs compared to nTregs indicated that the *in vitro* converted cells were in a more activated state and more endowed with surface molecules that would permit tissue migration (Fig 2.2B and 2C).

TGF-β modified CD103$^+$CD11c$^+$ DCs are involved in Foxp3 induction

It was of interest to note that the expression of some phenotypic markers observed at the end of the culture period was not evident on those Foxp3$^+$ cells that had been converted early during culture. The striking example of this was CD103, a molecule involved in tissue homing *in vivo* (15). Curiously after two days of culture, the majority of CD11c$^+$ dendritic cells (DCs) were already CD103$^+$ but only a minor fraction (up to 2-5%) of CD4$^+$Foxp3$^+$ was CD103$^+$ (Fig 2.3A and B). The dendritic cells isolated from cultures lacking TGF-β, after two days, did not show this phenotype. We suspected that the CD103$^+$ DCs could be the cells that were mainly responsible for causing the majority of CD4$^+$Foxp3$^+$ conversion observed in our culture system. In support of this notion, we could demonstrate that the DCs isolated from cultures after two days, could be used to drive Foxp3 expression in *de novo* cultures of purified CD4$^+$CD25$^-$Foxp3$^-$ T cells that were TCR stimulated in the presence of IL-2, but without additional TGF-β in the culture system. Accordingly, CD11c$^+$ cells were isolated by positive selection from 2 day stimulated splenocytes with anti-CD3, IL-2 and TGF-β (conversion medium). In addition, CD11c$^+$ cells were isolated from similar cultures that lacked TGF-β. The purity of CD11c$^+$ DCs was between 85 to 90% in different experiments.

As shown in Fig.2.3C, the addition of CD11c$^+$ cells from cultures with the conversion medium caused up to 50% of CD4$^+$ cells to become Foxp3$^+$ after 4 days further stimulation in the absence of TGF-β. In contrast, CD11c$^+$ cells from the two day culture that lacked TGF-β failed to induce significant numbers of Foxp3$^+$ converted cells in the secondary cultures. We interpret these experiments to mean that the CD11c$^+$ cells were intricately involved in the *in vitro* conversion process perhaps by being modified in their function during the initial culture period. This modification could include acting as a source of TGF-β. Thus, the addition of anti-TGF-β

antibody either in primary or secondary cultures markedly inhibited the induction of Foxp3 in purified $Foxp3^{-}CD25^{-}CD4^{+}$ (Fig 2.3C). Furthermore, the DCs isolated after two days in the conversion system had higher TGF-β mRNA levels than did DCs stimulated under non converting conditions (data not shown).

Functional activity of Foxp3 converted cells

At the end of the 5 days culture period, $CD25^{+}CD4^{+}$ T cells were isolated from both cultures stimulated with the conversion medium as well as cultures that were stimulated in the presence of IL-2 but without TGF-β. These cultures without TGF-β failed to generate significant numbers of $Foxp3^{+}CD4^{+}$ T cells as already stated. Both sets of $CD25^{+}$ cells were added to anti-CD3 stimulated naïve $CD4^{+}CD25^{-}$ OVA-specific T cells isolated from pooled spleens and LNs of DO11.10Rag2-/- mice and irradiated T depleted splenocytes from the same strain of mice, to measure their anti-proliferation activity. As shown in Fig. 2.4A, only the $Foxp3^{+}$ containing population showed a dose dependent suppressive activity. Experiments were also performed to determine possible mechanisms involved in the suppressive activity of iTregs. Neutralizing antibodies to either TGF-β, IL-10, ICOS or PD-1 were added in suppression assays. The suppressive activity was only diminished significantly when PD-1 blocking antibody was added to the cultures (Fig. 2.4B. We interpret these experiments to mean that the suppression did not involve either TGF-β or IL-10 cytokines and that PD-1 engagement was likely involved in the suppression mechanism as previously observed by others (16).

In a second approach, $CD25^{+}CD4^{+}$ T cells from *in vitro* conversion cultures and splenic $CD25^{+}CD4^{+}$ nTregs isolated from immunocompetant BALB/c animals were compared for their ability to suppress the proliferation of $CD25^{-}CD4^{+}$ T cells. The latter were isolated from pooled spleens and LNs of naïve Thy1.1 BALB/c animals (hence polyspecific population) stimulated

with anti-CD3 antibody and T depleted irradiated splenocytes as a source of APCs. Proliferation of Thy1.1^{+}CD4^{+} T cells was analyzed by dilution of CFSE. In such experiments, the Ova-specific converted cells showed activity that was somewhat enhanced compared to the nTreg population (Fig 2.4C).

***In vivo* activity of *in vitro* converted cells in ocularly infected DO11.10RAG2-/- mice**

The major objective of our investigation was to determine if *in vitro* converted Foxp3^{+}cells could influence the severity of ocular immunoinflammatory lesions induced in mice by HSV infection. The first model used TCR transgenic mice on a RAG-/- background which were shown previously to develop stromal keratitis (SK) upon ocular infection with HSV even though their CD4^{+} T cells were almost all reactive with OVA$_{323\text{-}339}$ peptide and not detectably cross-reactive with HSV antigens (17).The T Cells in the ocular lesions of such animals were shown to react with the KJ1-26 monoclonal antibody noted by others to react with the TCR of H-2^{d} CD4^{+} T cells that recognize the OVA323-339 peptide (18). Since this KJ^{+} TCR had no demonstrable reactivity with HSV, we surmised that the activation of KJ^{+}CD4^{+} T cells was not TCR mediated but involved activation by one or more cytokines (19). We have referred to this as a bystander model of SK (11).

As already described the Foxp3 converted cells in the *in vitro* system used in this communication were all KJ^{+} and hence OVA-specific. Consequently, we speculated that such cells adoptively transferred into the infected DO11.10RAG2-/- animals should modulate the severity of the ocular lesions. To test this, DO11.10 RAG2-/- mice were ocularly infected with 5x10^{5} pfu of HSV KOS (which routinely induces SK in these animals) and some were given intravenously 5x10^{5} Foxp3 converted cells either 24hrs before or at 6 day p.i. The severity of

lesions was then followed in control and adoptive transfer recipients over a 11-12 day observation period (animals began to die after day 11 from herpetic encephalitis).

The cumulative results of three similar experiments are shown in Fig 2.5A, and B. As is evident, the average severity of ocular lesions was significantly reduced in animals that received cells 24 hours before infection. Similarly, lesion severity was also reduced significantly in the day 6 recipients. Samples were collected from both groups of animals to determine if the adoptively transferred Foxp3$^+$ T cells could be demonstrated in the recipient tissues. It became possible to detect transferred cells since the DO11.10 RAG2-/- animals do not have detectable Foxp3$^+$ cells even following infection with HSV. The results of such experiments revealed that both in day-1 and day -6 transfer recipients, appreciable numbers of Foxp3$^+$ cells could be demonstrated by FACS analysis of collagenase digested ocular samples as well as in the draining LN and spleen (Fig 2.5C). Reduced percentages as well as absolute numbers of PMNs such as neutrophils (CD11b$^+$Gr1$^+$) were found in the corneal tissues of transfer recipients as compared to control animals (Fig 2.5D).These experiments clearly demonstrated that the *in vitro* Foxp3 converted cells may function *in vivo* to diminish herpetic lesions although in the model studied we could not establish how the inhibition was achieved or if this occurred by the action of Foxp3$^+$ cells in the ocular tissue themselves or in the DLN in some way. It remains unclear how HSV infection causes SK in the DO11.10RAG2-/- model but we suspect the mechanism involves the activation of CD4$^+$ T cells by inflammatory mediators generated by the infection. In support of this, CD4$^+$ T cells collected from lymphoid tissues as well as cornea showed high frequencies of CD69$^+$ cells indicating they are activated. Transfer of iTregs into such animals significantly reduced the frequencies of activated cells (data not shown).

Inhibitory effects of Treg on SK in reconstituted SCID mice

Whereas SCID animals ocularly infected with HSV fail to develop SK lesions, they do so if reconstituted with $CD4^+CD25^-$ T cells even if such cells are taken from naïve animals (6). In a previous study, we demonstrated that SK severity in such animals was inhibited if $CD4^+CD25^+$ T cells were cotransferred with the $CD4^+CD25^-$ population. As shown in Fig. 2.6A, this observation was repeated but in addition we were able to show that Ova-specific iTregs were equally capable of modulating lesion severity. This occurred despite the fact the iTregs population was KJ^+ and hence ova-specific. Such experiments indicate that iTregs may act in a bystander inhibitory fashion but how such an effect was mediated requires further exploration. The effect appeared to involve inhibition of proliferation of effectors. This was shown in experiments wherein CFSE labeled $CD4^+CD25^-$ (effectors) cells were transferred alone or with either iTregs or nTregs in SCID animals 24 hrs before ocular HSV I infection. As shown in Fig. 2.6B, the frequencies of proliferating cells, when measured at day 7 p.i., were reduced in recipients of both iTregs and nTregs as indicated by dilution of CFSE.

Inhibition of SK by iTregs in immunocompetent BALB/c animals

The final approach used to measure the efficacy of *in vitro* converted $Foxp3^+$ T cells was to use the immunocompetent ocularly infected BALB/c animals as the transfer recipients. In these experiments, animals were infected with $5x10^5$ pfu of HSV RE, a dose which was expected to cause lesions in the majority of recipients and the outcome was compared in control animals with those given different numbers of *in vitro* converted cells day-1 prior to infection. The numbers of donor cells varied from $2x10^6$ to $5x10^4$ of $Foxp3^+$ T cells which it must be emphasized were KJ^+ and hence Ova-specific. The results of a representative experiment are shown in Fig 2.7A. As can be seen, lesions were markedly reduced in recipients given $2x10^6$

donor cells but inhibition was also significant in those that received 5 fold less cells. Using the $5x10^5$ donor cell dose for transfer, we also compared the suppressive effects of donor cells that were fractionated into $CD4^+$ and $CD4^-$ populations prior to transfer. As is evident in Fig 2.7B, only the $CD4^+$ fraction suppressed lesion severity in the BALB/c recipient animals.

In subsequent experiments, HSV infected BALB/c animals were given adoptive transfers of $Foxp3^+$ converted cells ($5x10^5$ $Foxp3^+$ cells) either one day before, or 3 or 6 days p.i. Animals were then scored for both the extent of angiogenesis and SK lesion severity scores over a 15 day observation period. The data in Fig 2.7C and D, show the cumulative data of individual animal scores of three separate experiments. As is evident, significant levels of inhibition occurred in the early transfer recipients. Transfers at day 6 provided suppression in some animals but overall the results were not significant especially at day 15 p.i. probably because of lower sample size and large variations. Interestingly, transfer of Tregs at day 3 invariably failed to produce suppression of SK or levels of angiogenesis. This might be explained by the fact that levels of proinflammatory cytokines, such as IL-6 are high at this time in the DLN and cornea (Fig 2.7E) Thus cytokines such as IL-6 are known to blunt the function of Tregs (20). Inhibition could be achieved in the day 3 transfer model if ten fold more cells were transferred on day 3 (Fig 2.7F and G). Conceivably, the inhibition was evident because insufficient IL-6 was present to blunt the function of all of the transferred Tregs, but this issue needs to be formally explained.

Our data demonstrate that early transfer of Foxp3 converted OVA-specific T cells are fully capable of inhibiting lesions caused by HSV provided sufficient cells are transferred and if performed early after infection. It is far from clear how the Treg which are OVA-specific, act against an inflammatory lesion caused by HSV or in fact where the inhibitory effect is mediated. Experiments showed that adoptively transferred cells (based on determining the KJ^+ markers)

could be demonstrated to be present at least in appreciable numbers in the eye as well as lymphoid tissues at 16 day post transfer (Fig 2.8A). In long term studies, transferred cells (2-3% of recovered $CD4^+$ T cells) could be found in corneal tissues even after three month post transfer but such cells were undetectable in lymphoid tissues at this time point (data not shown). It would be interesting to investigate if corneal resident iTregs could prevent the recurrence of stromal keratitis.

Although some adoptively transferred iTregs could be demonstrated in ocular tissues, their polyspecific suppressive activity could be mediated mainly in lymphoid tissues. Thus, one consequence of HSV infection is an increase in the spleen size as well as the DLN. Curiously, as shown in the Fig. 2.8B and C, the spleen size and total cellularity in iTreg recipients was reduced approaching near to that of normal animals depending on the number of iTregs given. In addition, iTregs recipients showed lower numbers of HSV-specific IFN-γ producing $CD4^+$ T cells (Fig 2.8D), those principally responsible for mediating SK (21) than was evident in control infected animals that did not receive iTregs. Accordingly one outcome of the early iTreg transfer was suppression of the response of $CD4^+$ T cells to HSV although how this was achieved mechanistically remains to be explained. Regulatory cells transferred at day 6 had no effect on the magnitude of the anti-HSV immune response (data not shown).

We considered the possibility that iTreg adoptive transfers might serve to inhibit the division of host effectors while at the same time causing the expansion of the hosts' own nTregs as was reported in a diabetes model by the Steinman group (22). To support such a possibility, we performed adoptive transfers with KJ^+Treg and then compared the proliferative capacity of the host's $CD4^+Foxp3^+$ and $Foxp3^-$ populations. Our results showed reduced proliferative responses of host $CD4^+Foxp3^-$ cells compared to infected controls not given iTregs, but an

increase in the proliferative response of the host $CD4^+Foxp3^+$ population. Thus, the donor KJ^+Tregs appeared to mediate the suppression in at least two ways. These included inhibitory effects on the host effectors as well as an expansion of the host's own nTregs population. Curiously, the donor KJ^+ cells themselves underwent proliferation in the host which may also help explain how the minimal cell transfers were effective at modulating lesions.

Discussion

In this report we have confirmed the observations of others that $Foxp3^+$ regulatory T cells can be generated *in vitro* from $Foxp3^-$ naïve $CD4^+$ cells (7, 10). We developed a simplified technique that used unfractionated splenocytes as a precursor population and showed that stimulation under optimized conditions for 5 days with solid phase anti-CD3 mAb in the presence of TGF-β and IL-2 could induce up to 90% of $CD4^+$ T cells to become $Foxp3^+$ and able to mediate suppression *in vitro*. The converted cells had undergone cell division and the majority of them expressed activation markers along with surface molecules that would facilitate their migration into tissue sites. The primary reason for our study was to determine if such *in vitro* converted Tregs could be used *in vivo* to influence the outcome of a virus induced immunoinflammatory lesion in the eye caused by HSV infection. We could show in 3 separate models of herpetic stromal keratitis (SK) that adoptive transfers of *in vitro* converted Treg effectively diminished lesion severity especially when given in the initial phases of infection. The protocol we have developed, which is novel for a viral induced inflammatory disease, could provide a useful additional approach to control a chronic virus induced lesion.

Previous studies on adoptive transfers with *in vitro* converted Tregs have focused on the control of autoimmune lesions or to facilitate the acceptance of transplants (23-27). Moreover,

the techniques used to produce the Treg populations were usually complex requiring purification of both responders and stimulators to achieve success. We have found that unfractionated splenocytes can suffice as a responder population. In our case, these were transgenic T cells from naïve DO11.10 RAG2-/- mice which normally lack a population of $Foxp3^+$ T cells (28). The T cells from such animals are predominantly $CD4^+$ and express a TCR that recognizes the ova peptide that can be conveniently identified with the KJ26 monoclonal antibody. We could generate $Foxp3^+KJ^+$ cells either by stimulating splenocytes with ova peptide in the presence of TGF-β and IL-2, or more effectively by stimulating with solid phase anti-CD3 mAb. It was not necessary to use additional costimulators such as anti-CD28 as is used in most other studies (7, 10, 29). The Foxp3 conversion process involved cell division and appeared to depend on the presence of APC that responded initially to the TGF-β. Thus, we could show that 2 day stimulation of splenocytes in the conversion medium caused $CD11c^+$ cells in the culture to become $CD103^+$. Moreover, such cells could be used to drive Foxp3 expression in TCR stimulated cultures of naive $CD4^+Foxp3^-$ T cells without the addition of TGF-β to cultures. We interpret these observations to mean that the $CD103^+CD11c^+$cells could be a source of TGF-β and possibly costimulation to the $CD4^+$ cells that convert to become $Foxp3^+$. Curiously, recent studies on Foxp3 conversion in the gut had demonstrated an essential role of $CD103^+$ TGF-β producing dendritic cells (30-34).

The principal objective of our studies was to explore the value of adoptive transfers with regulatory T cells as a means to influence the pathogenesis of a viral induced immunoinflamatory lesion. In prior studies, we had shown that lesions caused by HSV infection in the eye were more severe in animals depleted of nTregs (6) so it was anticipated that adoptive transfers of Tregs might prove valuable to suppress the severity of SK lesions. However, as is

well known when using Treg therapeutically to control autoimmunity, the most potent Treg populations are those that are reactive with the same antigens as the effectors cells that drive the lesions (22, 23, 25). Unfortunately, we have no simple means of generating HSV-specific Tregs *in vitro* although this issue is under further investigation. There is, however, an SK model using TCR transgenic x Rag or SCID mice where the animals' T cells lack demonstrable cross-reactivity with HSV (17). Nonetheless, they develop stromal inflammatory lesions upon ocular infection with HSV. This has been referred to as the bystander model of SK (19). Using this model we could show that the adoptive transfer of *in vitro* generated $Foxp3^{+}KJ^{+}$ T cells could inhibit the severity of SK in HSV infected DO11.10RAG2-/- mice wherein lesions are orchestrated by $CD4^{+}$ effectors that are also KJ^{+} (19). In this instance therefore, the Treg control could be antigen-specific.

Inhibition was most effective when the Tregs were given around the time of infection but significant effects were also evident in animals in which therapy was delayed until day 6. We consider this latter observation particularly interesting since it indicates that the adoptive transfer of Treg may represent a potential means of controlling ongoing viral inflammatory disease. We suspect that even greater efficacy in the day 6 therapeutic model could be observed if animals were kept alive for longer than the usual time of their dying of herpetic encephalitis (around day 11). We are currently pursuing such experiments in animals treated with anti-virals or protected by neutralizing antibody. In longer living animals multiple Treg administration will also be feasible, perhaps a necessary protocol to fully control lesions.

Unexpectedly, we were also able to show that *in vitro* generated Ova-specific $Foxp3^{+}$ Tregs could modulate lesions in HSV infected BALB/c mice where certainly the $CD4^{+}$ T cells involved in orchestrating lesions were not KJ^{+} and ova specific. Such observations could mean

that adoptively transferred Tregs can act non-specifically. This could be possible since the cells used for adoptive transfer were highly activated and in fact showed enhanced regulatory effects *in vitro* compared to nTregs isolated from normal animals. In fact if the iTregs were transferred a week or more in animals prior to infection, control was not achieved (data not shown). During this time we presume that cells may have lost their activation status although this issue needs to be investigated.

Evidence for a non-specific regulatory effect of the *in vitro* converted Tregs was also seen in a second model of SK. In this model, SK can be induced in ocularly infected SCID mice as long as they are provided with adoptive transfers of normal or HSV immune $CD4^+$ T cells (6). We could demonstrate that co-transfer of the Ova-specific converted Tregs along with the polyspecific $CD4^+$ $CD25^-$ T cells resulted in significantly reduced lesions. These results provide evidence of non-specific bystander regulatory effects since the lesion-inducing effector cells would not be reactive to Ova peptide. Others too, have reported instances where Tregs could mediate bystander suppression (35-37). This is especially evident for regulatory cells that are abundant producers of cytokines such as IL-10 (26). However, we suspect that the bystander suppression effect in our system may not involve IL-10 because only a minor fraction of the cells could be shown to be IL-10 producers and the suppressive activity *in vitro* was found largely independent of cytokines. Nevertheless, this issue requires further investigation.

Although our results demonstrate that *in vitro* converted Tregs could suppress the severity of SK lesions, the location and mechanism by which the inhibition occurs *in vivo* remains unclear. Thus, the cell population expressed several molecules that permitted the cells to access tissue sites as well as lymphoid tissue. In fact, adoptively transferred cells could be demonstrated at both the ocular inflammatory site as well as in lymphoid tissues. Some reports

advocate that TGF-β generated Foxp3$^+$ cells have only a short life span *in vivo* (26, 38). This was not our experience since some KJ$^+$Foxp3$^+$ cells could still be recovered from the eyes of BALB/c mice three months after their administration. Finding cells in the eye does not mean that they exert their regulatory effect in that tissue. In fact, we strongly suspect that the suppressive effects of the early cell transfers were mediated mainly in lymphoid tissues where the effector T cell response to the virus is being generated. Thus recipients of such transfers had reduced HSV-specific effector CD4$^+$ T cell responses. Moreover, few if any effector T cells appear in the stroma until 6 or 7 days post infection (39).

Another interesting observation was that the viral-induced immunopathology was suppressed by quite small numbers of adoptively transferred iTregs. Conceivably, the iTregs could in some way be causing the host's own nTregs to become activated and suppressive as has been suggested to occur with similar studies in a diabetes model (22). In this model, control of pancreatic inflammation appeared to be mediated by the increased population of host derived Tregs in the tissue. In fact, donor cells were not demonstrable in the recipient tissues. In our system we, could demonstrate the presence of transferred cells in the target tissues but, in addition we noted that host nTregs underwent increased proliferation in iTreg adoptive transfer recipients compared to controls. Such host nTregs could conceivably contribute to the suppression of lesions as ongoing studies are attempting to demonstrate.

Finally, although the results were variable we could demonstrate that even in the BALB/c model that transfer of Tregs at 6 days p.i., around the time of major access of effector T cells into the corneal stroma, could suppress lesion severity. Transfer of Treg at this time period had no effect on the magnitude of the anti HSV immune response which is approaching its peak at this time. Conceivably the suppressive activity of such transfers could result from effects in the

tissues themselves. It may be that multiple transfers would be a more effective way of controlling ongoing lesions as we are currently investigating.

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APPENDIX

Figure 2. 1 In vitro generation of Foxp3$^+$CD4$^+$ T cells from OVA-specific precursor Foxp3$^-$ CD4$^+$ T cells.

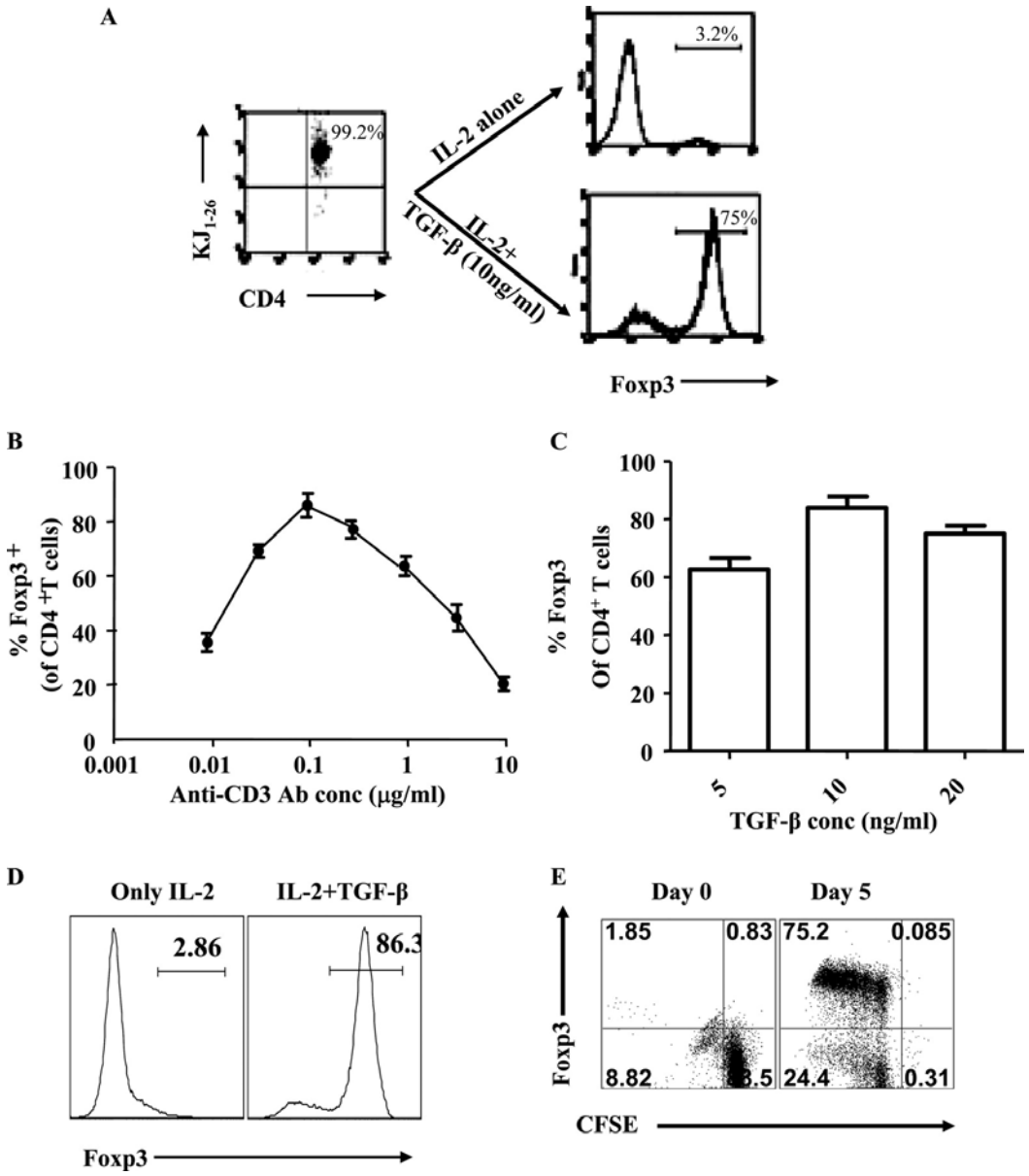
A. Splenocytes from DO11.10RAG2-/- mice were cultured in the presence of 0.3μg/ml of anti-CD3 antibody, 25U of rIL-2 and the indicated concentrations of TGF-β. More than 99 percent of CD4$^+$ T cells were KJ_{1-26} positive (gated on CD4$^+$ T cells). After five days of culture, cells were analyzed for the expression of CD4 and Foxp3. Using these conditions approx. 75% of CD4$^+$ T cells became Foxp3$^+$.

B. Dose response curve for Foxp3 induction with various conc. of anti-CD3 antibody, 10ng/ml of TGF-β and 25U of IL-2 is shown. A dose of 0.125μg/ml of anti-CD3 antibody (plate bound) was found to be optimal.

C. Dose response histogram for Foxp3 induction with various conc. of TGF-β, 25U of IL-2 and 0.125μg/ml of anti-CD3 is shown. At a dose of 10ng/ml of TGF-β 80 to 90 % of CD4$^+$ T cells converted to become Foxp3$^+$.

D. Representative histograms for gated CD4$^+$ T cells are shown to show the Foxp3 induction using optimal conditions (0.125μg/ml of plate bound anti-CD3 antibody, 25U of IL-2, 10ng/ml of TGF-β).

E. Splenocytes from DO11.10RAG2-/- animals were CFSE labeled and cultured with plate bound anti-CD3, IL-2 and TGF-β for five days. After five days cells were stained with CD4 and Foxp3. CFSE dilution and Foxp3 expression was shown in gated CD4$^+$ T cells. TGF-β induced CD4$^+$CD25$^+$Foxp3$^+$ T cells proliferate extensively.


A
99.2%
KJ1-26
CD4
IL-2 alone
IL-2+
TGF-β (10ng/ml)
3.2%
75%
Foxp3
B
% Foxp3 +
(of CD4 +T cells)
100
80
60
40
20
0
0.001
0.01
0.1
1
10
Anti-CD3 Ab conc (μg/ml)
C
% Foxp3
Of CD4+ T cells
100
80
60
40
20
0
5
10
20
TGF-β conc (ng/ml)
D
Only IL-2
IL-2+TGF-β
2.86
86.3
Foxp3
E
Day 0
Day 5
1.85
0.83
75.2
0.085
8.82
24.4
0.31
Foxp3
CFSE

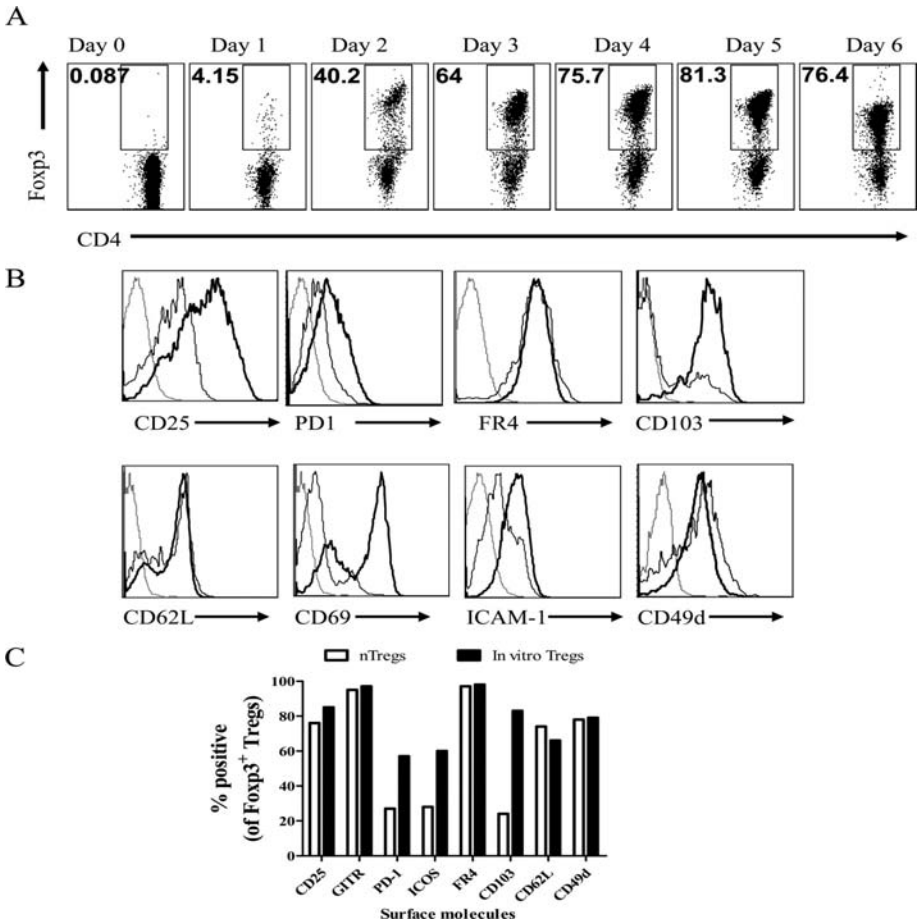


Figure 2. 2 Phenotypic characterization of in vitro generated Foxp3$^+$ T cells.

A. Representative FACS plots out of three similar experiments are shown for the kinetic analysis of Foxp3 induction in CD4$^+$CD25$^-$Foxp3$^-$ T cells in *in vitro* cultures. Maximum conversion was observed at day 5 after initiation of culture. (Gated on CD4$^+$ T cells). B. Expression of surface markers on *in vitro* converted Foxp3$^+$ cells was compared with that of nTregs isolated from spleens of naïve immunocompetent animals. Representative histograms are shown. (CD4$^+$Foxp3$^+$ T cells were gated, doted lines represent isotype control, solid lines represent expression on nTregs and solid but bold lines represent expression on iTregs). C. Bar diagram of percent positive of Foxp3$^+$ nTregs and iTregs for various surface markers is shown.

Figure 2. 3 Splenic CD11c$^+$ DCs are intricately involved in causing conversion of Foxp3$^-$ T cells into Foxp3$^+$ CD4$^+$ T cells.

Kinetics of CD103 expression on CD4$^+$Foxp3$^+$ T cells (A) and CD11c$^+$ DCs (B) in *in vitro* conversion culture is shown. The expression of CD103 was observed earlier on DCs than on CD4$^+$Foxp3$^+$T cells. C. CD11c$^+$ DCs were purified from 48hr splenocyte cultures in presence of anti-CD3, IL-2 and either without TGF-β as used in (C(a)) or with TGF-β as used in (C(b) and (C(d)) or with TGF-β + anti-TGF-β as used in (C(c)). These DCs were then co-cultured in 1:5 ratios with anti-CD3 stimulated purified CD4$^+$CD25$^-$Foxp3$^-$ T cells isolated from naïve DO11.10RAG2-/- animals in presence of IL-2 alone or with anti-TGF-β antibody. Representative FACS plot showing Foxp3 induction when the DCs were isolated from primary culture with no TGF-β (C(a)), primary culture in presence of TGF-β (C(b)), primary cultures in presence of TGF-β and anti-TGF-β and when DCs were from primary culture with TGF-β but anti-TGF-β antibody was added in secondary cultures.

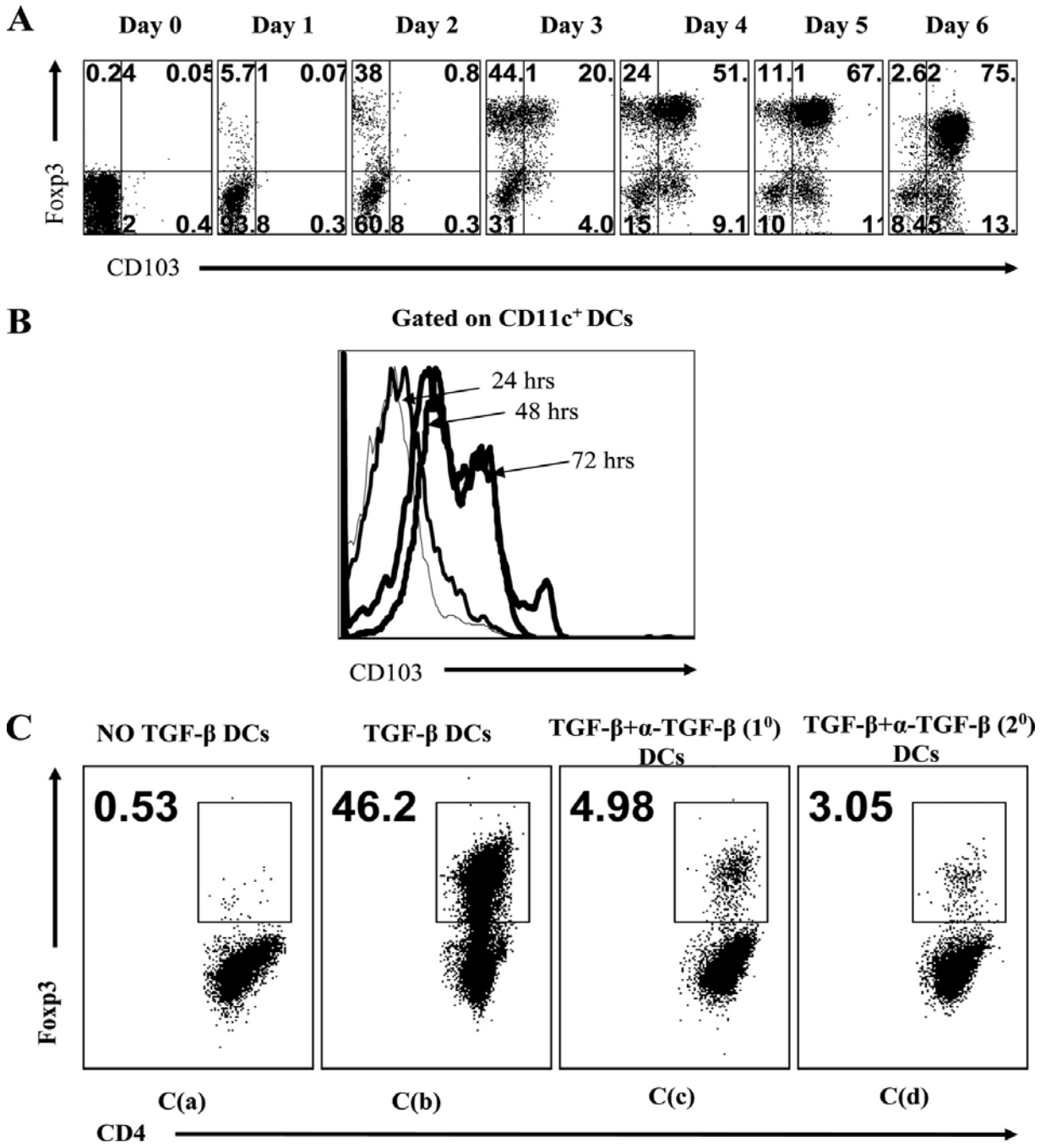

A
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Foxp3
CD103
B
Gated on CD11c+ DCs
24 hrs
48 hrs
72 hrs
CD103
C
NO TGF-β DCs
TGF-β DCs
TGF-β+α-TGF-β (1⁰) DCs
TGF-β+α-TGF-β (2⁰) DCs
0.53
46.2
4.98
3.05
Foxp3
C(a)
C(b)
C(c)
C(d)
CD4

Figure 2. 4 In vitro generated Tregs inhibit the proliferation of antigen-specific and polyspecific CD4+CD25- T cells.

$CD25^+CD4^+$ T cells were isolated from both cultures stimulated with the conversion medium (Tregs) as well as cultures that were stimulated in the presence of IL-2 only ("control cells"). Additionally, $CD4^+CD25^+$ T cells were also isolated from pooled spleens and LNs of BALB/c animals. These cells, in two fold serial dilutions, were used in suppression assays against the cultures of anti-CD3 antibody stimulated CFSE labeled $CD4^+CD25^-$T ($1x10^5$) cells from naïve DO11.10RAG2-/- (i.e. to measure antigen-specific effect) and Thy1.1 BALB/c animals (i.e. to measure polyspecific effect) with irradiated T depleted splenocytes ($2x10^5$) from homologous system as described in Material and Methods section.

A. The extent of CFSE dilution as an indication of suppressive activity of *in vitro* generated $CD4^+CD25^+$ regulatory T cells against anti-CD3 stimulated labeled $CD4^+CD25^-$ T cells from naïve DO.11.10 RAG2-/- animals is shown. Of the $CD4^+CD25^+$ T cells, about 90% and 3% were $Foxp3^+$ from cultures in the presence or absence of TGF-β, respectively. A first gate was applied on $CD4^+$ T cells and then extent of CFSE dilution in $CD4^+CFSE^+$ T cells was analyzed.

B. Blocking antibodies to either TGF-β (10μg/ml) or IL-10 (10μg/ml) or PD-1 (10μg/ml), or ICOS (10μg/ml) were added to the suppression cultures and the extent of division of CFSE in labeled cells was determined. Representative FACS plots at two dilutions of Tregs to Teffectors are shown for PD-1 and ICOS neutralized cultures. Dashed line represents dilution of CFSE when no Tregs were added, solid line represents dilution of CFSE when Tregs were added, solid but bold line represents dilution of CFSE when along with Tregs either anti-PD1 or anti-ICOS antibodies were added.

C. Representative histograms indicating CFSE dilution in the Thy1.1 gated population is shown. $CD4^{+}CD25^{+}$ T cells were isolated from iTreg culture, control cells and splenic nTregs from BALB/c animals and were used against anti-CD3 stimulated labeled $CD4^{+}CD25^{-}$ T cells from Thy1.1 animals. The markers show the percentages of cells that underwent less than two divisions.

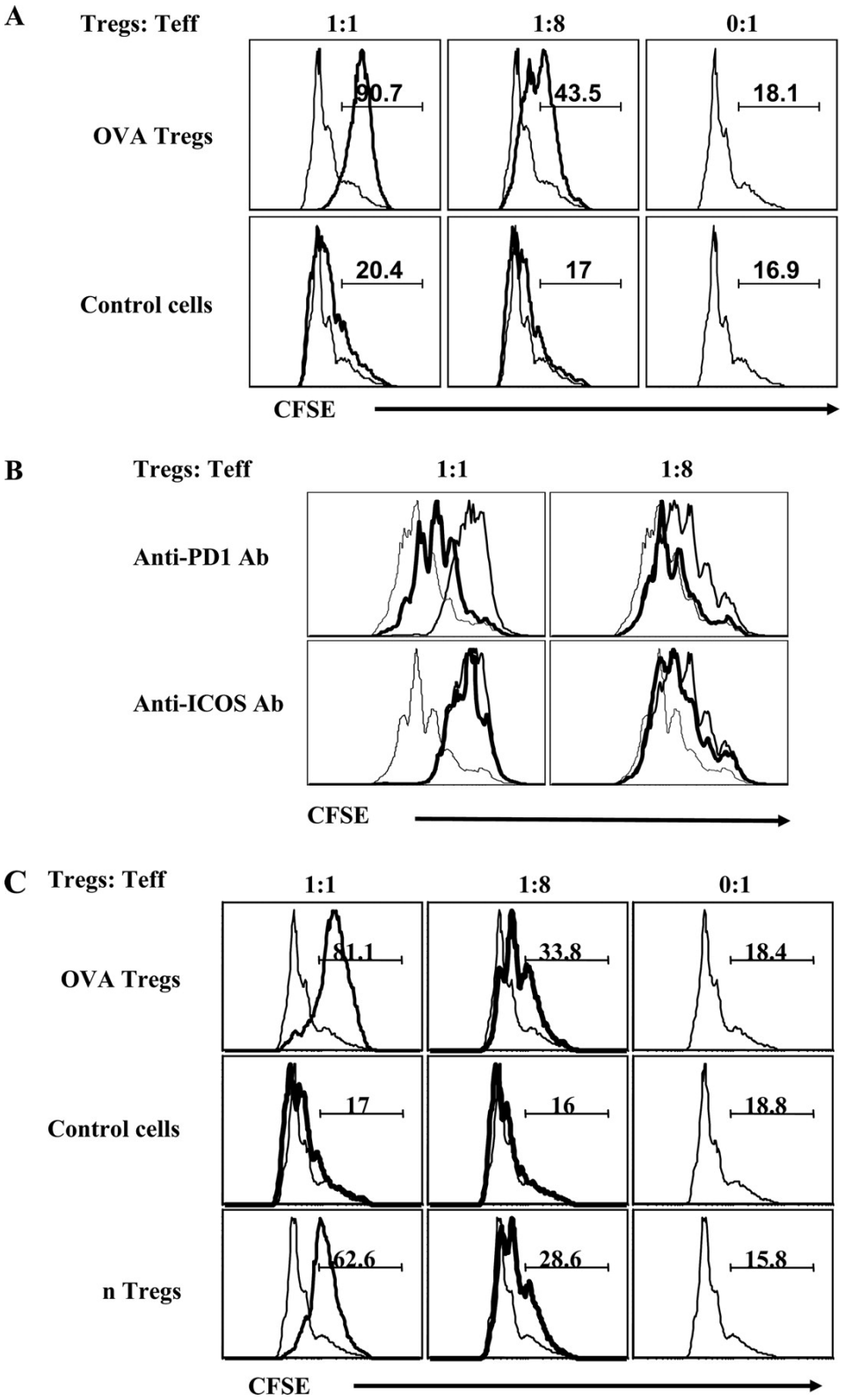

A
Tregs: Teff
1:1
1:8
0:1
OVA Tregs
90.7
43.5
18.1
Control cells
20.4
17
16.9
CFSE
B
Tregs: Teff
1:1
1:8
Anti-PD1 Ab
Anti-ICOS Ab
CFSE
C
Tregs: Teff
1:1
1:8
0:1
OVA Tregs
81.1
33.8
18.4
Control cells
17
16
18.8
n Tregs
62.6
28.6
15.8
CFSE

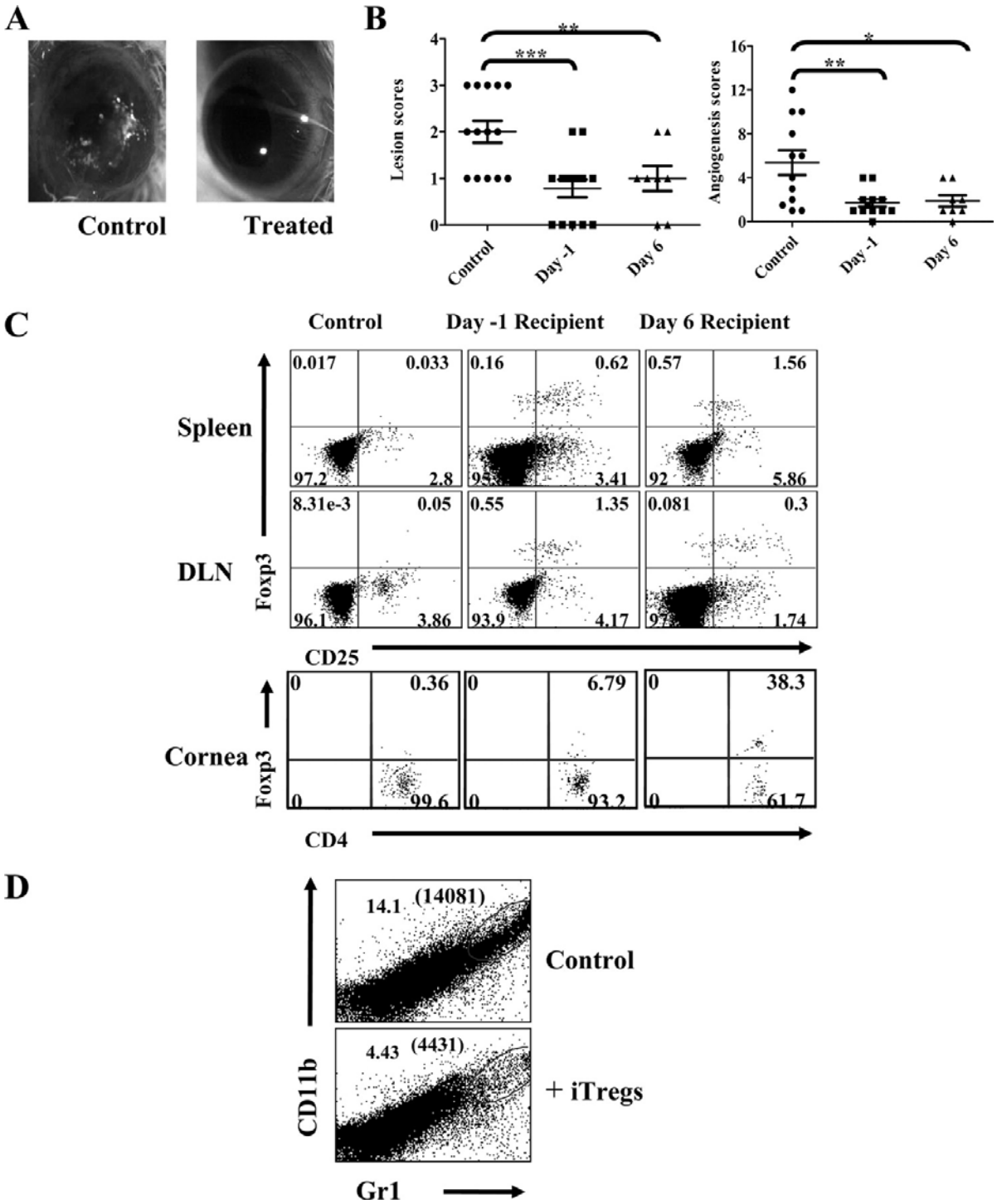
Figure 2. 5 In vitro generated OVA Tregs control SK severity in a bystander disease model. $5x10^5$ $Foxp3^+CD4^+$ T cells were adoptively transferred to DO11.10 RAG2-/- animals 24 hours before or 6 days post ocular HSV I infection. The disease severity and angiogenesis was recorded.

A. Gross eye pictures of control and transfer recipient animals from a representative experiment when $5x10^5$ $Foxp3^+$ cells were transferred 24 hours before infection are shown.

B. Cumulative data on SK severity and angiogenesis from different experiments at 11 days p.i. is shown. $5x10^5$ $Foxp3^+$ T cells were transferred 24 hours before or 6 days post infection. P values were calculated with one way ANOVA using a Dunnet post test settings.

C. Distribution of adoptively transferred $Foxp3^+$ T cells in lymphoid organs (spleen and draining cervical LN) and ocular tissues at 11 dpi is shown.

D. Representative FACS plots showing the infiltration of neutrophils ($CD11b^+Gr1^+$) in collegenase digested cornea from control and transfer recipient animals given $5x10^5$ $Foxp3^+$ cells were transferred 24 hours before infection are shown. Absolute numbers of neutrophils/cornea are shown in parenthesis.


A
Control
Treated
B
Lesion scores
Angiogenesis scores
Control
Day -1
Day 6
C
Control
Day -1 Recipient
Day 6 Recipient
Spleen
DLN
Cornea
Foxp3
CD25
CD4
0.017
0.033
97.2
2.8
0.16
0.62
3.41
0.57
1.56
92
5.86
8.31e-3
0.05
96.1
3.86
0.55
1.35
93.9
4.17
0.081
0.3
97
1.74
0
0.36
99.6
6.79
93.2
38.3
61.7
D
14.1
(14081)
Control
4.43
(4431)
+ iTregs
CD11b
Gr1

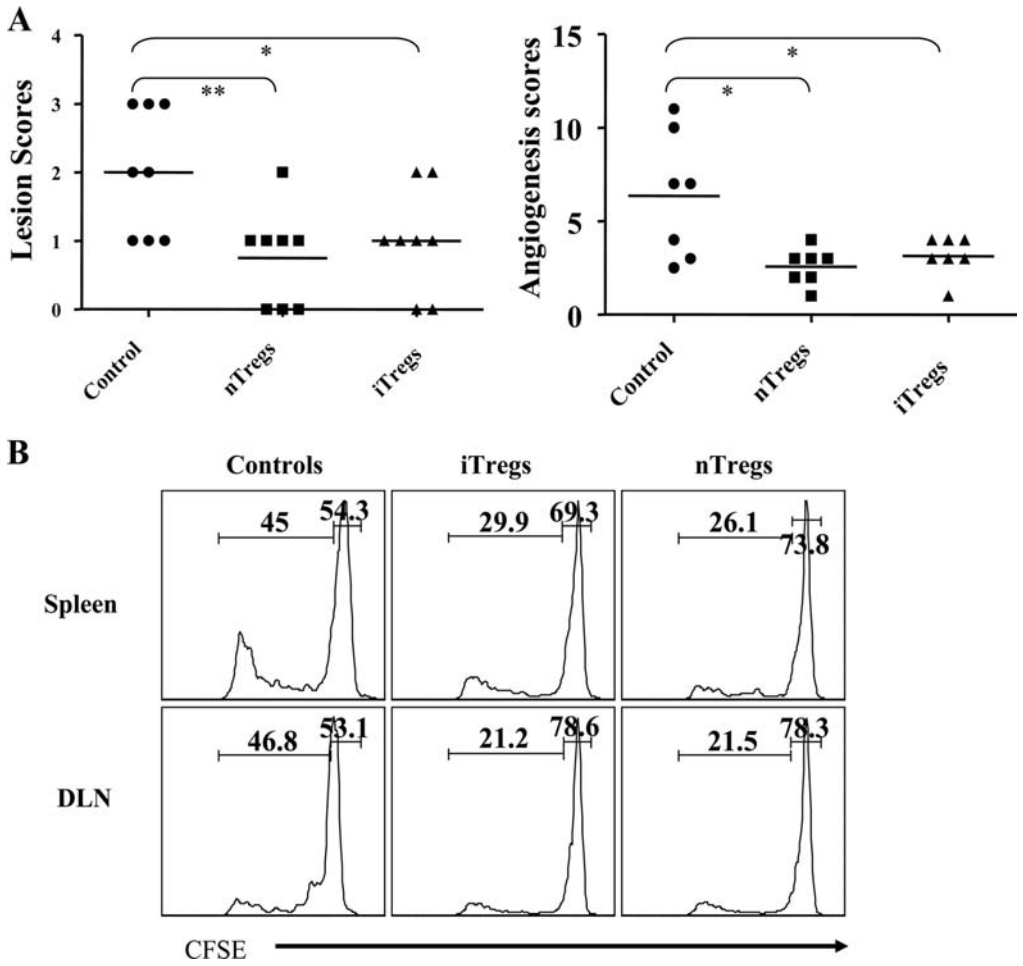


Figure 2. 6 Co-transfer of in vitro generated Tregs with polyspecific CD4+CD25- T cells in SCID animals reduces the severity of SK.

$1x10^6$ $CD4^+CD25^+$ T cells isolated from *in vitro* cultures and splenic nTregs ($CD4^+CD25^+$ T cells) from naïve BALB/c animals were co transferred with $5x10^6$ $CD4^+CD25^-$ T cells 24 hours before ocular HSV I infection ($5x10^5$ pfu). The disease severity and angiogenesis was recorded over a 12 days period.

A. The SK lesion scores and angiogenesis at 12 days p.i. are shown.

B. Proliferation of polyspecific CFSE labeled $CD4^+CD25^-$ T cells in spleen and DLN in the presence iTregs or splenic nTregs is shown.

Figure 2. 7 In vitro generated OVA-specific Tregs diminishes the severity of SK in immunocompetent BALB/c transfer recipients in a dose dependent manner.

A. Indicated doses of unfractionated $Foxp3^+$ T cells were adoptively transferred in BALB/c animals 24 hours before ocular HSV infection and the disease severity was monitored until day 15. A bar diagram indicating the average SK lesion scores at different doses of transferred $Foxp3^+$ cells at 15 days p.i. is shown. P values were calculated with one way ANOVA using a Dunnet post test settings taking no Tregs as a control. B. $5x10^5$ of fractionated $CD4^+$ and non-$CD4^+$ cells isolated from *in vitro* conversion cultures were transferred in BALB/c animals. Average lesion scores is shown at 15 days p.i. Only $CD4^+$ T cells could control the severity of SK. Student's t test (unpaired) was used for calculating the level of significance

C. $5x10^5$ $Foxp3^+$ cells were transferred 24 hours before, 3 days or 6 days p.i. and SK severity was recorded. Lesion scores and angiogenesis is shown at 10 days p.i. and 15 days p.i. No SK modulatory activity was shown by Tregs at 3 dpi transfer. P values were calculated with one way ANOVA using a Dunnet post test settings.

D. Eye pictures of control and iTregs transfer recipient animals from a representative experiment when $5x10^5$ and $1x10^6$ $Foxp3^+$ cells were transferred 24 hours before infection are shown. E. The kinetics of the levels of proinflammatory cytokine IL-6 in cornea and DLN after ocular HSV I infection as determined by sandwich ELISA is shown. F-G. Indicated doses of iTregs were transferred at 3dpi in BALb/c animals and lesion severity (F) and angiogenesis (F) were recorded at day 15 and compared with one way ANOVA using a Dunnet post test settings.

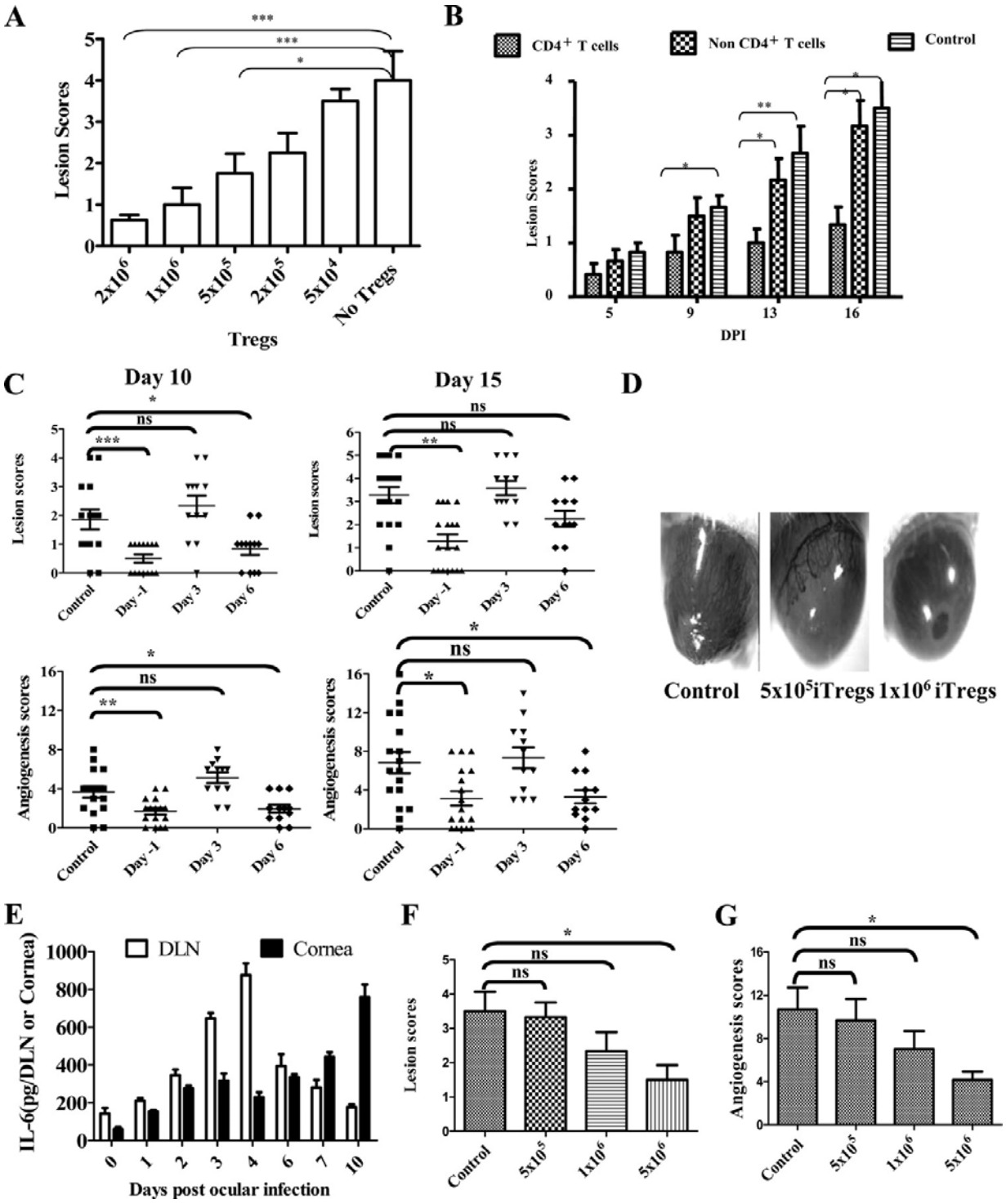
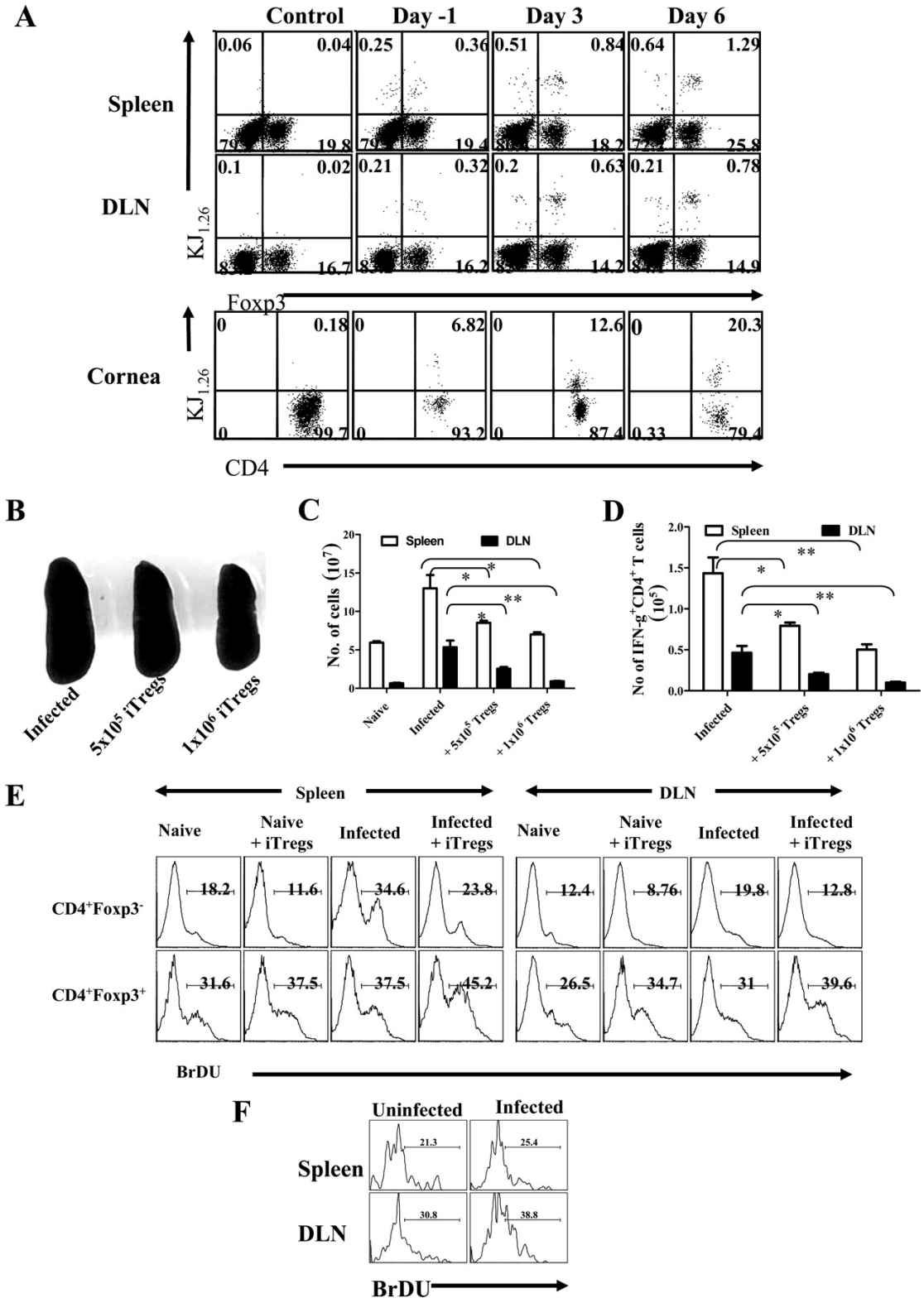

A
Lesion Scores
Tregs
2x10^6
1x10^6
5x10^5
2x10^5
5x10^4
No Tregs
B
CD4+ T cells
Non CD4+ T cells
Control
Lesion Scores
DPI
C
Day 10
Day 15
Lesion scores
Angiogenesis scores
Control
Day -1
Day 3
Day 6
D
Control
5x10^5iTregs
1x10^6 iTregs
E
DLN
Cornea
IL-6(pg/DLN or Cornea)
Days post ocular infection
F
Lesion scores
Control
5x10^5
1x10^6
5x10^6
G
Angiogenesis scores
Control
5x10^5
1x10^6
5x10^6

Figure 2. 8 Adoptively transferred Foxp3+KJ1.26+T cells were present in lymphoid and ocular tissues and diminished HSV-specific CD4+ T cell immune response.

BALB/c animals were given $5x10^5$ of iTregs before 24 hrs or 3 days or 6 days p.i. of ocular HSV infection and 15 days pi DLNs, spleens and ocular tissues were examined for the presence of CD^+Foxp3^+ and KJ^+ T cells. A. Representative FACS plots are shown. B-D. BALB/c animals were given indicated numbers of iTregs before 24 hrs of ocular HSV infection and 15 days pi immune parameters were studied B. Spleen size as an indication of generated immune response is shown in control and iTreg recipient animals. C. Total cellularity in the spleen (blank bars) and DLN (filled bars) of controls and Treg recipient animals is shown. D. Total number of IFN-γ^+CD4^+ T cells in Spleen and LN of control and Treg recipients is shown. E-F. $5x10^5$ iTregs were transferred into 12 animals which were then divided into four groups: naive, naïve + iTregs, Infected and Infected + iTregs. Animals in Naïve + iTregs and Infected + iTregs were given $5x10^5$ iTregs. After 24 hrs, animals from Infected and Infected + iTregs were infected with HSV 1. All animals were given BrDU in DW for next 10 days. After 10 days spleens and draining cervical LNs were analyzed for the frequencies of host $CD4^+Foxp3^+$ or $CD4^+Foxp3^-$ and donor $CD4^+KJ1\text{-}26^+Foxp3^+$ cells that incorporated BrDU. Representative FACS plots for host cells (E) and donor cells (F) are shown.


A
Control
Day -1
Day 3
Day 6
Spleen
DLN
Cornea
KJ1.26
Foxp3
CD4
B
Infected
5x10^5 iTregs
1x10^6 iTregs
C
Spleen
DLN
No. of cells (10^7)
Naive
Infected
+5x10^5 Tregs
+1x10^6 Tregs
D
No of IFN-g+CD4+ T cells (10^5)
E
Naive
Naive + iTregs
Infected
Infected + iTregs
CD4+Foxp3-
CD4+Foxp3+
BrDU
F
Uninfected
Infected
Spleen
DLN
BrDU

PART-III

ANTI-INFLAMMATORY EFFECTS OF FTY720 AGAINST VIRAL INDUCED IMMUNOPATHOLOGY: ROLE OF DRUG INDUCED CONVERSION OF T CELLS TO BECOME FOXP3^{+} REGULATORS

This chapter is a slight modification of the research paper published in 2008 in The Journal of immunology by Sharvan Sehrawat and Barry T Rouse.

Sehrawat, S and B. T. Rouse 2008. Anti-Inflammatory Effects of FTY720 Against Viral Induced Immunopathology: Role of Drug Induced Conversion of T Cells to Become Foxp3^{+} Regulators. *J. Immunol.* 180(11): 7636-47. *Copyright © 2008 by The American Association of Immunologists, Inc.*

In this chapter 'we' and 'our' refer to co-author and me. My contributions in the paper include(1) Selection of the topic (2) Data analysis and interpretation (3) planning experiments (4) compiling and interpretation of literature (5) understanding how results fit into literature (6) providing structure to the paper (7) making graphs and figures (8) writing and editing

Abstract

FTY720 has been used to control inflammatory lesions but the mechanisms by which the drug acts *in vivo* are poorly understood. They may result primarily from effects on lymphocyte and dendritic cell homing to lymphoid and inflammatory sites. We demonstrate that FTY720 may also act by causing the conversion of antigen stimulated non-regulatory CD4^{+} T cells to Foxp3^{+}CD4^{+} regulatory T cells and enhances their suppressive activity. In a model in which mice were ocularly infected with HSV, daily treatment with FTY720 resulted in significantly diminished ocular lesions. The treated animals showed increased frequencies of Foxp3^{+} T cells in lymphoid organs and at two inflammatory sites viz. cornea and trigeminal ganglia. In a second series of experiments, immunized DO11.10RAG2-/- animals, normally lacking endogenous

Foxp3$^+$ T cells, given FTY720 treatment developed high frequencies of Foxp3$^+$ regulatory T cells in lymph nodes. Some converted cells persisted in treated animals for several weeks after drug administration was discontinued. Finally, FTY720 could effectively induce Foxp3 expressing cells from Foxp3$^-$ cells *in vitro*, an effect inhibited by anti-TGF-β or the proinflammatory cytokine IL-6. Accordingly, the anti-inflammatory effects of FTY720 could be mediated at least in part by its ability to cause the conversion of antigen stimulated conventional T cells to become Foxp3$^+$ regulators. The use of FTY720 along with antigen administration could represent a useful therapeutic means to selectively expand antigen-specific regulators which could be valuable in many clinical situations such as allotransplants, some autoimmunties as well as with some chronic infections.

Introduction

The fungal metabolite drug FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol, has been shown to control some autoimmunities and allergic diseases as well as to suppress transplant rejection and graft-vs-host disease (1). The drug is currently in phase III clinical for the treatment of multiple sclerosis (2). FTY720, upon phosphorylation, is known to mimic the action of sphingosine-1-phosphate (S1P) and acts as an agonist for four of the five S1P receptors (3). One outcome of such binding to lymphocytes is a change in their trafficking patterns, with cells migrating more into lymph nodes (LN) and their egress being impeded (4, 5). This may result in lymphopenia and limited access of lesion-orchestrating lymphocytes to inflammatory sites (6). Additionally, FTY720 may hamper dendritic cell migration into LNs as well as cytokine production, effects that could result in immunosuppression (7). There is also evidence that

FTY720 treatment may serve to increase the function of regulatory T cells (Tregs) (8, 9). Accordingly, it was shown that the exposure of $CD4^+CD25^+$ T cells from mice to the phosphorylated form of FTY720 resulted in their enhanced suppressive activity in an Ag-specific manner. Additionally, in a model of Th1-induced autoimmunity, animals treated with FTY720 showed control over the onset and development of colitis. In the same study, an increase in the Foxp3 mRNA at the site of inflammation was also noted, but preferential migration of $Foxp3^+$ T cells from elsewhere could not be excluded (9).

In the present report, we have sought to determine whether FTY720 could cause the conversion of conventional $Foxp3^-$ T cells to $Foxp3^+$ Tregs. In a model of virus-induced inflammatory disease caused by HSV infection of the mouse cornea, treatment with FTY720 resulted in significantly diminished lesions. Furthermore, treated animals developed an expanded population of $Foxp3^+CD4^+$ T cells, although in this model it was not possible to define whether these cells were derived from preexisting $Foxp3^+$ T cells or were converts from the $Foxp3^-$ nonregulatory $CD4^+$ T cells. More direct evidence that FTY720 could function to cause the conversion of TCR-stimulated cells to $Foxp3^+$ regulators was obtained in a TCR transgenic x $RAG2^{-/-}$ model, which lacked $Foxp3^+$ T cells (10). Treatment of such animals after immunization with cognate Ag recognized by the TCR resulted in the induction of substantial numbers of $Foxp3^+$ cells that were shown to express regulatory activity in vitro. Experiments in vitro with conventional T cells also showed that TCR activation in the presence of FTY720 and IL-2 resulted in the conversion of most surviving cells into $Foxp3^+$ T cells. This conversion did not require the addition of TGF-β in the cultures, although the mechanism by which FTY720 induced the conversion appeared to depend on TGF-β because the process was inhibited when anti-TGF-β Ab was added to cultures.

Our results show that an additional means by which FTY720 succeeds in controlling inflammatory reactions is to cause the conversion of conventional T cells to become Foxp3$^+$ regulators. The use of the drug along with Ag stimulation would represent a valuable means to achieve the selective expansion of a population of regulatory cells, which would be useful in clinical situations such as some autoimmunities, allotransplantation, and allergic diseases, as well as in some chronic infections.

Materials and Methods

Mice, virus, biological and pharmacological reagents

Female 6- to 8-wk-old BALB/c DO11.10RAG2$^{-/-}$ mice were purchased from Taconic Farms, and Thy1.2$^+$ BALB/c and CB.17 SCID mice were purchased from Charles River Laboratories. Foxp3-GFP knock-in animals were kindly provided by Dr. M. Oukka of Harvard Medical School. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facilities. BALB/c DO11.10RAG2$^{-/-}$ and CB.17 SCID mice were kept in our specific-pathogen free facility. HSV-1 RE was provided by Dr. Robert Hendricks (University of Pittsburgh). It was propagated and titrated on Vero cells (ATCC CCL81) using standard protocols. The virus was stored in aliquots at –80°C until use. All Abs were purchased from BD Pharmingen unless otherwise stated. The Abs used for flow cytometery were DO11.10-PE (KJ1–26), CD4-APC (RM4–5), CD25-FITC (7D4), Foxp3-PE (FJK-16s), CD62L-FITC (MEL-14), CD103-FITC (M290), glucocorticoid-induced TNF receptor (GITR)-FITC (DTA-1), CD45-APC (30-F11), and annexin V-APC. CD4-FITC (H129.9) was used for confocal microscopy. rhTGF-β1, rIL-6, anti-TGF-β1, 2, 3 Ab (1D11), and anti-CTLA-4 Ab (clone 6382) were obtained from R&D Systems. Anti-CD3 (145.2C11) and anti-CD28 (37.51)

were from BD Biosciences. rhIL-2 was obtained from Hemagen Diagnostics and FTY720 from Calbiochem. FTY720 was dissolved in ethanol at a concentration of 10 mg/ml, and before injecting into mice, a fresh solution was made in distilled water. For in vitro assays, FTY720 was dissolved in ethanol at 10 mg/ml concentration, and further dilution was made in RPMI 1640 medium without additives at the time of use. SEW2871 and S1P were obtained from Cayman Chemical and were dissolved in DMSO and 0.3 N NaOH, respectively. $OVA_{323–339}$ peptide was obtained from GenScript. CFSE was obtained from Molecular Probes and used at a final concentration of 0.5 μM for 15 min at 37°C in PBS.

Corneal HSV-1 infections, clinical observations and treatment of mice with FTY720

Six- to 8-wk-old BALB/c mice were ocularly infected under deep anesthesia with 5×10^5 PFU HSV RE and divided randomly into four groups. Aminals in each group were treated with three doses (0.3, 1.0, and 3.0 mg/kg body weight (BW)) of FTY720 i.p. daily starting from 24 h postinfection (PI) until day 15 PI, respectively. In some experiments, FTY720 treatment of infected animals was done until day 5 or day 9. Mice were observed for the development and progression of herpetic stromal keratitis (SK) lesions and angiogenesis from day 5 until day 15, as described elsewhere (11). The eyes were examined on different days PI and the clinical severity of keratitis and angiogenesis of individually scored mice was recorded. The scoring system was as follows: 0, normal eye; 1, mild corneal haze; 2, moderate corneal opacity, iris visible; 3, severe corneal opacity, iris invisible; 4, opaque cornea, ulcer formation; and 5, necrotizing SK. All experiments were repeated at least three times. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Induction of Foxp3 in Foxp3$^-$CD4$^+$ T cells by FTY720 in-vivo in immunized DO11.10 RAG2-/- mice

Six- to 8-wk-old DO11.10RAG2$^{-/-}$ mice were immunized i.p. with 50–100 μg of OVA$_{323–339}$ in CFA and divided into four groups. Animals in three groups were given 0.3, 1, and 3 mg/kg BW of FTY720 i.p., respectively, every alternate day for 15 days while the fourth group was given same volume of diluent. Another group of mice was injected with PBS with CFA and was given the above-mentioned doses of FTY720. Additionally, some immunized and FTY720-(0.3 mg/kg BW) treated animals ($n = 3$) were given 250 μg of anti-TGF-β (1D11) Ab i.p. at days 3, 6, and 10. For some experiments, immunized animals were also treated with SEW2871 (0.3, 1, and 5 mg/kg BW) following the same protocols as those with FTY720. For some of the experiments, DO11.10RAG2$^{-/-}$ mice were immunized in the foot pad with 5 μg of OVA emulsified with CFA in 30 μl volume. Lymphoid tissue samples were collected at different intervals and analyzed for the expression of Foxp3, CD25, and CD4$^+$ T cells.

In-vitro suppression assay

DO11.10RAG2$^{-/-}$ mice were immunized with an emulsion of OVA$_{323–339}$ and CFA and treated with FTY720 as described in the previous section. In vitro suppression assays were done with CD4$^+$CD25$^+$ T cells isolated from the proximal (iliac and mesenteric) and distal (cervical, axillary, and superficial inguinal) LNs of immunized and FTY720-treated DO11.10RAG2$^{-/-}$ mice using homologous CD4$^+$CD25$^-$ T cells and T-depleted splenocytes. Additionally, to examine the enhancement of suppressive activity of Tregs by FTY720, DO11.10 animals were immunized and some were treated with FTY720 for 15 days. CD4$^+$CD25$^+$ T cells were isolated from pooled LNs (cervical, axillary, superficial inguinal, mesenteric, and iliac) of all of these animal groups using a regulatory T cell isolation kit (Miltenyi Biotec) as per the manufacturer's instructions.

$CD4^+CD25^-$ T cells were isolated either from pooled LNs (cervical, axillary, superficial inguinal, mesenteric, and iliac) and spleens of naive DO11.10RAG2$^{-/-}$ mice or from those of DO11.10 animals and labeled with CFSE (0.5 μM). $CD4^+CD25^-$ T cells (1×10^5) from either DO11.10RAG2$^{-/-}$ or DO11.10 mice were cultured with a 2-fold serial dilution of $CD4^+CD25^+$ T cells and 2×10^5 irradiated Thy1.2-depleted splenocytes isolated from either DO11.10RAG2$^{-/-}$ or DO11.10 mice, respectively, in the presence of 1 μg/ml soluble anti-CD3. Dilution of CFSE in stained $CD4^+$ T cells was analyzed by flow cytometry after 72 h of incubation. For analysis of CFSE dilution, the first gate was applied on $CD4^+$ T cells. Of these cells, $CFSE^+CD4^+$ T cells were then gated and the dilution of the intensity of CFSE was analyzed. In some of the experiments, 1 μCi of tritiated thymidine was added after 48 h of incubation, and levels of incorporation were measured 16 h later in a PerkinElmer Top Counter.

***In vitro* generation of Foxp3$^+$ T cells**

A modification of Chen et al.'s in vitro culture system (12) was developed for the induction of Foxp3 in naive precursor $CD4^+CD25^-$ T cells isolated from DO11.10RAG2$^{-/-}$ mice, which lack their own Foxp3$^+$ T cells (10). Total splenocytes (2×10^6) after RBC lysis and several washings were cultured in 1 ml volume with previously optimized doses of plate-bound anti-CD3 Ab (0.125 μg/ml in 200 μl volume), rIL-2 (25 U/ml), and TGF-β (10 ng/ml) for 5 days at 37°C in a 5% CO_2 incubator in 48-well plates. In other cultures, in place of TGF-β, various concentrations of FTY720 added daily along with IL-2 (25 U/ml) were used. In some of the experiments, $CD4^+CD25^-$ T cells purified from DO11.10RAG2$^{-/-}$ animals and T-depleted irradiated splenocytes were cultured with plate-bound anti-CD3, 1 μg/ml soluble anti-CD28 Ab, rIL-2, and FTY720 (10 ng/ml added daily). After 5 days, cells were characterized phenotypically by flow cytometry. In some experiments, the induction of Foxp3 in $Foxp3^-CD4^+$ T cells was

analyzed at different time points after the initiation of culture. Some of the cultures began with CFSE-labeled splenocytes. In such cultures, dilution of CFSE was analyzed after 5 days of incubation. In some experiments involving Foxp3 induction, anti-TGF-β1, 2, 3 Ab at a concentration of 15 μg/ml was used to effectively neutralize TGF-β production (13). In other experiments, rIL-6 (35 ng/ml) was used in an attempt to abrogate Foxp3 induction (14). For some experiments, various doses of SEW2871 (1, 10, and 100 ng/ml) and S1P (10^{-6}, 10^{-7}, 10^{-8} M) were added every 24 h instead of FTY720 into the cultures of splenocytes.

Cell sorting and transfer

$CD4^+$ T cells were first purified from Foxp3-GFP knock-in animals using a $CD4^+$ T cell isolation kit, and 2×10^6 cells were transferred into nine CB.17 SCID animals. One group of three animals was then treated with 0.3 mg/kg BW of FTY720 and another group with 3 mg/kg BW for 15 days daily. All animals were subsequently analyzed for the proportion of GFP^+CD4^+ and GFP^-CD4^+ T cells in various lymphoid tissues. In some experiments, purified $CD4^+$ T cells were sorted into Foxp3-GFP^+ and GFP^-CD4^+ T cells by a FACSVantage cell sorter (BD Biosciences) and were then activated in vitro for 2 days using anti-CD3 and anti-CD28 mAbs in the presence of IL-2. These cells were then mixed in 1:10 ratio (GFP^+ and GFP^-) and 2×10^6 cells transferred into CB.17 SCID animals, which were then treated with FTY720 and analyzed as described above.

Flow cytometry

In vitro cultured cells, LN cells, splenocytes, peripheral blood cells, and peritoneal exudate cells were first blocked with anti-CD32/16 mAb for 30 min and then were reacted with fluorochrome-labeled Abs as per the manufacturer's instructions. For Foxp3 staining, a kit from eBioscience was used. Annexin V staining was done using a kit from BD Biosciences. For some

of the experiments, corneas and trigeminal ganglias (TGs) were excised, pooled groupwise, and digested with 60 U/ml Liberase (Roche Diagnostics) for 60 min at 37°C in a humidified atmosphere of 5% CO_2 as described earlier (15). After incubation, the corneas and TGs were disrupted by grinding with a syringe plunger on a cell strainer, and a single-cell suspension was made in complete RPMI 1640 medium. Cells were then stained as described above and were acquired and analyzed by flow cytometery on a BD FACSCalibur using CellQuest Pro or FlowJo softwares.

Immunflorescence and ELISA

Eyes were removed and frozen in OCT compound at 15 days p.i. Six-micrometer-thick sections were cut, air dried, and fixed in cold acetone for 5 min. The sections were then blocked with 3% BSA and analyzed by confocal microscopy for the presence of $CD4^+$ T cells.

The concentrations of TGF-β and IL-17 produced in in vitro cultures were quantified by sandwich ELISA using kits from R&D Systems. Culture supernatants were acidified before use in the TGF-β ELISA.

Statistical analysis

Statistical significance was determined by Student's *t* test. A *p* value of <0.05 was regarded as a significant difference between groups *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. GraphPad Prism software was used to calculate the statistical significance.

Results

FTY720 administration diminishes the severity of herpes virus induced ocular lesions

We evaluated the disease-modulating activity of FTY720 against SK induced by ocular infection by HSV. As is evident in Fig. 3.1, *A* and *B*, infected animals treated daily with various

doses (0.3, 1, and 3 mg/kg BW) of FTY720 (starting 24 h PI and continued until the experiments were terminated on day 15) developed significantly fewer stromal lesions and angiogenesis than did untreated infected controls in a dose-dependent manner, with maximum suppression being evident at 3 mg/kg BW dose of FTY720. For most of the subsequent experiments, a dose of 3 mg/kg BW was used. The kinetics of lesions and angiogenesis expression at a dose of 3 mg/kg BW are shown in Fig. 3.1, *C* and *D*. The incidence of infected eyes with a lesion severity score of ≥3.0 was significantly higher in controls as compared with FTY720-treated animals (Fig. 3.1*E*). An analysis of serial corneal sections by confocal microscopy revealed diminished $CD4^+$ T cell infiltration in FTY720-treated animals compared with untreated controls (Fig. 3.1*F*). Four corneas from eyes with scores representing the group average were pooled from both treated and control animals at day 10 and day 16 PI. These were analyzed by flow cytometry (after collagenase digestion) for the presence of $CD4^+$ T cells. Such experiments were done separately at least three times and the data are shown in Fig. 3.1, *G* and *H*, at 16 days PI (DPI). Reduced total numbers of $CD4^+$ T cells were present in FTY720-treated animals, but the proportion of the $CD4^+$ T cells that were $Foxp3^+$ was increased (Fig. 3.1, *G* and *H*). In the same experiment, infiltration of $CD4^+$ T cells was found to be reduced in the trigeminal ganglion while the proportion of $Foxp3^+$ T cells increased, showing that FTY720 treatment decreases the infiltration of $CD4^+Foxp3^-$ T cells but increases that of $CD4^+Foxp3^+$ at both sites of inflammation caused by HSV-1.

FTY720 treatment after infection results in increased frequencies of $CD4^+CD25^+Foxp3^+$ T cells

That $CD4^+$ T cell infiltrates were diminished and lesions suppressed in treated animals could well be the consequence of the known ability of FTY720 to limit access of inflammatory T

cells to lesion sites (6). However, as mentioned above, in ocular tissues there was an increased frequency of Foxp3$^+$ T cells in treated animals. Accordingly, the anti-inflammatory effect of FTY720 might be mediated, at least in part, by a differential effect on Foxp3$^+$ T cells. To assess this possibility, spleens and LNs were collected at different time points PI from treated and control animals to quantify and measure the phenotypes of CD4$^+$ T cells. The results of a typical experiment when the animals were treated with 0.3 (Fig. 3.2*B*) and 3.0 mg/kg BW (Fig. 3.2, *A* and *B*) are depicted. As is apparent at both days 9 and 16 PI, Foxp3$^+$CD4$^+$CD25$^+$ T cells were increased in frequency in both the draining cervical LN as well as distal LNs, but not in the spleen, especially at the early time point (Fig. 3.2, *A* and *B*). These frequency differences were more apparent at earlier time points and were in fact already evident by 5 days PI (see Fig. 3.3). Other experiments also measured and compared the expression of additional phenotypic markers involved in lymphocyte homing on both Foxp3$^+$ and Foxp3$^-$CD4$^+$ T cells of treated and control LN cells. Of the markers measured (CD62L, CD103, and CD49d), the most dramatic differences were observed with CD103 expression on Foxp3$^+$ (but not Foxp3$^-$) cells. Expression was increased 6- to 7-fold in both draining LN and spleen on Foxp3$^+$ cells from treated animals (Fig. 3.2*C*). This observation could explain why Foxp3$^+$ cells were enriched in the ocular and TG inflammatory tissues of treated animals, because CD103 is known to be a tissue-homing molecule (16). Another homing molecule, CD49d, shown previously to be expressed on most inflammatory cells that infiltrate the eye (15), showed no significant changes in expression levels as a consequence of FTY720 treatment (Fig. 3.2*C*).

Additional experiments measured the numbers of Foxp3$^+$ and Foxp3$^-$CD4$^+$ T cells recoverable from the spleen and LNs at various times after infection of control and FTY720-treated animals. In such experiments, FTY720 treatment resulted in decreased numbers of CD4$^+$

T cells, especially in the draining LNs and spleen, but the ratio of Foxp3$^+$:Foxp3$^-$ cells increased (Fig. 3.3*A*). These results could mean either that FTY720 caused greater retention of Foxp3$^+$ cells than conventional T cells in LNs (an unexpected outcome because Foxp3$^+$ cells were reported by others to express low levels of the S1P receptors involved in LN retention (5)) or that events such as differential apoptosis or the conversion of some TCR stimulated Foxp3$^-$ into Foxp3$^+$ cells. To look for the preferential retention of Foxp3$^+$ cells over Foxp3$^-$ cells in lymphoid organs under the influence of FTY720 treatment, CD4$^+$ T cells (having both fractions of CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^-$) purified from Foxp3-GFP knock-in animals were transferred into SCID animals, which were then treated with two different doses (0.3 and 3 mg/kg BW) of FTY720 daily for 15 days. The lymphoid organs were analyzed after 15 days for the proportion of GFP$^+$ and GFP$^-$ CD4 T cells. Such experiments showed a trend for the preferential retention of Foxp3$^+$ cells over Foxp3$^-$CD4 T cells, but the differences were not significant (Fig. 3.3*B*). This result could be because Foxp3$^+$ T cells could undergo more homeostatic proliferation than Foxp3$^-$CD4$^+$T cells under the influence of FTY720. In some of the experiments, sorted Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells were separately activated in vitro for 2 days in the presence of anti-CD3, anti-CD28 mAbs, and IL-2 and were then transferred in a 1:10 ratio (Foxp3$^+$-Foxp3$^-$) into SCID recipients. Subsequently, the proportions of Foxp3$^-$ and Foxp3$^+$ cells were analyzed in lymphoid tissues after 15 days of FTY720 treatment. Such experiments revealed no significant differences in the proportions of Foxp3$^-$ and Foxp3$^+$ T CD4$^+$ T cells in the FTY720-treated and control animals, indicating that differential retention of either cell types in the lymphoid organs did not occur (data not shown).

Looking at the differential apoptosis of Foxp3$^-$ cells over Foxp3$^+$ cells, we used Foxp3-GFP knock-in mice that were infected with HSV-1, with some being treated with FTY720 (either

0.3 or 3.0 mg/kg BW). After 15 days, draining and distal (superficial inguinal) LNs were analyzed for evidence of apoptosis in Foxp3$^+$ and Foxp3$^-$CD4$^+$ T cells. We could find no evidence for differential apoptosis of either Foxp3$^-$ or Foxp3$^+$ cells in treated vs control animals (Fig. 3.3*C*). However, a trend in increased apoptosis of Foxp3$^-$CD4$^+$ T cells was observed in the nondraining LNs, which could result from the suppressive effects of Tregs on effector T cells, as the former are present more abundantly in the nondraining LNs than in draining cervical LNs. In a separate experiment, when HSV-1-infected animals were treated with FTY720 after day 8 PI, a time when viral Ags were no longer present, we did not find increased frequencies of Foxp3$^+$CD4$^+$ T cells. This finding could support the idea that FTY720 causes the conversion of TCR-stimulated conventional T cells to become Foxp3$^+$ regulators

Animals without Foxp3$^+$ T cells exposed to antigen in the presence of FTY720 develop Foxp3$^+$ T cells

Evidence that TCR-stimulated Foxp3$^-$CD4$^+$ T cells may convert to Foxp3$^+$ regulatory cells was obtained in TCR transgenic x RAG2 $^{-/-}$ mice, which are well known to lack Foxp3$^+$ T cells (10). This observation was also confirmed in our studies (Fig. 3.4*A*). In these experiments, DO11.10RAG2$^{-/-}$ mice were immunized i.p. with OVA$_{323–339}$ peptide in CFA, and some animals were additionally treated on alternate days with various doses (0.3, 1.0, or 3.0 mg/kg BW) of FTY720 starting 24 h after immunization. Experiments were usually terminated on day 15 to assess the presence of Foxp3$^+$CD4$^+$ T cells in various lymphoid tissues. Whereas a few Foxp3$^+$ T cells were induced in immunized but untreated animals, Foxp3$^+$ cells accounted for a major percentage of CD4$^+$ T cells in those animals given FTY720 (Fig. 3.4*A*). In fact, such cells were present in surprisingly high frequencies (Fig. 3.4*B*) both in LNs proximal to the site of immunization (iliac and mesenteric) as well as in distal (cervical, axillary, and superficial

inguinal) LNs. The increase in Foxp3$^+$ T cell percentages followed a dose dependency of FTY720 treatment, with the highest frequencies being observed at 3 mg/kg BW. Among CD4$^+$ T cells, the average frequencies of Foxp3$^+$CD4$^+$ T cells were 63% in cervical (range 55–75%), 60% in axillary (range 52–65%), 48% in superficial inguinal (range 36–52%), 15% in iliac (range 12–25%), and 10% in mesenteric (range 6.8–15%) LNs (Fig. 3.4*B*). Lesser frequencies of CD4$^+$Foxp3$^+$ T cells were evident in the spleen (range 2–6%) (Fig. 3.4, *A* and *B*). The absolute numbers of Foxp3$^+$ T cells in various organs vary, but they were significantly higher in cervical, axillary, and superficial inguinal LNs of FTY720-treated animals as compared with controls (Fig. 3.4*C*). Some experiments were terminated at day 5 after FTY720 treatment. Some converted cells were already present at this time (up to 20% of CD4$^+$ T cells were Foxp3$^+$). In other experiments, lymphoid tissues were examined at 40 DPI (FTY720 treatment ended at day 30) and at day 75 PI (FTY720 treatment ended at day 15). In such animals up to 20–30% and 5–10%, respectively, of CD4$^+$ T cells were Foxp3$^+$ in all lymphoid organs including the spleen, indicating that the converted cells may redistribute among all LNs and spleen in the absence of FTY720 treatment and persist for a prolonged period.

Some experiments were performed with DO11.10RAG2$^{-/-}$ mice to investigate the mechanisms involved in the induction of Tregs with FTY720. Thus, DO11.10RAG2$^{-/-}$ mice immunized with OVA peptide were treated every alternate day with SEW2871 (0.3, 1 and 5 mg/kg BW), a specific agonist of the S1P$_1$ receptor. After 15 days, animals were sacrificed and lymphoid tissues were analyzed for CD4, CD25, and Foxp3. As shown in Fig. 4.4*D*, animals develop increased frequencies of Foxp3$^+$ T cells as compared with untreated animals, but these frequencies were far less than those found in FTY720-treated animals. This may be because FTY720 engages more than just the S1P$_1$ receptor. Additional experiments were done to see

whether TGF-β blocking in vivo could have some effects on the accumulation of Tregs. Anti-TGF-β1, 2, 3 mAb (250 μg/ml) was injected i.p. at days 3, 6, and 10 in immunized and FTY720-treated animals. Lymphoid tissues were collected after 15 days and analyzed for $CD4^+CD25^+Foxp3^+$ T cells. TGF-β-neutralized animals developed significantly fewer Tregs as compared with control animals (Fig. 3.4*B*).

Curiously, the highest frequencies of $Foxp3^+CD4^+$ T cells induced in immunized FTY720-treated animals were usually in LNs that were not considered as draining LNs to the immunization site. This pattern of events was also evident as early as 5–7 days after immunization and was also seen when the site of immunization was in the neck region (data not shown). At present, we have no explanation for these observations, but they may reflect Ag dissemination to distal sites, especially following i.p. immunization along with inhibition of Tregs by inflammatory cytokines that are likely to be more abundant in the local LNs. In additional experiments, Ag was given in the foot pad, which we surmised might limit the spread of Ag to distal LNs. However, even with these experiments, increased frequencies of $Foxp3^+$ T cells were found in non-draining LNs (36 ± 8% in CLN, 28 ± 4% in sup Ig LN) as compared with draining popliteal LNs (10 ± 4.3%). Understanding why distal tissues develop more $Foxp3^+$ cells requires further investigation.

***In vivo* converted $Foxp3^+CD25^+CD4^+$ T cells express regulatory activity**

To demonstrate that FTY720-converted $Foxp3^+$ cells in DO11.10RAG2$^{-/-}$ mice expressed regulatory activity in vitro, $CD4^+CD25^+$ T cells were isolated from both the proximal and distal LNs 15 days after immunization of FTY720-treated animals. Of these $CD25^+$ cells, >90% were additionally $Foxp3^+$ (Fig. 3.5*A*). As is evident from Fig.3.5*B*, the $CD4^+CD25^+$ T cells isolated from both proximal and distal LNs suppressed in a dose-dependent manner the proliferation of

anti-CD3-stimulated, CFSE-labeled $CD4^+CD25^-$ T cells isolated from pooled spleens and LNs of DO11.10RAG2$^{-/-}$ naive animals. It was interesting to observe differences in the levels of CD25 on $Foxp3^+$ T cells among proximal and distal LNs, with cells isolated from distal LNs showing lower levels of CD25 expression. This observation might be explained by the differential availability of cytokines in the draining vs non-draining LNs that drive CD25 expression, but these issues require further investigation. Despite differences in CD25 levels, the in vitro suppressive activity of Tregs isolated from these sites was not significantly different. Experiments were also done to compare the in vitro regulatory activity of $CD4^+CD25^+$ T cells isolated from immunized and immunized plus FTY720-treated immunocompetant animals, which do have naturally occurring Tregs. For this purpose, DO11.10 animals were used. $CD4^+CD25^+$ T cells were isolated and pooled from cervical, axillary, superficial inguinal, mesenteric, and iliac LNs of both groups. Approximately 80% of these cells also expressed Foxp3 (Fig. 3.5*C*). The responder cells ($CD4^+CD25^-$) were isolated from pooled spleens and LNs of DO11.10 naive animals. As shown Fig. 3.5*D*, the $CD4^+CD25^+$ T cell population from FTY720-treated animals showed higher in vitro activity than did those from untreated animals in a dose-dependent manner. Thus, in addition to expanding the population of Tregs, the cells also appear to show enhanced regulatory activity when measured in vitro. In a previous report, FTY720 treatment of $CD4^+CD25^+$ Tregs in vitro was shown to enhance their regulatory activity (8).

FTY720 can induce Foxp3 expression in antigen stimulated $CD4^+$ T cells *in vitro*

Our above in vivo experiments indicate that $CD4^+$ T cells may be converted to express Foxp3 and become regulatory when Ag stimulated in the presence of FTY720. To establish more directly whether FTY720 can cause $Foxp3^-$ cells to convert to $Foxp3^+$, in vitro experiments were performed. In such experiments, whole splenocytes from naive DO11.10RAG2$^{-/-}$ animals were

stimulated with plate-bound anti-CD3 either in the presence of optimal amounts of TGF-β (our unpublished data) or with varying concentrations of FTY720, both along with human rIL-2. FTY720, which is less stable in aqueous solution, was added to the cultures every 24 h. As shown in Fig. 3.6*A*, 77% (range for >10 experiments of 75–94%) of viable $CD4^+$ T cells became $Foxp3^+$ after 5 days of culture in the presence of TGF-β. In cultures containing FTY720, ~54% (range for five experiments of 30–55%) of $CD4^+$ T cells became $Foxp3^+$. The optimal FTY720 concentration was found to be 10 ng/ml when added daily (Fig. 3.6*B*). The in vitro-generated $Foxp3^+$ T cells were also analyzed for other phenotypic markers. Most cells were additionally $CD25^+$, $CD62L^{high}$, and $GITR^+$, showing essentially the same phenotype as TGF-β-converted cells (Fig. 3.6*E*). However, the expression of CD103 was delayed, and maximal numbers of $Foxp3^+$ cells become $CD103^+$ after 6 days of incubation. In some experiments, cells were tested for Foxp3 conversion at different times after culture initiation. As shown in Fig. 3.6*C*, some conversion could be detected at day 2, but numbers increased over the culture period, reflecting perhaps the conversion of a new subpopulation each time the FTY720 was added. However, the observation could also reflect the proliferation of already converted cells. That proliferation of $Foxp3^+$ cells was occurring was shown when CFSE-labeled splenocytes were stimulated with anti-CD3 and IL-2 in the presence of FTY720. The newly differentiated $Foxp3^+$ T cells underwent multiple rounds of divisions (Fig. 3.6*D*). Therefore, FTY720 in the presence of IL-2 causes differentiation as well as proliferation of Tregs in vitro.

FTY720 mediated Foxp3 induction is TGF-β dependent

At present, we have no understanding as to the mechanism by which FTY720 induces TCR-stimulated T cells to express Foxp3. It is known, however, that FTY720 binding to the $S1P_1$ receptor may trigger some downstream events that are in common with those induced by TGF-β

(17, 18). The results expressed in Fig. 7*A* indicate that the mechanism by which FTY720 acts may in fact involve TGF-β. Thus, the addition of neutralizing anti-TGF-β1, 2, 3 Abs to culture stimulated with FTY720 markedly inhibited the percentage of $CD4^+$ T cells that became $Foxp3^+$ (Fig. 3.7, *A* and *B*). Additionally, supernatants of FTY720-stimulated cultures were shown to contain higher concentrations of TGF-β than the control supernatants, an effect that was dependent on the dose of FTY720 used (Fig. 3.7*C*). Accordingly, the mechanism by which FTY720 induces Foxp3 expression in $CD4^+$ T cells may proceed via the induction of TGF-β. The source of TGF-β in the splenocyte culture seems to be accessory cells, as no conversion was observed when purified populations of $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 Abs in the presence of FTY720 and IL-2 (data not shown). This notion was further supported by experiments wherein neutralization of CTLA-4 was achieved using anti-CTLA-4 Ab in the FTY720 induction cultures. With CTLA-4 neutralization, the frequencies of cells expressing Foxp3 were reduced significantly (Fig. 3.7*B*). However, the cell type involved in secreting TGF-β remains to be identified.

It has been suggested that proinflammatory cytokines such as IL-6 could neutralize the effect of Foxp3 induction and, along with TGF-β, induce the IL-17-producing cells (14). Therefore, we examined the effect of IL-6 addition in the FTY720 Foxp3-converted cultures. In such experiments, the addition of IL-6 to such FTY720-induced cultures markedly reduced the formation of $Foxp3^+$ T cells, while at the same time such addition led to the induction of some cells in the cultures that produced IL-17 (Fig. 3.7, *B* and *D*). The observation that IL-6, and perhaps other inflammatory cytokines, may inhibit the induction of $Foxp3^+$ T cells may explain in part why the frequency of $Foxp3^+$ cells in the draining LNs, where cytokines are more likely to

be present especially early after infection or immunization, was less than in some distal LNs. These issues are under further investigation.

Some experiments were done to investigate the role of other S1P receptor agonists such as SEW2871 and S1P in the Foxp3 induction process. These compounds were added daily to the in vitro cultures of anti-CD3- and IL-2-stimulated splenocytes, which were then analyzed after 5 days for the expression of Foxp3$^+$ in CD4$^+$ T cells. Such experiments showed a small but significant increase in the Foxp3$^+$ T cells with SEW2871 treatment, but these numbers were not significant with S1P (Fig. 3.7*E*).

Discussion

The present report documents the efficacy of FTY720 in inhibiting the severity of immunoinflammatory lesions caused by ocular infection with HSV. Our results showed that the use of the drug after infection significantly reduced disease, an effect that could be the consequence of the well-documented ability of FTY720 to retain T cells and some other cell types in LNs, thereby diminishing their access to tissue sites of inflammation (6). However, we also noted an expansion of Foxp3$^+$ Tregs in the FTY720-treated animals, which could also explain, at least in part, the reduced lesions in the FTY720-treated animals because SK lesion severity is known to be influenced by naturally occurring Tregs (15). We interpreted our observations to mean that the source of the expanded Foxp3$^+$ population could represent conversion of Ag-stimulated conventional CD4$^+$ T cells to become Foxp3$^+$ and regulatory in function.

More direct evidence that FTY720 could cause the conversion of Foxp3$^-$ to Foxp3$^+$ T cells was obtained by additional in vivo and in vitro studies. The in vivo evidence came from the

use of TCR transgenic x RAG2$^{-/-}$ mice, which are known to possess few if any Foxp3^{+} Tregs (10). Exposure of such animals to Ag along with FTY720 treatment resulted in the development of high frequencies of Foxp3^{+}CD4^{+} T cells in many LNs. Finally, the most convincing evidence that exposure of Ag-stimulated Foxp3^{-}CD4^{+} T cells could be converted by exposure to FTY720 to become Foxp3^{+} Treg came from in vitro studies. Accordingly, the addition of FTY720 daily to TCR-stimulated Foxp3^{-} T cells in the presence of IL-2 resulted in the conversion of substantial numbers of cells to Foxp3^{+} over a 5-day culture period. This conversion did not require the addition of extraneous TGF-β, although the conversion process could involve the induction of TGF-β because the addition of anti-TGF-β Ab to cultures markedly diminished the production of Foxp3^{+} T cells. The effect of TGF-β neutralization on accumulation of Foxp3^{+} T cells was also evident in in vivo experiments. The use of FTY720 given along with Ag could represent a useful way to achieve the selective expansion of Ag-specific regulators, which could be valuable in many clinical situations such as allotransplants, some autoimmunities, as well as with some chronic infections.

One curious observation we made in both FTY720-treated HSV infected and immunized DO11.10RAG2$^{-/-}$ animals was that the frequency of Foxp3^{+} T cells was usually higher in LNs distal to the site of infection or immunization than was evident in the proximal LNs, which likely took up most of the Ag. At present, we have no explanation for this observation, but it could reflect differential redistribution of Foxp3^{+} and CD4^{+} effectors from the proximal LN site of induction. This might occur, as others have reported that naturally occurring Tregs express lower levels of the S1P receptors involved in LN retention than other T cell subsets (8). This could mean that Tregs are less likely than activated effectors to be retained in the proximal LNs, especially during FTY720 treatment, and hence are more able to disseminate to other sites. An

alternative idea is that Tregs at proximal sites may be partially blunted by proinflammatory cytokines that could be present at higher concentrations in proximal LNs, especially early after infection and immunization. A third explanation may relate to the levels of Ag available to induce Foxp3$^+$ Tregs at proximal and distal sites. In this context, others have shown that very low levels of Ag may be more effective at inducing Foxp3$^+$ Tregs than are higher doses (19, 20). Such low levels, possibly conveyed there by dendritic cells, are likely to be present at distal sites compared with those in the proximal LN. Additional experiments are under way in an attempt to explain high frequencies of Foxp3$^+$-converted cells in distal LNs.

Although our in vitro studies demonstrate that FTY720 may induce the conversion of TCR-stimulated conventional T cells into Foxp3$^+$ regulators, the mechanism by which this occurs remains to be explained. The conversion process did not require the addition of TGF-β, but the mechanism could involve the induction of TGF-β either in the converting T cells themselves or, as we consider more likely, in accessory cells in the cultures. Such accessory cells might also be responsible for phosphorylation of the drug, which appears to be a necessary step for it to bind effectively to the S1P receptors (3, 21). We are currently attempting to determine which cells types in our culture system act as the source of the sphingosine kinases involved in the FTY720 phosphorylation or whether this activity is independent of the phosphorylation state of the drug, as has been reported for some activities of FTY720 (22).

Our studies also indicate that one means by which the FTY720-induced Foxp3 conversion occurred could involve TGF-β induction as an intermediate step. In support of this, fluids in FTY720-treated cultures contained higher levels of TGF-β than found in control cultures. Moreover, the addition of neutralizing Abs to TGF-β markedly diminished the FTY720-induced conversion process. It was also of interest that in cultures that contained IL-6, but no

TGF-β, the addition of FTY720 resulted in the induction of increased amounts of IL-17 production compared with cultures lacking FTY720. This observation may also argue that FTY720 functions by causing the production of TGF-β from some cell types, because this cytokine, along with IL-6, is known to be a stimulus for Th-17 cell induction (14). The observation might also mean that FTY720 will be a better inducer of Foxp3$^+$ Tregs if used when levels of proinflammatory cytokines are low.

Sakaguchi et al.'s seminal observations in the mid-1990s (23) reawakened interest in Tregs and opened up the prospect of using these cells immunotherapeutically. However, in normal individuals, most Foxp3$^+$ Tregs are considered to be thymus-derived and are largely reactive to a range of self Ags (24). For therapeutic purposes, it would be preferable to use Tregs of known Ag specificity so as to increase potency and avoid potential side effects of inhibiting desirable immune responses (25). Some have expanded specific self-reactive Tregs in vitro and demonstrated in vivo efficacy using adoptive transfer approaches (25). Such approaches, however, are cumbersome and extremely expensive. A better way would be to expand the Treg population in vivo to the Ag of choice. This may be accomplished by approaches such as the one we have described in the present report wherein Foxp3$^+$ cells with regulatory function are converted from conventional Ag-stimulated nonregulatory precursors. That such conversion can be accomplished was appreciated some time ago by the Horwitz and Wahl groups who showed that TGF-β stimulation was a key event for the conversion process (12, 26). This was supported by elegant studies from Bettelli and colleagues, who defined in vitro conditions to generate Ag-specific Foxp3$^+$ Tregs as well as proinflammatory IL-17-producing cells (14). More recently, several independent groups observed that retinoic acid may also be involved in the Foxp3 conversion process (13, 27, 28, 29). At least with mouse T cells, conversion by retinoic acid

additionally requires TGF-β stimulation (13, 27, 28, 29), but this may not be the case with human cells (30). Recently, other molecules have also been shown to facilitate the conversion of Ag-stimulated conventional T cells to become Foxp3$^+$ regulators (31, 32).

We would argue that the approach we have described in this report represents a valuable one in terms of therapy for chronic inflammatory diseases. Thus, as is well known, FTY720 has a potent anti-inflammatory activity because of its known effect on lymphocyte sequestration (4). However, its ability to expand and activate Foxp3$^+$ Tregs to an Ag of choice could prove particularly useful, because this should avoid the unwanted side effects that polyclonal Treg populations might exert. It will be particularly important to determine how long FTY720-converted cells remain in the body as functional regulators after treatment has been discontinued. So far, we have only studied animals up to 10 wk posttreatment and found that some cells with the converted phenotype are still present. Further long-term studies are currently under way.

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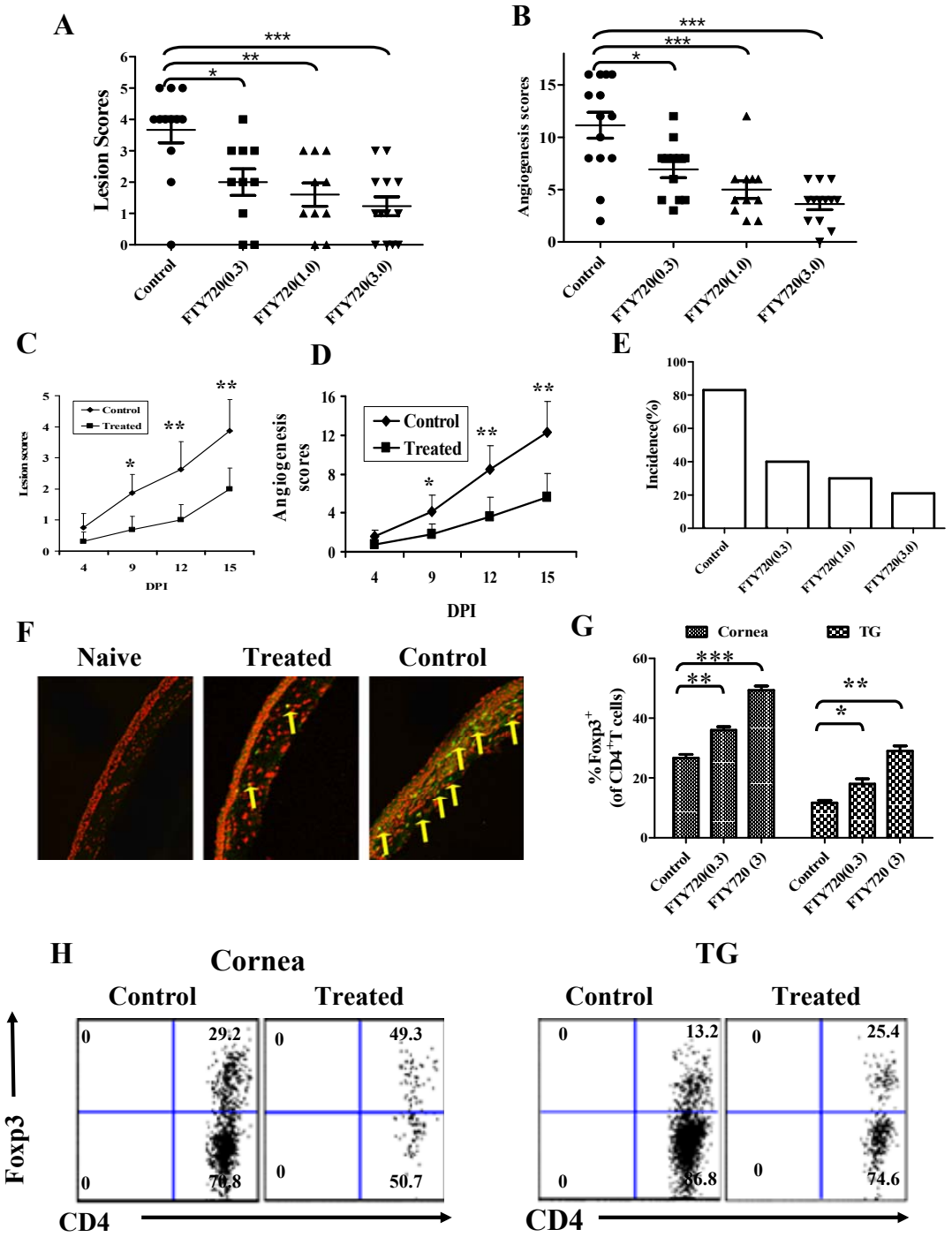
APPENDIX

Figure 3. 1 FTY720 treatment diminishes SK lesion severity and increases the representation of Foxp3^{+}CD4^{+} T cells at inflammatory sites

BALB/c mice were infected ocularly with 5x10^{5} HSV I (RE) and some were additionally treated with FTY720 daily from 24 hrs until day 15 p.i. as described in Materials and Methods. Lesion severity (A) and angiogenesis (B) were scored. The lesion severity and angiogenesis were diminished in FTY720 treated animals. The incidence of HSK 15dpi is shown (positive if score ≥ 3.0) (C). D. Representative corneal sections were stained for CD4^{+} T cells (see arrows) and analyzed by confocal microscopy 15dpi. (E) Single cell suspension was made from the cornea and trigeminal ganglias (TGs) digested with liberase and stained for Foxp3, CD4 and CD45 (leukocyte marker) 10 days post infection. Representative FACS plots from pooled corneas (left panel) and TGs (right panel) of control and FTY720 treated animals are shown (Gated on CD45^{+}CD4^{+} cells). The proportions of Foxp3^{+}CD4^{+} T cells was increased both in corneas and TGs of FTY720 treated animals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Lesion Scores
Control
FTY720(0.3)
FTY720(1.0)
FTY720(3.0)
B
Angiogenesis scores
C
Lesion scores
Control
Treated
DPI
D
Angiogenesis scores
E
Incidence(%)
F
Naive
Treated
Control
G
Cornea
TG
% Foxp3+ (of CD4+T cells)
H
Cornea
TG
Control
Treated
Foxp3
CD4
29.2
70.8
49.3
50.7
13.2
86.8
25.4
74.6

Figure 3. 2 FTY720 treatment early after ocular HSV infection increases the frequencies and alters the phenotype of $CD4^+CD25^+Foxp3^+$ T cells in lymphoid organs.

Lymphoid organs (spleens and LNs) from control and FTY720-treated BALB/c animals (as in Fig. 1) were processed at day 9 and day 16 PI and analyzed by flow cytometry. *A*, Representative FACS plots for the staining of CD4, CD25, and Foxp3 from spleen and draining (cervical), and distal (superficial inguinal) LNs of control and FTY720-treated (3 mg/kg BW) animals are shown at days 9 (*left panel*) and 16 PI (*right panel*) (gated on $CD4^+$ T cells). *B*, Relative frequencies of $CD4^+CD25^+Foxp3^+$ T cells in spleen and draining (cervical) and distal (superficial inguinal) LNs as measured by flow cytometry at 9 (*left panel*) and 16 DPI (*right panel*) are shown. The frequencies of $CD4^+CD25^+Foxp3^+$ T cells are increased in the lymphoid organs. The statistical significance was determined by Student's *t* test. *C*, Expression of CD62L, CD103, and CD49d was examined at day 16 PI on $Foxp3^+$ and $Foxp3^-CD4^+$ T cells obtained from the spleens and draining LNs of control and FTY720-treated animals (as in Fig. 2*A*) by flow cytometry. FTY720 treatment changes the expression pattern of some of the homing molecules specifically on $Foxp3^+$ T cells.

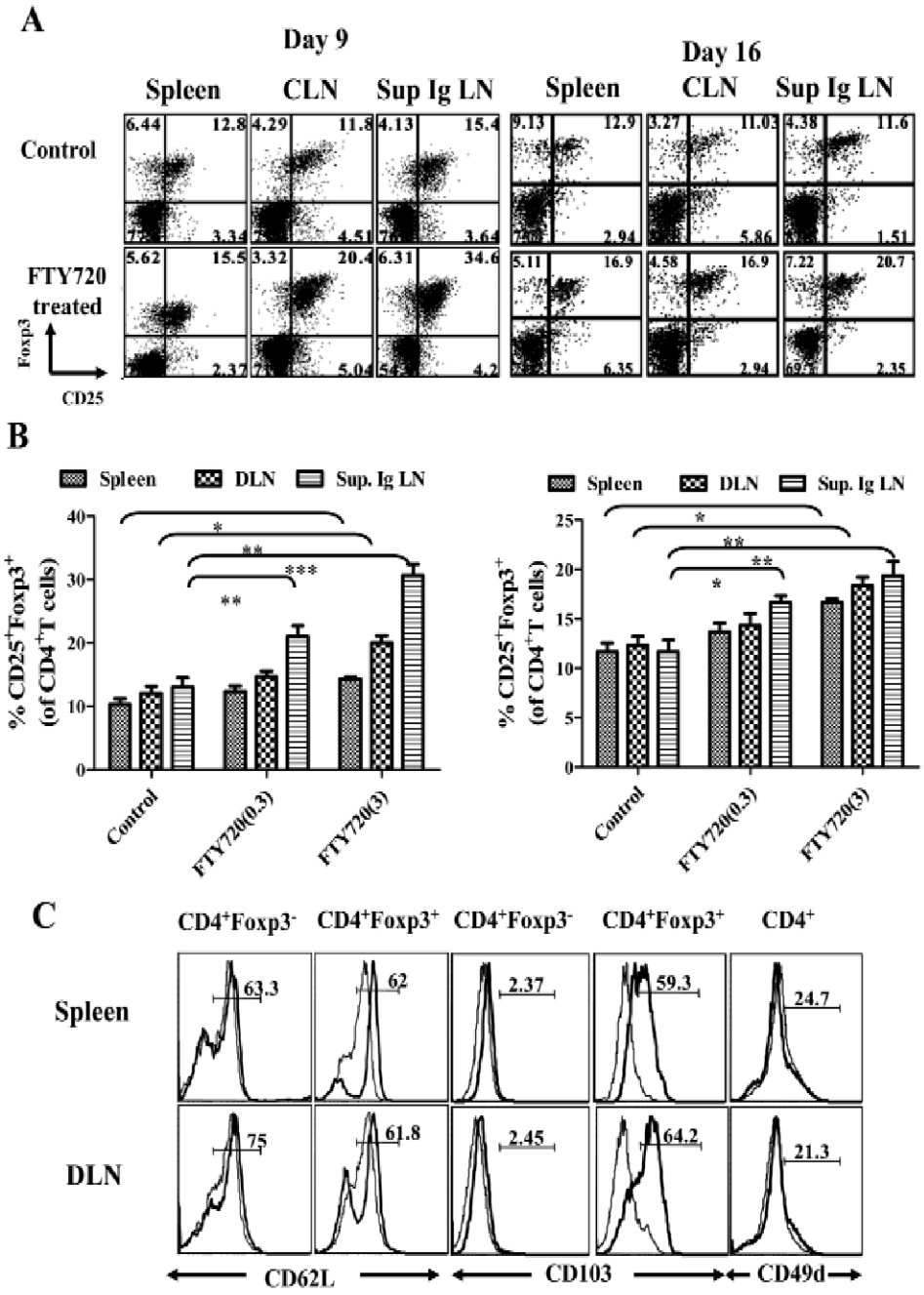

A
Day 9
Day 16
Spleen
CLN
Sup Ig LN
Spleen
CLN
Sup Ig LN
Control
FTY720 treated
Foxp3
CD25
B
Spleen
DLN
Sup. Ig LN
% CD25+Foxp3+ (of CD4+T cells)
Control
FTY720(0.3)
FTY720(3)
C
CD4+Foxp3-
CD4+Foxp3+
CD4+Foxp3-
CD4+Foxp3+
CD4+
Spleen
DLN
63.3
62
2.37
59.3
24.7
75
61.8
2.45
64.2
21.3
CD62L
CD103
CD49d

Figure 3. 3 FTY720 treatment increases the representation of Foxp3$^+$ T cells over CD4$^+$Foxp3$^-$ T cells in the lymphoid organs.

A, Ratios of absolute numbers of Foxp3$^+$CD4$^+$ T cells and Foxp3$^-$CD4$^+$ T cells in spleen, cervical LNs, and superficial inguinal LNs at days 5, 10, and 16 are shown. *B*, CD4$^+$ T cells (2 x 10^6) (containing both Foxp3$^+$ and Foxp3$^-$) from Foxp3-GFP knock-in animals were transferred into SCID animals, which were then treated with FTY720 (0.3 and 3.0 mg/kg BW) daily for 15 days, and lymphoid organs were then analyzed for percentages of GFP$^+$CD4$^+$ and GFP$^-$CD4$^+$ T cells. No significant differences were found in treated and untreated animals. *C*, Foxp3-GFP knock-in animals were infected with HSV-1 (5 x 10^3 PFU) ocularly and treated with FTY720 (0.3 and 3.0 mg/kg BW) for 15 days. On day 16, draining cervical (*upper panel*) and distal superficial inguinal (*lower panel*) LNs were analyzed for evidence of annexin V$^+$GFP$^+$ and GFP$^-$ CD4$^+$ T cells. Representative FACS plots from three animals studied are shown.

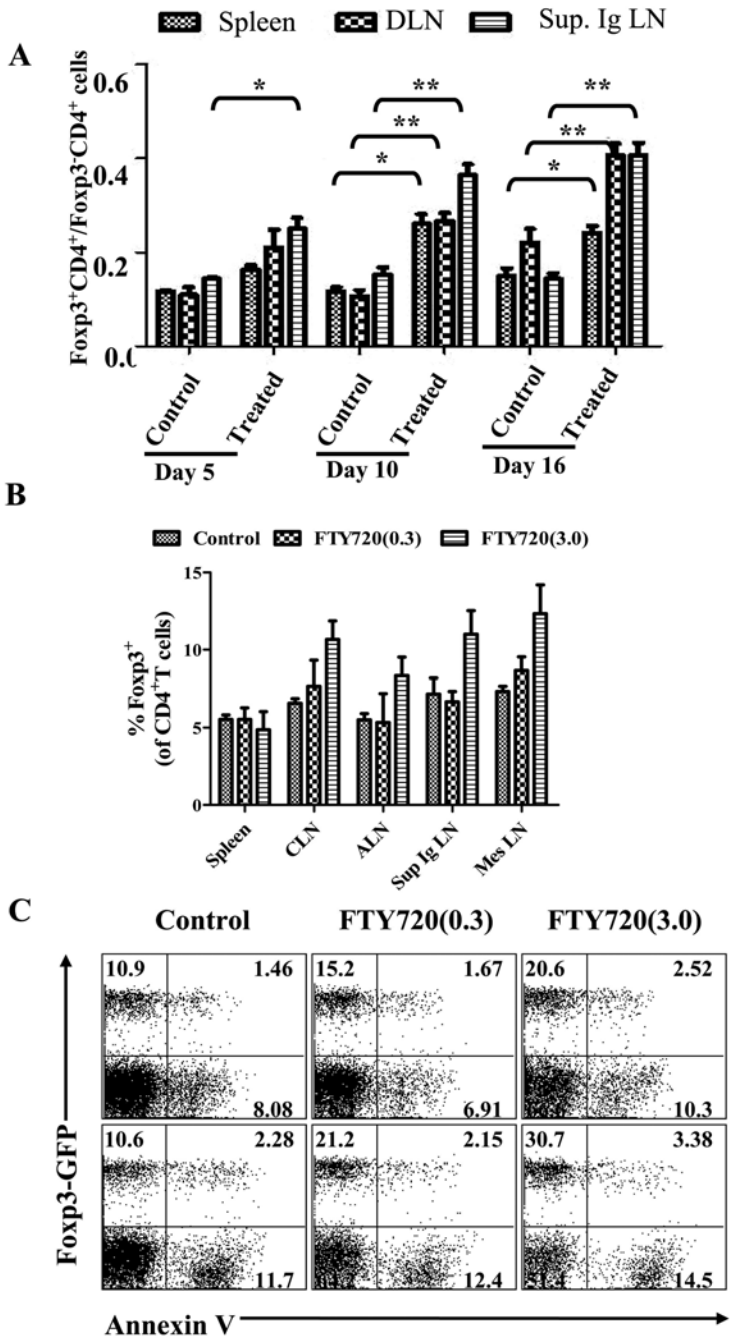

A
Spleen
DLN
Sup. Ig LN
Foxp3+CD4+/Foxp3-CD4+ cells
0.6
0.4
0.2
0.0
*
**
**
*
**
**
*
Control
Treated
Control
Treated
Control
Treated
Day 5
Day 10
Day 16
B
Control
FTY720(0.3)
FTY720(3.0)
% Foxp3+ (of CD4+T cells)
15
10
5
0
Spleen
CLN
ALN
Sup Ig LN
Mes LN
C
Control
FTY720(0.3)
FTY720(3.0)
10.9
1.46
8.08
15.2
1.67
6.91
20.6
2.52
10.3
10.6
2.28
11.7
21.2
2.15
12.4
30.7
3.38
14.5
Foxp3-GFP
Annexin V

Figure 3. 4 FTY720 administration along with Ag immunization induces Foxp3 expression in $CD4^+$ T cells in vivo.

$DO11.10RAG2^{-/-}$ mice were immunized i.p. with OVA in CFA and some (*n* = 6–7/group) were additionally treated with FTY720 (0.3, 1.0, and 3.0 mg/kg BW) on alternate days starting from 24 h postimmunization and continued until day 15. At 16 days of treatment, spleen and proximal (iliac and mesenteric) and distal (cervical, axillary, and superficial inguinal) LNs were isolated and analyzed for $CD4^+CD25^+Foxp3^+$ T cells by flow cytometry (gated on $CD4^+$ T cells). *A*, Representative FACS plots for percentages of $CD4^+CD25^+Foxp3^+$ T cells from six to seven different experiments are shown when animals were treated with 3.0 mg/kg BW of FTY720. *B*, Bar diagram for percentages of $CD4^+Foxp3^+$ T cells from six to seven different experiments is shown. Three animals immunized and FTY720-treated (0.3 mg/kg BW) were additionally given 250 μg of anti-TGF-β Ab at days 3, 6, and 10 and percentages of $CD4^+Foxp3^+$ T cells in lymphoid organs analyzed at 15 day are shown. *C*, Absolute numbers of $CD4^+Foxp3^+$ T cells in lymphoid tissues of immunized controls and immunized plus FTY720-treated (0.3 and 3.0 mg/kg BW) animals are shown. *D*, $DO11.10RAG2^{-/-}$ animals were immunized and treated with SEW2871 (0.5, 1.0, and 5.0 mg/kg BW) as described for FTY720 treatment. After 15 days, percentages of $Foxp3^+CD4^+$ T cells in various lymphoid tissues were analyzed.

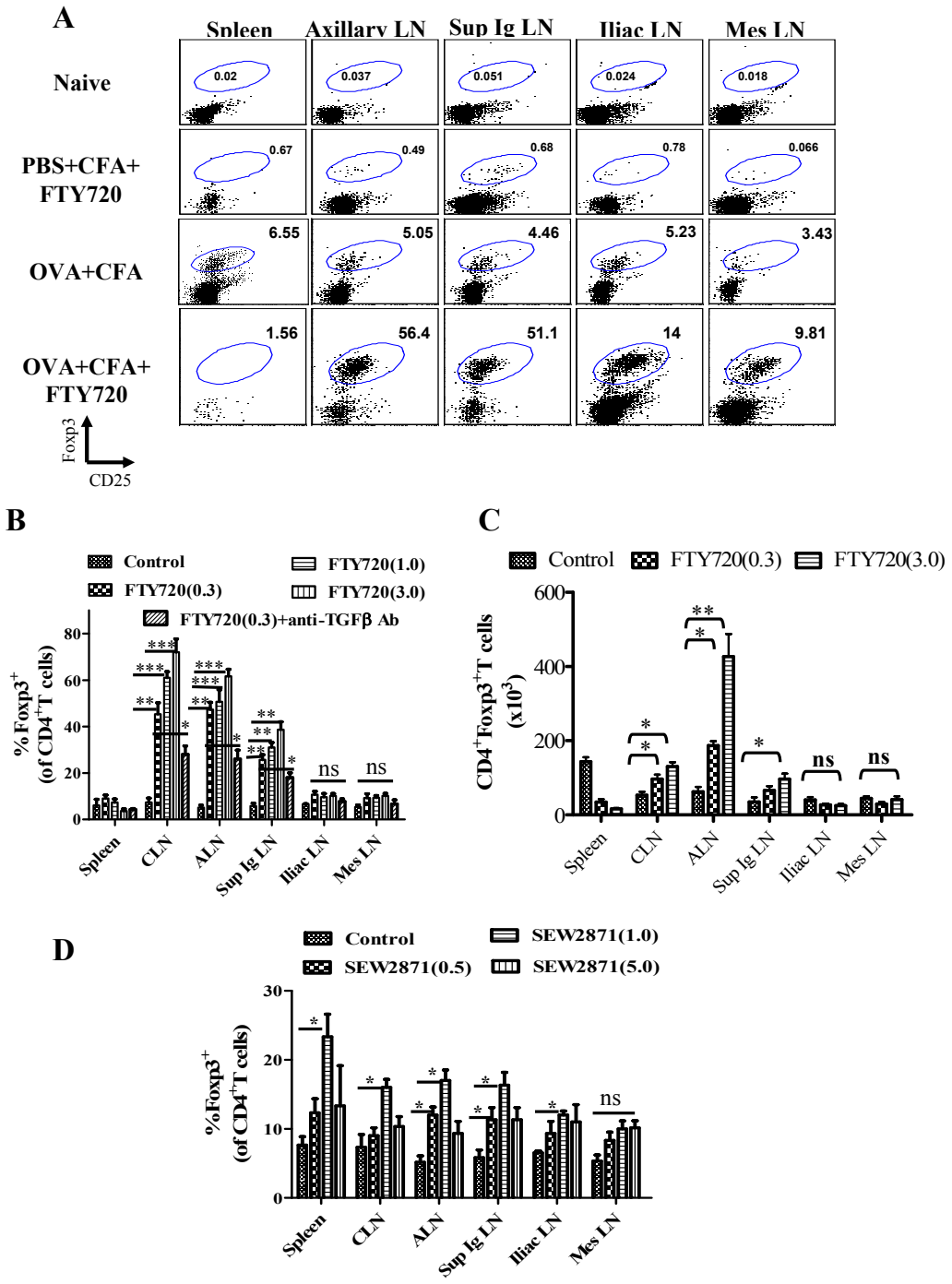

A
Spleen
Axillary LN
Sup Ig LN
Iliac LN
Mes LN
Naive
0.02
0.037
0.051
0.024
0.018
PBS+CFA+ FTY720
0.67
0.49
0.68
0.78
0.066
OVA+CFA
6.55
5.05
4.46
5.23
3.43
OVA+CFA+ FTY720
1.56
56.4
51.1
14
9.81
Foxp3
CD25
B
Control
FTY720(1.0)
FTY720(0.3)
FTY720(3.0)
FTY720(0.3)+anti-TGFβ Ab
% Foxp3+ (of CD4+T cells)
Spleen
CLN
ALN
Sup Ig LN
Iliac LN
Mes LN
ns
C
Control
FTY720(0.3)
FTY720(3.0)
CD4+Foxp3+T cells (x10^3)
D
Control
SEW2871(1.0)
SEW2871(0.5)
SEW2871(5.0)
% Foxp3+ (of CD4+T cells)

Figure 3. 5. FTY720-induced Foxp3$^+$CD25$^+$CD4$^+$ T cells are immunosuppressive.

CD4$^+$CD25$^+$ T cells were isolated from immunized and immunized plus FTY720-treated DO11.10RAG2$^{-/-}$ and DO11.10 animals were used in suppression assays against the cultures of CD4$^+$CD25$^-$ T cells from naive DO11.10RAG2$^{-/-}$ and DO11.10 mice, respectively, stimulated with anti-CD3 Ab as described in *Materials and Methods*.

In *A* and *B*, DO11.10RAG2$^{-/-}$ animals were used; in *C* and *D*, DO11.10 animals were used. *A*, CD4$^+$CD25$^+$ T cells were purified from pooled proximal (iliac and mesenteric) and distal (cervical, axillary, and superficial inguinal) LNs of FTY720-treated immunized DO11.10RAG2$^{-/-}$ animals to the extent of ~90% (*left panel*). FACS plot for purified CD4$^+$CD25$^+$ cells from proximal LNs is shown. A representative histogram of CD4$^+$CD25$^+$ T cells that were Foxp3$^+$ is shown (*right panel*). Ninety to 95% of CD4$^+$CD25$^+$ T cells were Foxp3$^+$ from both pooled proximal and distal LNs. Isotype control staining is shown (*middle panel*). *B*, Representative FACS plots are shown to demonstrate the suppressive activity of CD4$^+$CD25$^+$ T cells from immunized and FTY720-treated DO11.10RAG2$^{-/-}$ animals. CD4$^+$CD25$^-$ T cells showing that intensity of CFSE is decreased when these cells were cocultured with CD4$^+$CD25$^+$ T cells at 1:1 and 1:4 ratios (Treg-to-effector T cells) from proximal (prox) LN (*upper panel*) and distal (dis) LN (*middle panel*). *Lower panel*, Overlap of FACS plot for comparison of suppressive activity of Tregs isolated from draining LNs and peripheral LNs. *C*, Proportions of Foxp3$^+$ T cells among CD4$^+$CD25$^+$ T cells isolated from immunized and immunized plus FTY720-treated animals are shown. *D*, Inhibition of CD4$^+$CD25$^-$ T cell proliferation in presence of CD4$^+$CD25$^+$ T cells from immunized and immunized plus FTY720-treated animals is shown (% inhibition = cpm of CD4$^+$CD25$^-$ T cells – cpm of CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^-$ coculture/cpm of CD4$^+$CD25$^-$ T cells x 100).

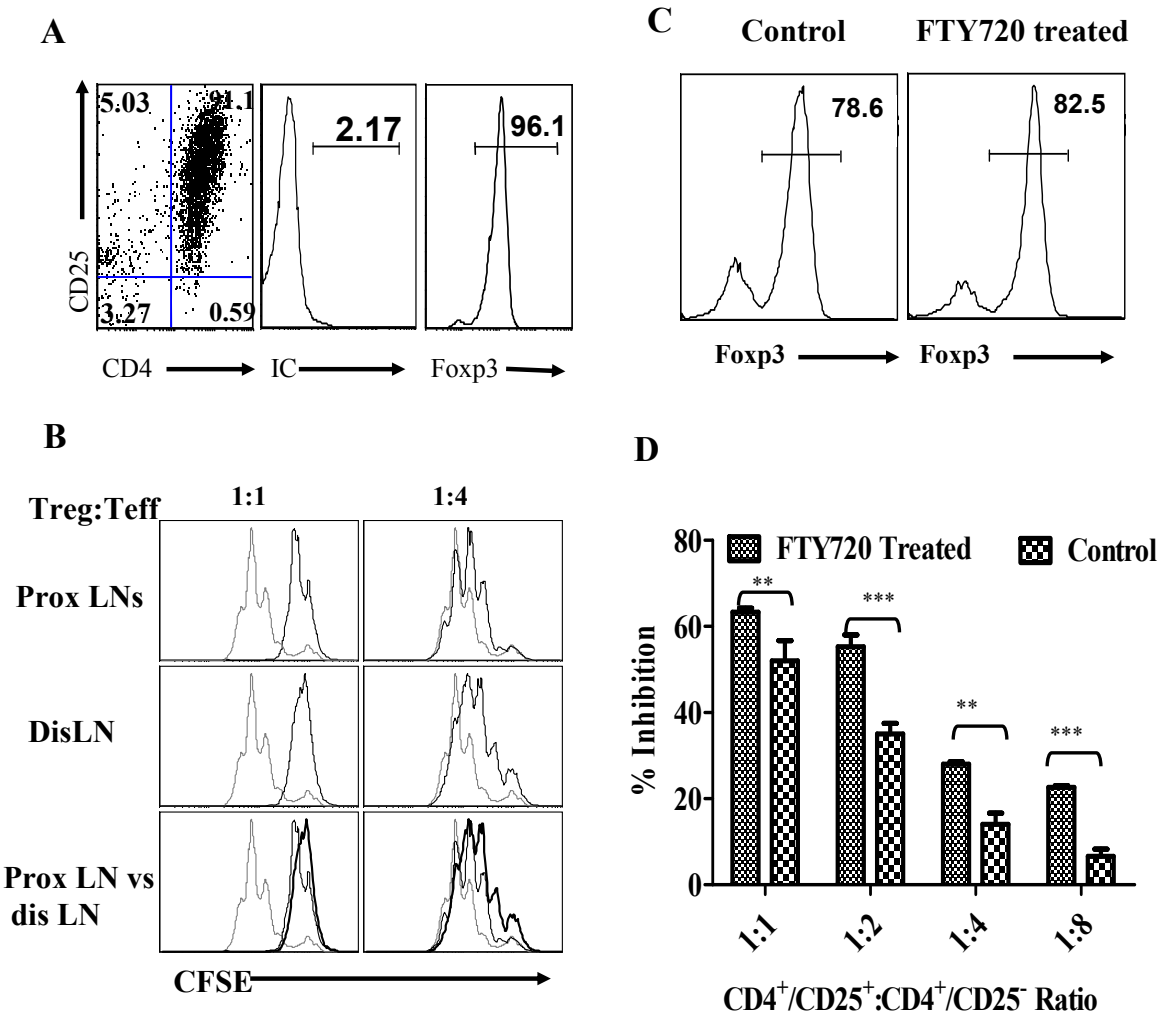

A
5.03
91.1
3.27
0.59
CD25
CD4
2.17
IC
96.1
Foxp3
C
Control
FTY720 treated
78.6
82.5
Foxp3
Foxp3
B
Treg:Teff
1:1
1:4
Prox LNs
DisLN
Prox LN vs dis LN
CFSE
D
FTY720 Treated
Control
% Inhibition
80
60
40
20
0
**
***
**
***
1:1
1:2
1:4
1:8
CD4+/CD25+:CD4+/CD25- Ratio

Figure 3. 6 In vitro generation and phenotypic characterization of FTY720-induced Foxp3$^+$ T cells.

A, Splenocytes were cultured in the presence of 25 U of rIL-2 and the indicated concentrations of TGF-β as a positive control or FTY720. More than 99% of CD4$^+$ T cells were $KJ_{1–26}$ positive (gated on CD4$^+$ T cells). After 5 days of culture, cells were analyzed for the expression of CD4, Foxp3, and CD25. *B*, Dose response histogram for Foxp3 induction with FTY720 from a typical experiment is shown. A dose of 10 ng/ml FTY720 when added daily in the culture was found to convert optimally Foxp3$^–$ cells into Foxp3$^+$CD4$^+$ T cells. *C*, Kinetic analysis of in vitro induction of Foxp3 in CD4$^+$CD25$^–$Foxp3$^–$ T cells with TGF-β (10 ng/ml) and FTY720 (10 ng/ml) is shown. Representative FACS plots of three similar experiments are shown. *D*, Splenocytes from DO11.10RAG2$^{–/–}$ animals were CFSE labeled and cultured with plate-bound anti-CD3, IL-2, and FTY720 or TGF-β as a positive control for 5 days. After 5 days, cells were stained with CD4 and Foxp3. CFSE dilution and Foxp3 expression were shown in gated CD4$^+$ T cells. FTY720-induced CD4$^+$CD25$^+$Foxp3$^+$ T cells proliferate extensively. *E*, The phenotype of in vitro-generated Foxp3$^+$ T cells by FTY720 and TGF-β as percentage positive for indicated surface marker is shown.

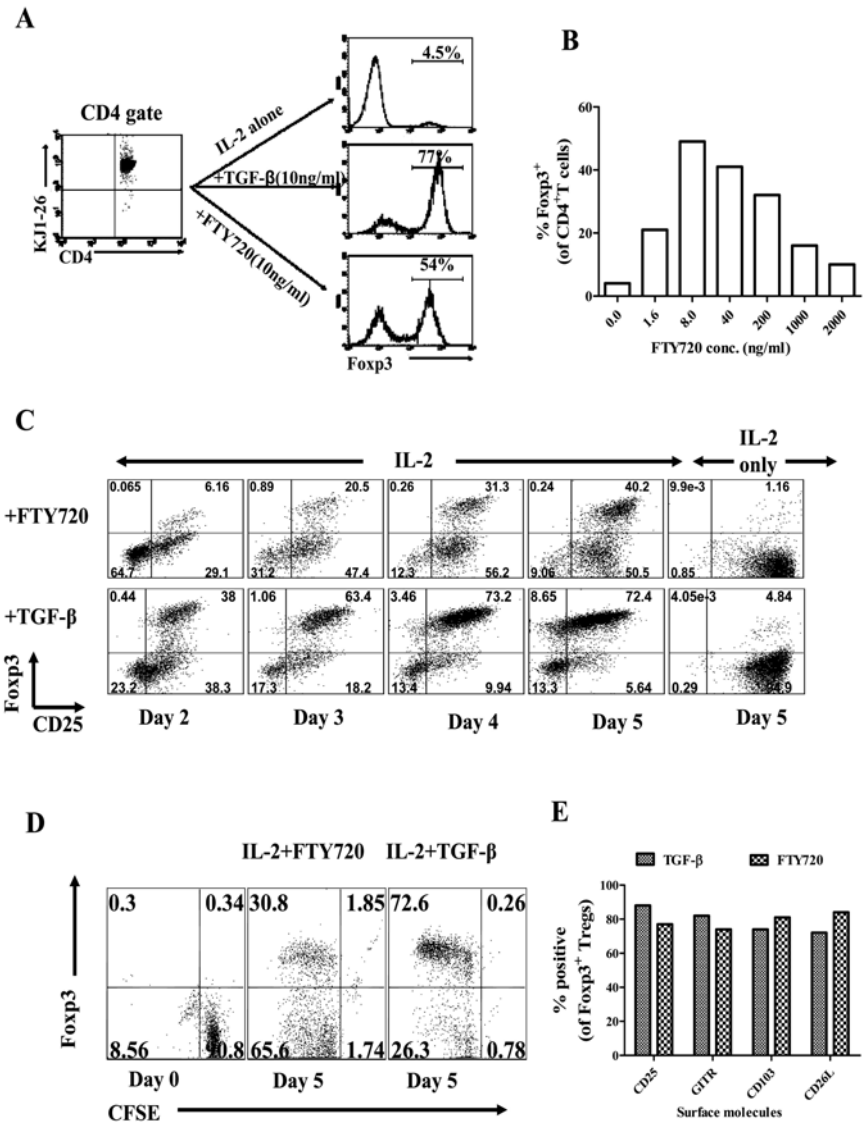
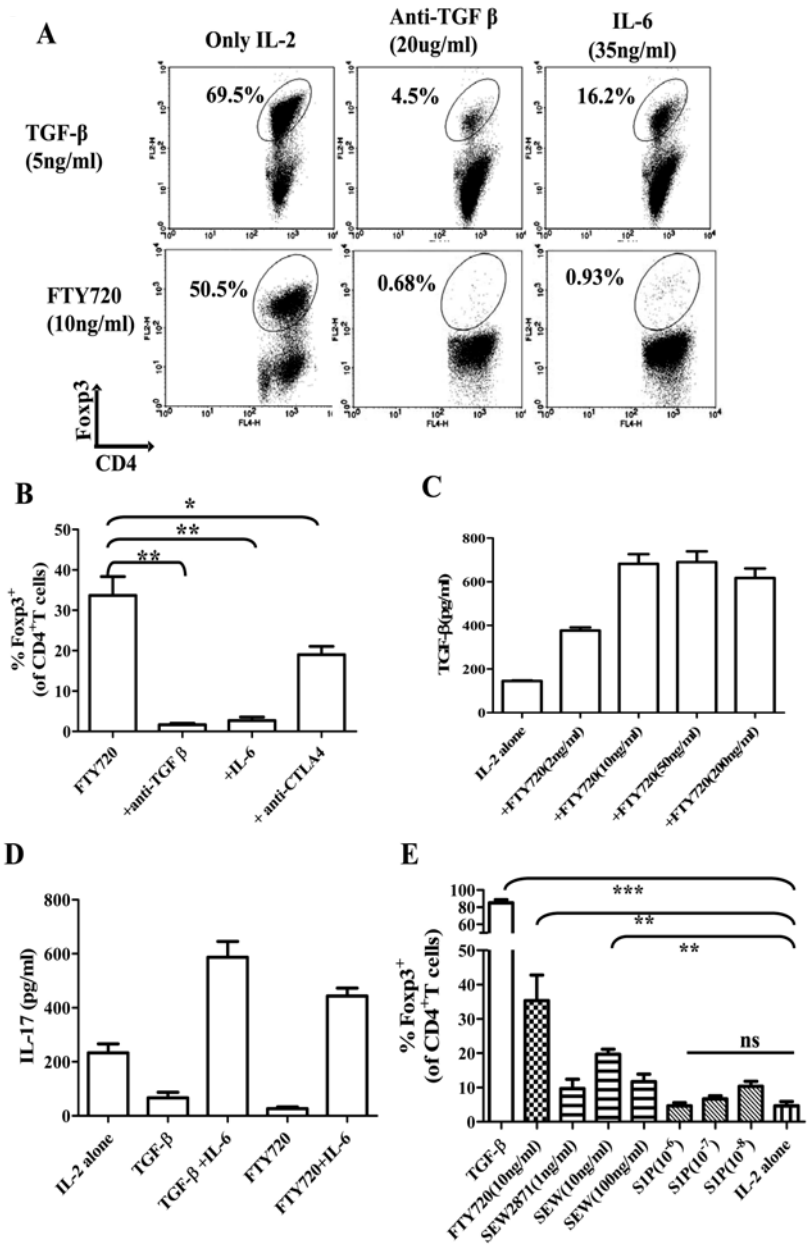

A
CD4 gate
KJ1-26
CD4
IL-2 alone
+TGF-β(10ng/ml)
+FTY720(10ng/ml)
4.5%
77%
54%
Foxp3
B
% Foxp3+ (of CD4+T cells)
FTY720 conc. (ng/ml)
C
IL-2
IL-2 only
+FTY720
+TGF-β
Foxp3
CD25
Day 2
Day 3
Day 4
Day 5
Day 5
D
IL-2+FTY720
IL-2+TGF-β
Foxp3
Day 0
Day 5
Day 5
CFSE
E
TGF-β
FTY720
% positive (of Foxp3+ Tregs)
CD25
GITR
CD103
CD26L
Surface molecules

Figure 3. 7 TGF-β is involved in FTY720-mediated Foxp3 induction in $CD4^+Foxp3^-$ cells.

A, Anti-TGF-β1, 2, 3 Abs or IL-6 was added in the splenocyte cultures in the presence of TGF-β (*upper panel*) and FTY720 (*lower panel*), and surviving $CD4^+$ T cells were analyzed for Foxp3 expression (CD4 gate). *B*, Percentages of $Foxp3^+$ cells of $CD4^+$ T cells are shown in splenocyte cultures added with anti-TGF-β (20 μg/ml), IL-6 (35 ng/ml), and anti-CTLA-4 Ab (20 μg/ml). *C*, Dose response bar diagram of TGF-β concentration in culture supernatants of splenocytes added with different doses of FTY720 is shown. *D*, IL-17 concentrations from culture supernatants of splenocytes in the presence of IL-2 only and IL-2 with TGF-β or TGF-β + IL-6 or FTY720 or FTY720 + IL-6 as measured by sandwich ELISA are shown. *E*, Bar diagram showing the percentages of $Foxp3^+$ cells induced with TGF-β, FTY720, SEW2871, and S1P is shown.


A
Only IL-2
Anti-TGF β (20ug/ml)
IL-6 (35ng/ml)
TGF-β (5ng/ml)
FTY720 (10ng/ml)
69.5%
4.5%
16.2%
50.5%
0.68%
0.93%
Foxp3
CD4
B
% Foxp3+ (of CD4+T cells)
FTY720
+anti-TGF β
+IL-6
+ anti-CTLA4
C
TGF-β(pg/ml)
IL-2 alone
+FTY720(2ng/ml)
+FTY720(10ng/ml)
+FTY720(50ng/ml)
+FTY720(200ng/ml)
D
IL-17 (pg/ml)
IL-2 alone
TGF-β
TGF-β +IL-6
FTY720
FTY720+IL-6
E
% Foxp3+ (of CD4+T cells)
TGF-β
FTY720(10ng/ml)
SEW2871(1ng/ml)
SEW(10ng/ml)
SEW(100ng/ml)
S1P(10-6)
S1P(10-7)
S1P(10-8)
IL-2 alone
ns

PART IV

ROLE OF TIM-3/GALECTIN-9 INHIBITORY INTERACTION IN VIRAL INDUCED IMMUNOPATHOLOGY: SHIFTING THE BALANCE TOWARDS REGULATORS

Research described in this chapter is from a paper submitted for publication in *Journal of Imunology* by Sharvan Sehrawat, Amol Suryawanshi, Mitsuomi Hirashima and Barry T. Rouse.

Sehrawat S, Suryawanshi A, Hirashima M and Rouse BT. Role of TIM-3/galectin-9 inhibitory interaction in viral induced immunopathology: Shifting the balance towards regulators. (Journal of Immunology-Submitted)

In this chapter "our" and "we" refers to me and co-authors. My contribution in the paper includes (1) Selection of the topic (2) Compiling and interpretation of the literature (3) Designing experiments (4) understanding the literature and interpretation of the results (5) providing comprehensible structure to the paper (6) Preparation of graphs and figures (7) Writing and editing

Abstract

Controlling chronic immunoinflammatory diseases such as lesions in eye caused by infection with herpes simplex virus (HSV) represents a major therapeutic challenge. Since $CD4^+$ T cells are the primary orchestrators of lesions caused by HSV, targeting activated $CD4^+$ T cell subsets and increasing the representation of cells that express regulatory function would be a logical therapeutic approach. We show in this report that this outcome can be achieved by therapy, systemic or local, with the lectin-family member galectin-9. This molecule, which is a natural product of many cell types, acts as a ligand to the inhibitory molecule TIM-3 that is expressed by activated but not naïve T cells. We show that 50% or more of T cells in ocular

lesions caused by HSV in mice express TIM-3 and that blocking signals from its natural ligand with a monoclonal antibody results in more severe lesions. More importantly the provision of additional galectin-9, either systemically or more effectively by local subconjuctival administration, diminished the severity of SK lesions as well as the extent of corneal neovascularization. Multiple mechanisms were involved in inhibitory effects. These included apoptosis of the orchestrating effector T cells with consequent reduction of proinflammatory cytokines, an increase in the representation of two separate subtypes of regulatory cells as well as inhibitory effects on the production of molecules involved in neovascularization, an essential component of SK pathogenesis. Our results indicate that galectin-9 therapy may represent a useful approach to control HSV induced lesions, the commonest cause of infectious blindness in the Western World.

Introduction

One of the dire consequences of herpes simplex virus (HSV) infection is blindness resulting from infection in the eye and a subsequent chronic inflammatory reaction in the corneal stroma. This lesion is considered to be immunopathological orchestrated by T lymphocytes that recognize peptides derived from viral proteins or perhaps from altered self proteins of the damaged cornea (1, 2). Currently, herpetic stromal keratitis (HSK) is mainly controlled by combinations of drugs that include antivirals and steroids with the latter being administered for prolonged periods of time (3). Future therapies are expected to emerge from a better understanding of the disease pathogenesis so that critical steps can be counteracted more precisely. Identifying such steps has come mainly from studies in animal models, especially the

mouse, where lesions that closely resemble those in humans can routinely be induced following primary infection with appropriate strains of virus (4). Such studies have revealed a critical role of $CD4^+$ T cells of the Th1 subset as mediators of lesions (5, 6). In consequence, either preventing the access of Th1 cells to the eye or blunting their activity once at ocular sites represents potentially a valuable form of therapy. Recent studies on some autoimmune lesions caused by pathogenic T cells have indicated that one means of terminating the activity of such T cells is to engage receptors expressed by activated cells that deliver an inhibitory or lethal signal to the cell (7-11). This effect was achieved in some situations by engaging the TIM-3 (T cell immunoglobulin and mucin-3) receptor, a member of the T cell immunoglobulin and mucin family of proteins, with its recently identified ligand galectin-9 (8). Accordingly, the resolution of autoimmune lesions in collagen arthritis (a $CD4^+$ Th1 subset mediated autoimmune lesion) occurred following treatment with galectin-9 (12). Some measure of control was also achieved with galectin-9 treatment in other immunoinflammatory lesions such as experimental autoimmune encephalomyelitis and graft versus host disease (8, 13, 14). To our knowledge, a role for TIM-3 galectin-9 interaction in controlling inflammatory lesions caused by microbial agents has yet to be explored. The present studies were designed, therefore, to evaluate if lesions in the eye caused by HSV were subject to control by manipulating the TIM-3/galectin-9 system on one or more cell types involved in causing HSK.

Our studies demonstrate that galectin-9 and TIM-3 interaction does influence the expression of lesions in the eye following ocular infection with HSV. Accordingly, lesions were significantly more severe if the signals delivered to TIM-3 were interrupted using anti-TIM-3 antibody. Moreover if galectin-9 was supplied in excess, either by systemic or local administration, lesion severity, which included particularly the extent of ocular

neovascularization, was diminished. The mechanisms by which galectin-9 acted in vivo were likely multiple. These included induction of apoptosis of pathogenic effector Th1 cells, induction or the expansion of two types of regulatory cells as well as the diminished production of some factors involved in corneal neovascularization. Influencing the function of the TIM-3/galectin-9 pathway holds promise as a means to control the severity of HSK lesions.

Materials and Methods

Mice, Virus, cell lines

Female 6- to 8-wk-old C57B/6 mice were purchased from Harlane Sprague-Dawley (Indianapolis, IN). GFP-Foxp3 Knock-in mice were a kind gift from Dr. M. Oukka of Brigham and Women Hospital, Harvard Medical School. All animals were housed in the animal facilities at the University of Tennessee. BALB/c DO11.10 RAG2-/- mice were purchased from Taconic Farm and kept in our specific pathogen-free facility where food, water, bedding, and instruments were autoclaved. All manipulations were done in a laminar flow hood. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. HSV-I RE Tumpey and HSV-I RE Hendricks was propagated and titrated on Vero cells (ATCC CCL81) using standard protocols. The virus was stored in aliquots at -80°C until use.

Antibodies and reagents

CD4-APC (RM4-5), DO11.10-PE (KJ1.26), CD25-FITC (7D4), CD103-FITC (M290), CD62L-APC (MEL-14), CD44-FITC (1M7), Foxp3-PE(FJK-16s), Foxp3-FITC (FJK-16s), CD69-FITC (H1.2F3), CD11c-PE (HL3), CD11c-APC (HL3), anti-IFN-γ-FITC, anti-IL-17-PE, CD11b-PerCP (M1/70), Gr1-PE (RB6-8C5), Gr1-FITC (RB6-8C5), F4/80-FITC (BM8), Ly6C-

FITC (AL-21), anti-CD3 (145.2C11), and anti-CD28 mAb (37.51) were purchased from BD PharMingen (San Diego, CA). Anti-TIM3-APC (cat.# FAB1529A), anti-TIM-3-PE (cat.# FAB1529P), recombinant IL-6 and recombinant human TGF-β1 were obtained from R & D. Anti-TIM-3 monoclonal antibody (RMT2-23) and rat IgG2a isotype control antibody (2A3) was obtained from Bio-X-cell, West Lebanon, NH. Mouse and human recombinant galectin-9 was provided by Gal Pharma, Japan. Galectin-3 was obtained from Sigma Chemical Co. Recombinant human IL-2 and $OVA_{323-339}$ peptide were obtained from Hemagen and Genscript respectively. CFSE was obtained from Molecular Probe and used at a final concentration of 0.5 μM for 15 min. at 37^0C in PBS.

Corneal HSV-1 infection and clinical observations

Corneal infections of C57B/6 mice were conducted under deep anesthesia. Mice were scarified on their corneas with a 27-gauge needle, and a 3-μl drop containing the required viral dose was applied to the eye. The eyes were examined on different days postinfection (p.i.) with a slit-lamp biomicroscope (Kowa, Nagoya, Japan), and the clinical severity of keratitis and angiogenesis of individually scored mice was recorded as described elsewhere (15, 16).

Treatment of animals with anti-TIM-3 antibody and galectin-9

Six- to 8-wk-old C57B/6 mice were ocularly infected under deep anesthesia with either $5x10^5$ PFU HSV RE Hendricks or $5x10^3$ PFU of HSV I RE Tumpey and divided randomly into groups. One group of animals infected with HSV I RE Hendricks was administered with 100μg of anti-TIM-3 antibody intraperitoneally (i.p.) every alternate day starting from day 3 until day 13 p.i. Animals in control group were given isotype control antibody following same regimen. Animals infected with HSV I RE Tumpey were given galectin-9 (10μg, 50μg or 100μg) ip starting from day 3 until day 13 daily while control animals received diluent. In some

experiments, 6-8µl of galectin-9 (1, 5 or 10µg) was injected sub conjuctivally into eye daily starting either from day 4 or from day 8 until day 13. Galectin-9 was concentrated using Amicon ultracentriguge devices (Millipore). Tubes were treated with N/10 NaOH and washed with sterile PBS before using. Mice were observed for the development and progression of herpetic stromal keratitis (SK) lesions and angiogenesis from day 5 until day 14, as described elsewhere (15, 16). Most of the experiments were repeated at least three times unless stated

***In vitro* differentiation of $CD4^+CD25^+Foxp3^+$ regulatory T cells and Th17 cells**

Splenocytes isolated from DO11.10Rag2-/- mice were used as the precursor population for induction of Foxp3 in $CD4^+$ T cells as described elsewhere (17). Briefly, $2x10^6$ of total splenocytes after RBC lysis and several washings were cultured in 1 ml volume with previously optimized doses of plate-bound anti-CD3 Ab (0.125 µg/ml in 200 µl volume), rIL-2 (25-100 U/ml), and TGF-β (2.5 to 10 ng/ml) for 5 days at 37°C in a 5% CO_2 incubator in 48-well plates. In some cultures in addition to IL-2, TGF-β either alone or in combination with various concentrations of Galectin-9 was added. After 5 days, cells were characterized phenotypically by flow cytometry.

For the differentiation of $CD4^+$ T cells into Th17 cells, splenocytes isolated from DO11.10 Rag2-/- mice were cultured with 10 µg/ml of $OVA_{323-339}$ peptide, TGF-β (2.5 to 10 ng/ml), IL-2 (25U) and IL-6 (30-60 ng/ml) for five days. To look for the effect of galectin-9 on Th17 cells, various concentration of galectin-9 or galectin-3 were added into cultures at the beginning. After 5 days, cells were analyzed by intracellular cytokine staining for the production of IL-17 and IFN-γ using BD bioscience kit. Foxp3 intranuclear staining was done using a kit from eBioscience.

Proliferation assays

Splenocytes and DLN cells isolated from control and galectin-9 treated HSV infected animals 14 dpi were labeled with 0.5µM of CFSE at 37^0C for 10 minutes. $5x10^5$ of labeled cells were cultured in presence of IL-2 (100U/ml), anti-CD3 (1µg/ml) and anti-CD28 (1µg/ml) mAb for three days. After three days of incubation, dilution of CFSE was analyzed in stained $Foxp3^-$ and $Foxp3^+$ $CD4^+$ T cells.

Ex vivo apoptosis assay

Draining lymph node cells and splenocytes isolated from uninfected and HSV infected C57B/6 mice at 8 dpi were incubated with various concentrations of galectin-9 and galectin-3 for 8 hrs in 96 well flat bottomed plates in humidified incubators in presence of 5% CO_2. After incubation period was over cells, were stained for annexin V using a kit from BD Bioscience. Additionally, cells were also co-stained for TIM-3 and Annexin V. Stained cells were analyzed immediately by flow cytometry.

In vitro assays for VEGF production

A mouse stromal fibroblast cell line (MKT-1) kindly gifted by Dr Winston Kao, Department of Ophthalmology, University of Cincinnati, Cincinnati, OH, was used for studying the effect of galectin-9 on VEGF production. The cells were cultured in 10% DMEM and plated onto 24 well tissue culture plates. The cells at more than 90% confluency were infected with 5MOI of HSV KOS for one hour. Thereafter, galectin-9 was added into the cells at various concentrations. Untreated but infected cells served as positive control for these experiments. In initial experiments cells were harvested at different times and stored in RNA stabilizing solution obtained from Quiagen. For most of the subsequent experiments cells were harvested after 12 hrs.

Real time RT-PCR

RNA was extracted from the cells stored in RNA later using RNeasy mini kit (Quiagen). Total cDNA was made with 1µg of RNA using oligod(T) primer. Real time PCR was performed using SYBR Green PCR Master Mix with iQ5 Real-Time PCR Detection System (BioRad, Hercules, CA). VEGF expression levels of each samples were normalized to HPRT using ΔCt calculations. Relative VEGF expression between control and experimental groups were calculated using $2^{-\Delta\Delta Ct}$ formula. The sequences of the primers were: VEGF 5'-ACACAGGACCGCTTGAAGAT-3' and 5'-CTGCACCCACGACAGAAG-3'. HPRT 5'-GACCGGTCCCGTCATGC-3' and 5'-TCATAACCTGGTTCATCATCGC-3'.

ELISA

The corneal samples were pooled group wise and homogenized using a tissue homogenizer (Pellet pestle mortar, Kontes). The concentrations of various cytokines and VEGF was measured by sandwich ELISA kits from eBioscience (IL-6), and BD Bioscience (IL-12, TGF-β) and Quantikine (VEGF-A). For TGF-β quantitation, samples were first acidified as per manufacturer's instructions.

Flow cytometric analysis

Cell preparation Single-cell suspensions were prepared from the cornea, draining cervical lymph nodes (DLN), and spleen of mice at different time points p.i. Corneas and trigeminal ganglia were excised, pooled group wise and digested with 60 U/ml Liberase (Roche Diagnostics) for 60 min at 37°C in a humidified atmosphere of 5% CO_2 as described earlier (17, 18). After incubation, the corneas and trigeminal ganglia were disrupted by grinding with a syringe plunger on a cell strainer and a single-cell suspension was made in complete RPMI 1640 medium.

Staining for flow cytometry The single-cell suspension obtained from LNs, spleen, and corneal samples were stained for different cell surface molecules for FACS. All steps were performed at 4^0C. Briefly, a total of $1x10^6$ cells were first blocked with an unconjugated anti-CD32/CD16 mAb for 30 min. in FACS buffer. After washing with FACS buffer, fluorochrome-labeled respective Abs was added for 30 min. Finally, the cells were washed three times and resuspended in 1% paraformaldehyde.

To enumerate the number of IFN-γ and TNF-α producing T cells, intracellular cytokine staining was performed as previously described (18). In brief, 10^6 freshly isolated splenocytes, lymph node cells were cultured in U bottom 96-well plates. Cells were left untreated or stimulated with 2 MOI of UV inactivated HSV I and incubated overnight at 37°C in 5% CO_2. Brefeldin A (10 μg/ml) was added for the last five hours of the culture period. After this period, cell surface staining was performed, followed by intracellular cytokine staining using a Cytofix/Cytoperm kit (BD PharMingen) in accordance with the manufacturer's recommendations. The Ab used were anti-IFN-γ-PE and anti-TNF-α-FITC. The fixed cells were resuspended in 1% paraformaldehyde. For in vitro induced cultures, cells were stimulated with PMA (50ng) and ionomycin (500ng) for 5 hours in presence of brefeldin A (10 μg/ml). Subsequently, cells were first stained as described above for surface CD4 and intracellular cytokines (IL-17 or IFN-γ). The stained samples were acquired with BD FACSCalibur and the data were analyzed using the Flowjo software.

Statistical analysis

Most of the analyses for determining the level of significance were performed using Student's *t* test. $P \leq 0.001$ = *** $P \leq 0.01$ = ** and $P \leq 0.05$ = * were considered significant.

Results are expressed as mean ± SD. For some experiments, as mentioned in the fig. legends, one way ANOVA test was applied.

Results

TIM-3 expression is up regulated on T cells after HSV infection

As a prelude to exploring the value of manipulating TIM-3/galectin-9 interaction to influence the outcome of stromal keratitis, mice were ocularly infected with HSV and the expression pattern of TIM-3 on $CD4^+$T cells was measured at various times in lymphoid tissues, as well as the eye and trigeminal ganglion. Naïve animals lack inflammatory cells in the corneal stroma but invasion by many cell types, including $CD4^+$ T cells, is fully evident by day 8 p.i. At this time, replicating virus is usually cleared, although the extent of the ocular inflammatory response usually continues to progress and peaks in severity between 15 to 21 days (5). Spontaneous regression, especially to a fully resolved state, usually does not occur without treatment. The results in fig 4.1A and D record the frequency and numbers of $CD4^+$TIM-3^+ T cells in the draining lymph nodes (DLN) and spleens at various times after HSV infection. In naïve animals, few $CD4^+$ T cells expressed TIM-3 (between 0.5 to 3.0% in different experiments) and many that do were $Foxp3^+$ (around 30 % of $CD4^+$TIM-3^+) (Fig 4.1C). By day 8, significant increases in the frequencies of TIM-3^+CD4^+ T cells were evident in both lymph nodes and spleen. The pattern was similar on day 15 but by day 40 had declined markedly. Analysis of lymph node and spleen cells at the time of peak responses revealed that most of the TIM-3^+CD4^+ T cells expressed the activation marker $CD44^{hi}$ (Fig 4.1B). Although we lack the

reagents to prove it, we suspect that the increased populations of TIM-3$^+$CD4$^+$ T cells were HSV specific cells.

Ocular as well as trigeminal ganglion samples were also analysed using tissue pools from several animals after collagenase digestion (Fig. 4.1E). At both time points examined, around 50% of CD4$^+$ T cells were TIM-3$^+$. Taken together these results demonstrate that TIM-3 is up regulated on CD4$^+$ T cells following HSV infection. This was especially evident at the inflammatory ocular and trigeminal ganglia sites.

TIM-3 signaling may control stromal keratitis lesion severity

Studies on autoinflammatory lesions have indicated that TIM-3 signaling may represent a mechanism by which lesions are regulated under physiological conditions (8, 11, 19). The results expressed in figures 2A and B indicate that a similar situation likely occurs with HSV induced ocular lesions. This was shown by comparing the outcome of ocular infection in mice given a TIM-3 blocking monoclonal antibody (mAb) with those that received an isotype control mAb. Treatment was begun 24 hours pi and the severity of HSK, as well as the extent of neovascularization, compared over a 15 day observation period. The results were clear cut and supported the notion that blocking TIM-3 led to enhanced HSV lesions and increased levels of neovascularization (Fig. 4.2A and B). In two separate experiments involving 14 eyes, positive SK lesions (a scores of ≥ 2) were evident earlier in mice that received anti-TIM-3 (8 out of 14 eyes positive at day 7) than those given the isotype control Ab (0 out of 14 eyes at day 7). Lesions also became significantly more severe in anti-TIM-3 recipients with a higher percentage of eyes showing positive lesions overall (12 out of 14 with scores of ≥ 2 and 10 out of 14 with a score of ≥ 3) compared to isotype control Ab recipients (2 out of 14 with scores of ≥ 3) at day 14. A similar pattern of events was noted with the neovascularization scores.

At day 15, experiments were terminated and corneas were pooled and collagen digested to enumerate and phenotype the recovered cells. In addition, lymph nodes and spleens were collected from animals with positive lesions to measure HSV specific immune responses. As seen in figure 4.2C and D, the number of both $CD4^+$ T cells and granulocytes was far greater in the ocular samples from anti-TIM-3 treated animals than controls (a more than 10 fold increase in $CD4^+$ T cells and granulocytes). With regard to phenotype, the frequency of $CD4^+$ T cells that were TIM-3^+ was far higher in the anti-TIM-3 MAb group (72% in one experiment) than in controls (35%). The reason for this observation was not clear, but conceivably it could have reflected the finding that the magnitude of anti-HSV $CD4^+$ T cell responses was higher in the anti-TIM-3 MAb treated animals (Fig 4.2E and F). Thus cells isolated from individual spleens and draining LNs at 15 days p.i. of control Ab and TIM-3 antibody recipients, when stimulated with HSV antigens and the numbers of IFN-γ and TNF-α producing cells measured, revealed a 2.5 fold increase in the frequencies and numbers of each of the cytokine producing cell populations in anti-TIM-3 treated animals (Fig. 4.2E and F)

These results indicated that TIM-3 signaling of $CD4^+$ T cells in lymphoid organs or perhaps corneal lesions via its ligand under natural conditions may serve to regulate the extent of stromal keratitis and that this may proceed at least in part by influencing the magnitude of the immune response.

Galectin-9 Administration suppresses HSK lesions

Recently galectin-9 was shown to be the natural ligand of TIM-3 and that signaling via galectin-9, at least to some T cell subsets, may cause them to undergo apoptosis (8). To evaluate if the administration of galectin-9 could influence the expression of HSK, two types of experiments were preformed in HSV ocularly infected mice. Animals were either treated i.p.

with different doses of galectin-9 starting at day 4 or were given different doses of the drug injected subconjunctively starting at day 4 or day 8 until mice were sacrificed to evaluate different parameter at day 15. Both treatment modalities inhibited SK lesions as well as the extent of neovascularization, but the efficacy of the local subconjunctival administration was the greater (Fig 4.3A, B and C). The results shown in figure 4.3A demonstrated that following systemic administration, significant levels of lesion inhibition were only observed at the highest doses of galectin-9 evaluated (100μg) but a trend for suppressed lesions was evident at lower doses.

With local administration of galectin-9, a dose dependent inhibition of SK lesions and neovascularization also occurred with the differences compared to controls even more apparent then observed with systemic administration (Fig 4.3B and C). Accordingly, in animals given galectin-9 locally starting on day 4, lesions were reduced almost 3 fold on average compared to untreated controls with these differences being highly significant ($p \geq 0.01$). The numbers of $CD4^{+}TIM\text{-}3^{+}$ cells recovered from eyes were significantly reduced compared to untreated controls (Fig 4.4A). In addition the frequencies and numbers of PMNs were reduced 6-7 fold as a consequence of galectin-9 treatment (Fig 4.4B and C). Along with clinical changes occurring in response to local galectin-9 treatment, the levels of cytokines as well as the angiogenesis factor VEGF were measured in pooled corneal extracts. As shown in Fig 4.4D, whereas levels of the proinflammatory cytokines IL-6 and IL-12 were less, TGF-β levels were higher than in controls. VEGF levels were also lower in galectin-9 treated pooled samples.

Of notable interest, if local treatment was begun on day 8, the time when lesions become clinically evident, they failed to progress and even slightly diminished in severity compared to untreated animals (Fig 4.3F). Furthermore, as shown in fig. 4.4E, pooled corneal samples from

treated animals had lower levels of proinflammatory (IL-6) and higher levels of anti-inflammatory (TGF-β). These data strongly indicate that TIM-3/galectin-9 interaction serves to induce resolution of viral immunoinflammatory lesions.

Possible mechanisms by which galectin-9 therapy functions

Induction of apoptosis of TIM-3$^+$CD4$^+$ T cells

Prior in vitro studies have indicated that galectin-9 triggering of proinflammatory CD4$^+$ T cells causes them to undergo apoptosis (8). Since in the HSK model, lesions appeared to be mainly orchestrated by IFN-γ producing CD4$^+$ T cells (6), lesions could be reduced if orchestrating T cells were destroyed. We attempted to demonstrate such an effect in vivo by examining single cell suspensions from lymphoid organs by flow cytometry or tissue sections of treated eyes for signs of apoptotic cells. Such could not be demonstrated (data not shown). However, it was evident that the total numbers of CD4$^+$ T cells as well as HSV-specific CD4$^+$IFN-γ$^+$ T cells were significantly reduced in animals treated systemically with galectin-9 which could have been the consequence of apoptosis (Fig 4.5A). In addition, we were able to demonstrate CD4$^+$ T cells that express TIM-3 as a consequence of HSV infection *in vivo* could be induced to undergo apoptosis when exposed to galectin-9 *in vitro* (Fig 4.5B-D).

As TIM-3 expression was also evident on about 10-15% of Foxp3$^+$CD4$^+$ T in lymphoid organs, we investigated if these cells are also susceptible to killing by galectin-9 ligation. Thus, DLN cells were isolated from either infected or uninfected GFP-Foxp3 knock-in animals and treated with various doses of galectin-9 *in vitro* for eight hours. As shown in fig 4.5B, while more than 90% of CD4$^+$TIM-3$^+$Foxp3$^-$ cells underwent apoptosis, less than 10% of CD4$^+$TIM-3$^+$Foxp3$^+$ T cells were annexin V$^+$. Furthermore, CD4$^+$TIM-3$^+$Foxp3$^+$ T cells showed minimal level of expression of annexin V on their surface as measured by the mean fluorescence

intensity. This could mean that as a consequence of galectin-9 treatment with Foxp3$^+$ cells showing more resistance to apoptosis, the ratio of Tregs: Teffector would increase perhaps accounting in part for the anti-inflammatory effect of galectin-9.

Expansion or induction of Foxp3$^+$ Tregs

Prior experiments have established that the severity of HSK lesions can be modulated by Foxp3$^+$ regulatory T cells either induced in vivo or by adoptive transfers of such cells (15, 17). There is some indirect evidence that galectin-9 could be involved in the induction of Foxp3$^+$ cells since such cells were reduced in numbers in galectin-9 knockout animals (12). To determine if galectin-9 administration to HSV infected animals had any influence on the Treg response, experiments were done in which animals were infected with HSV after which from day 4 to day 14 one group was injected systemically with a dose of galectin-9 shown to be effective at diminishing lesion severity and the other group received diluent. Experiments were terminated on day 15 and the cell types in individual DLN and spleen were collected to quantify CD4$^+$ T cells that were Foxp3$^+$. As shown in Fig. 4.5E and F, significant increases ($P \geq 0.05$) in the frequencies and numbers of CD4$^+$Foxp3$^+$ T cells were evident in the spleens of galectin-9 treated animals. Additionally the numbers of Foxp3$^+$ cells in the DLNs of galectin-9 treated animals were also increased, but the numbers compared to controls were not significant. This effect of galectin-9 treatment might be the consequence of expansion of preexisting Foxp3$^+$ cells or perhaps conversion of some Foxp3$^-$ cells to become Foxp3$^+$.

Evidence that galectin-9 could be causing some expansion of Foxp3$^+$ cells was shown by comparing the proliferative capacity in vitro of Foxp3$^+$ cells from control and galectin-9 treated animals. As shown in fig. 4.5G, CFSE dilution in Foxp3$^+$ cells TCR stimulated for 3 days, in the presence of IL-2 was greater in the cell population from galectin-9 treated animals than from

controls. Curiously, the effect of galectin-9 treatment on the response of Foxp3$^-$ cells was to diminish their proliferation capacity compared to that occurring in the same cell population from controls. These observations support the concept that the enrichment of Foxp3$^+$ cells in galectin-9 treated animals could in part be explained by expansion of previously Foxp3$^+$ cells.

Galectin-9 may promote Foxp3$^+$ Treg conversion and limit Th17 generation *in vitro*

To directly assess whether or not galectin-9 can cause some TCR stimulated conventional T cells to become Foxp3$^+$, an in vitro culture system was used which in previous studies demonstrated Foxp3$^+$ conversion (17). Briefly, splenocytes from DO11.10 RAG$^{-/-}$ animals (that lack Treg) were stimulated *in vitro* with plate bound anti-CD3 antibody, optimal amounts of IL-2 and various doses of galectin-9. TGF-β at optimal amounts was used instead of galectin-9 as a positive control system for Foxp3$^+$ Treg induction. In such experiments galectin-9 did cause significant numbers of cells to convert to become Foxp3$^+$ although the conversion was around four to five fold less effective than occurred with TGF-β (Fig. 4.6A and B). When experiments were done using suboptimal amounts of TGF-β, the addition of galectin-9 increased the conversion beyond the various doses of galectin-9 in the absence of TGF-β. This observation may mean that galectin-9 might be inducing TGF-β in the cultures which in turn could enhance the conversion efficiency although this needs to be formally confirmed.

In another approach splenocytes from DO11.10 RAG-/- animals, were cultured in conditions that resulted in the induction of Th17 cells. Basically such cultures were OVA$_{323-339}$ stimulated whole splenocytes in the presence of IL-2, TGF-β and IL-6. Curiously, the addition of galectin-9 in these cultures inhibited the induction of Th17 cells in a dose dependent manner (Fig 4.6C and D). It was of particular interest to note that the addition of galectin-9 in these cultures increased the production of Foxp3$^+$ cells up to four fold. The results could mean, however, that

the anti-inflammatory effects of galectin-9 will not be negated by the presence of proinflammatroy cytokines such as IL-6 in the inflamed tissue. We did not investigate the mechanism of Th17 suppression, but conceivably it could involve galectin-9 apoptosis signals delivered to TIM-3 expressed by developing Th17 cells. We are currently attempting to verify this.

Expansion of Myeloid suppressor cells

It was of interest to note that galectin-9 treated animals, as compared to control animals showed significantly expanded populations of $CD11b^{+}$ cells especially in the spleens (Fig. 4.7A and B). As shown in the fig 4.7C-E, a significant proportion of these cells also expressed $Gr1^{lo}$,$F4/80^{+}$ and $Ly6C^{hi}$ a phenotype possessed by myeloid suppressor cells which have been shown to inhibit the function of conventional $CD4^{+}$ and $CD8^{+}$ T cells (20, 21). Interestingly, the frequencies of cells of this phenotype also increased in when galectin-9 treatment was performed locally in the corneas of infected animals (Fig. 4.4B and C). Future studies will attempt to measure if the myeloid suppressor cells play any regulatory effect in HSK.

Taken together our results may indicate that a consequence of galectin-9 therapy is that there is an overall increase in the frequency of regulatory cells that include both $Foxp3^{+}$ Tregs and $CD11b^{+}Gr1^{lo}F4/80^{+}Ly6C^{hi}$ myeloid suppressor cells. Expanded population of $Foxp3^{+}$ Tregs may represent conversion of conventional cells to become Treg and perhaps reflect a disappearance of $CD4^{+}$ Th1 cells because many TIM-3^{+} cells are triggered to die by apoptosis.

Galectin-9 may also inhibit the production of angiogenic factors

As indicated above, galectin-9 treatment of HSV infected mice also led to a reduction in the extent of ocular neovascularization. Indeed there was a trend for the effect on this necessary step in HSK pathogenesis to be greater than was observed on SK lesion severity (Fig 4.3D). This

effect could be explained by the fact that some angiogenic factors derive from the inflammatory cells themselves and these responses were reduced in galectin-9 treated animals. Alternatively, galectin-9 might have some direct inhibitory effects on cells in the eye that produce angiogenesis factors in response to infection. To mimic the latter possibility, the effects of galectin-9 on the induced production of VEGF-A mRNA in stromal fibroblast cell line was measured. These cells can be induced to produce the angiogenic factor VEGF-A upon exposure to IL-6 or HSV infection (22). Experiments were performed in which MKT-1 cells were exposed to a high multiplicity of HSV-KOS (5MOI) which resulted in cells upregulating VEGF-A mRNA (Fig. 4.8). This increase which reached 1800 fold in some instance, was suppressed up to 100 fold by galectin-9. Similar doses of galectin-3 were almost without inhibitory effects. These results support the possibility that galectin-9 can function as an angiogenesis factor inhibitor although it must mediate its function by binding to a receptor distinct from TIM 3 since the latter molecule was not detectable on the MKT-1 cells (data not shown).

Discussion

Controlling chronic immunoinflammatory diseases represents a major therapeutic challenge. Such an example is lesions in the eye caused by HSV infection which commonly result in blindness. These lesions are strongly suspected to result from T cell mediated responses to the infection. Since the main T cell subset responsible for orchestrating lesions of stromal keratitis appear to be $CD4^+$ T cells of Th1 type with perhaps some involvement by Th17$CD4^+$ T cells, a logical approach to therapy would be to suppress or delete the function of activated $CD4^+$ T cell subsets and increase the representation of cells that express regulatory function. We show in this report that this outcome can be achieved by therapy systemic or local with the lectin

family member galectin-9. This molecule, which is natural product of cell types such as several cells of innate immune system, endothelial, epithelia cells etc., acts as a ligand to the inhibitory molecule TIM-3 that is expressed by activated but not naïve T cells. We show that 50% or more of T cells in ocular lesions caused by HSV in mice express TIM-3 and that blocking signals from its natural ligand with a monoclonal antibody results in more severe lesions. More importantly, however, the provision of additional galectin-9 either systemically or more effectively by local subconjuctival administration diminished the lesion severity of SK lesions as were the extent of corneal neovascularization. The mechanisms by which the galectin-9 therapy functioned were shown to be multiple. These involved apoptosis of the orchestrating effecter T cells with consequent reduction of proinflammatory cytokines, an increase in the representation of two separate subtypes of regulatory cells as well as inhibitory effect on the production of molecules involved in ocular neovascularization, an essential component of HSK pathogenesis. Our results indicate that galectin-9 therapy may represent a useful approach to control HSV induced lesions, the commonest infectious cause of blindness in the Western World.

A number of previous reports have shown that activated T cells, both $CD4^+$ and $CD8^+$ T cells (8, 13), may up regulate TIM-3 and that the engagement of the receptor with its ligand galectin-9 causes cells to undergo apoptosis (23). Since galectin-9 is a product of several cell types with its production increased upon exposure to some cytokines released from activated T cells, the TIM-3/galectin-9 interaction may represent a physiological means by which effector T cell responses are terminated (8, 11). Thus the severity of some autoimmune inflammatory responses may be more severe in galectin-9 knock out animals (12). Also of therapeutic relevance, it has been observed that administration of galectin-9 may suppress the severity of some autoimmunities even when given quite late in the disease process (12). Our studies too,

which we believe are the first to investigate the relevance of TIM-3/galectin-9 in a chronic viral induced inflammatory disease, galectin-9 administration caused the suppression of HSK lesions even when the drug was given 8 days after virus infection. At this stage, virus is usually no longer present in the eye although without treatment lesions continue to advance in severity and usually do not resolve spontaneously. In some instances we observed that galectin-9 therapy did cause almost complete resolution.

We presume that the efficacy of galectin-9 therapy resulted from causing apoptosis of the $CD4^+$ T cells responsible for orchestrating lesions. However, we failed to show any evidence of T cell apoptosis in vivo which may not be too surprising since apoptotic cells are rapidly phagocytosed in vivo (24, 25). We could readily show apoptosis of TIM-3^+ cells in populations of lymphoid cells taken from HSV infected animals when exposed to galectin-9 in vitro. However in such experiments, many TIM-3 positive cells resisted apoptosis even at highest doses of galectin-9 investigated. Curiously, in our in vitro studies we observed that TIM-3^+Foxp3^+CD4^+ T cells showed more resistance to galectin-9 induced apoptosis than did Foxp3^- CD4^+TIM-3^+ T cells. The molecular explanation for this interesting observation is currently under further investigation. However, if it turns out that Foxp3^+ regulatory T cells are more resistant to galectin-9 induced apoptosis in vivo, this might explain why the treatment resulted in suppressed SK lesions. Thus, as we have shown previously, the Treg response serves to modulate the severity of SK lesions (18). Differential susceptibility of T effectors to Treg would result in Treg enrichment. In line with this, we did observe that frequencies of Foxp3^+ T cells in lymphoid tissues as well as ocular inflammatory populations were increased in galectin-9 treated infected animals. That galectin-9 administration may result in the apparent induction of Foxp3^+

regulatory T cells was also reported recently by Seki et al although the mechanisms responsible for the effect were not investigated (12).

In our studies, we observed that galectin-9 could affect the Treg responses by mediating at least two effects in addition to the differential susceptibility to apoptosis. Accordingly, we could show that the proliferative capacity in vitro of TCR stimulated Foxp3 cells was greater in population taken from galectin-9 treated compared to control animals. However, perhaps of more importance we could also demonstrate that galectin-9 was able to induce conventional naïve TCR stimulated $CD4^+$ T cells to convert to become $Foxp3^+$. This was shown using TCR transgenic T cells TCR stimulated in vitro in the presence of galectin-9. Significant levels of conversion were observed, although this was less than that could be achieved with optimal doses of TGF-β. In fact our results could mean that the galectin-9 conversion effect involved the production of TGF-β, since when cells were stimulated in the presence of suboptimal levels of TGF-β, galectin-9 addition caused higher levels of conversion than could be achieved with galectin-9 or TGF-β alone. However, at present other possibilities can not be ruled out.

Whether or not galectin-9 administration succeeds in converting conventional cells to become $Foxp3^+$ and their expansion in vivo is currently under investigation. If it does occur, it might help explain why some parasitic infections appear to be potent inducers of Treg response, which in turn play an important role during pathogenesis (26, 27). Thus, many parasites express high levels of lectins that include galectin-9 in their surface components (27). Furthermore, galectin-9 is expressed abundantly in the gut mucosa (28) which is a site where the peripheral generation of regulatory T cells readily occurs (29-31). The presence of galectin-9 may help counteract an inflammatory environment that favors the induction of Th17 cells as we were able to demonstrate in our in vitro studies.

Whereas previous reports have noted that the TIM-3/galectin-9 interaction can have a notable effect on inflammatory cell function, any effect on angiogenesis was not noted. In our study, we observed that galectin-9 administration had an even greater effect on angiogenesis than it did on the severity of the SK lesions. The reasons for this may be that galectin-9 may be inhibiting the production of angiogenesis factors such as VEGF responsible for causing the new blood vessel development that plays an essential part of SK pathogenesis. Exactly how galectin-9 causes its inhibitory effects on angiogenesis factor induction still require further investigation. Of particular interest in our model systems, we demonstrated an inhibitory effect of galectin-9 on the induced production of VEGF from cells that lacked demonstrable TIM-3.

Taken together our results are consistent with the observation that the TIM-3/galectin-9 interaction plays a critical role at influencing the expression of HSV induced ocular lesions. It seems likely that the interaction affects lesion severity under normal circumstances since lesions become more severe if signals from endogenous galectin-9 are blocked with anti-TIM-3 mAb. Moreover, the interaction can be exploited for therapeutic purposes since treatment with galectin-9 can diminish lesions and can even result in their resolution. The mechanisms by which therapy succeeds are multiple and involve a change in balance between proinflammatory effector cells and regulators as well as effects on the production of angiogenesis factors responsible for causing neovascularization of the eye.

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APPENDIX

Figure 4. 1 Kinetic analysis of TIM-3 expression on $CD4^+$ T cells after ocular HSV infection.

C57B/6 mice were infected with $5x10^3$ of HSV. Three animals were sacrificed at each indicated time point and their spleens and draining cervical LN cells were analyzed for surface expression of CD4 and TIM-3 by flow cytometry. A. Histogram showing the percent of $CD4^+TIM\text{-}3^+$ T cells in spleen and DLN at indicated time points is shown. Data are shown from one representative experiment. B. Dot plot shows the expression of TIM-3 on $CD4^+CD44^{hi}$ T cells. C. The expression of TIM-3 on $CD4^+$Foxp3-GFP^+ and $CD4^+$Foxp3-GFP^- from uninfected and HSV infected animals at 8dpi. D. Absolute numbers of $TIM\text{-}3^+CD4^+$ T cells in spleen and DLNs at indicated time point are shown. The expression of TIM-3 on $CD4^+$ T cells isolated from inflamed pooled corneas and trigeminal ganglia at day 8 and day 15 post infection is shown. All kinetic experiments were repeated at least two times.

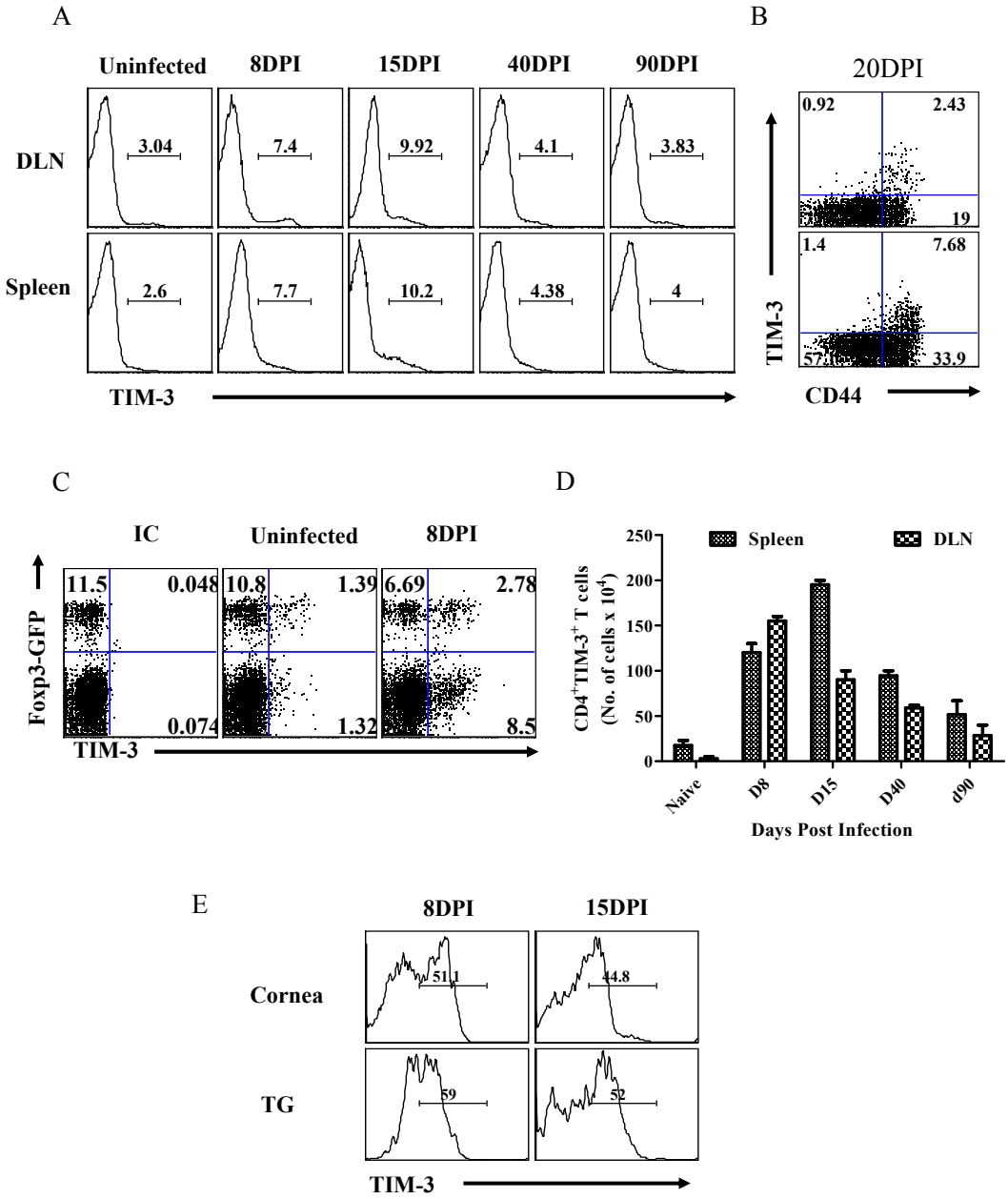

A
Uninfected
8DPI
15DPI
40DPI
90DPI
DLN
3.04
7.4
9.92
4.1
3.83
Spleen
2.6
7.7
10.2
4.38
4
TIM-3
B
20DPI
0.92
2.43
19
1.4
7.68
57.1
33.9
TIM-3
CD44
C
IC
Uninfected
8DPI
11.5
0.048
0.074
10.8
1.39
1.32
6.69
2.78
8.5
Foxp3-GFP
TIM-3
D
Spleen
DLN
CD4+TIM-3+ T cells (No. of cells x 10^4)
250
200
150
100
50
0
Naive
D8
D15
D40
d90
Days Post Infection
E
8DPI
15DPI
Cornea
51.1
44.8
TG
59
52
TIM-3

Figure 4. 2 Effect of TIM-3 blockade on corneal inflammation and $CD4^+$ T cell immune response.

C57B/6 animals (n=8) infected with $5x10^5$ PFU of HSV were given either anti-TIM-3 monoclonal antibody (n=4) or isotype control antibody (n=4) every alternate day starting from day 3 until day 13. The disease progression and immune parameters at day 14 were analyzed. A. The SK lesion severity and magnitude of angiogenesis is shown. B. Cumulative scores of lesion severity and angiogenesis at 14 days post infection. C. Percentages and phenotype (surface TIM-3) of $CD4^+$ T cells in the corneas of control (upper panel) and antibody treated (lower panel) animals is shown D. $CD11b^+Gr1^+$ PMN infiltrated into cornea of control (upper panel) and antibody treated (lower panel) animals is shown. E. HSV-specific $CD4^+$ T immune response in control and anti-TIM-3 antibody treated animals is shown at day 14. Dot plots depicting the percentages of IFN-γ^+ TNF-α^+ $CD4^+$ T cells is shown. F. Total number of HSV-specific $CD4^+$IFN-γ^+ and $CD4^+$IFN-γ^+TNF-α^+ T cells in spleen and DLN at day 14 is shown. The experiments were repeated three times. Student 't' test was used to calculate the level of significance.

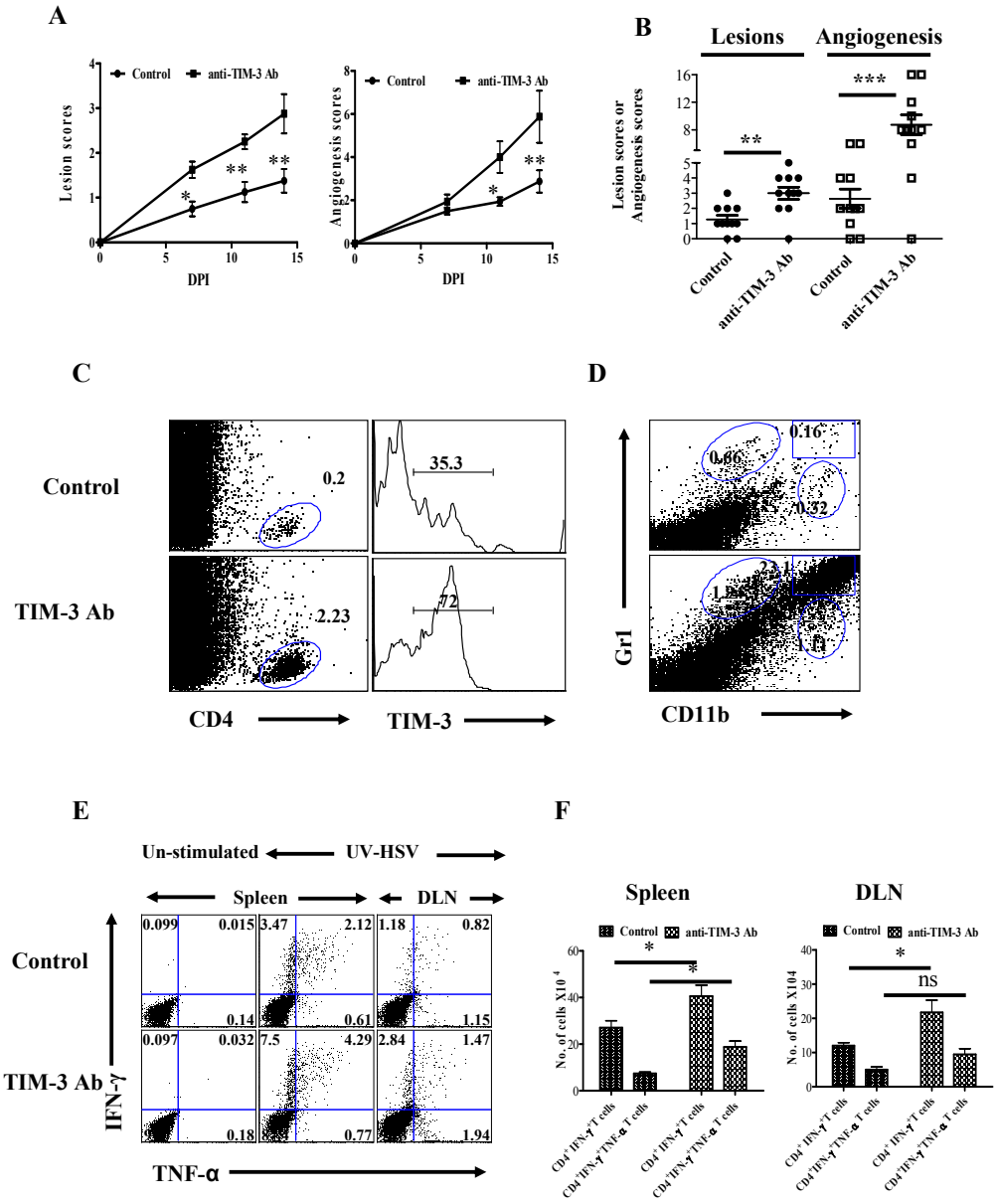
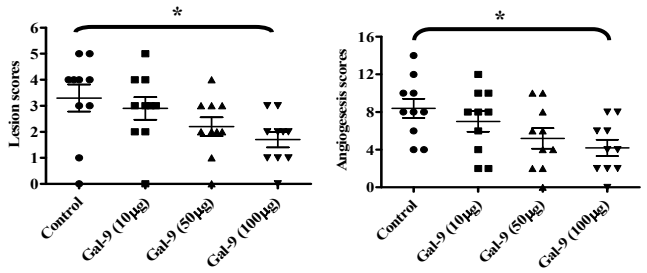

A
Control
anti-TIM-3 Ab
Lesion scores
DPI
Angiogenesis scores
B
Lesions
Angiogenesis
Lesion scores or Angiogenesis scores
Control
anti-TIM-3 Ab
C
Control
TIM-3 Ab
0.2
35.3
2.23
72
CD4
TIM-3
D
0.16
0.60
0.32
Gr1
CD11b
E
Un-stimulated
UV-HSV
Spleen
DLN
Control
TIM-3 Ab
IFN-γ
TNF-α
0.099
0.015
0.14
3.47
2.12
0.61
1.18
0.82
1.15
0.097
0.032
0.18
7.5
4.29
0.77
2.84
1.47
1.94
F
Spleen
DLN
Control
anti-TIM-3 Ab
No. of cells X10 4
No. of cells X104
ns
CD4+IFN-γ+T cells
CD4+IFN-γ+TNF-α+T cells

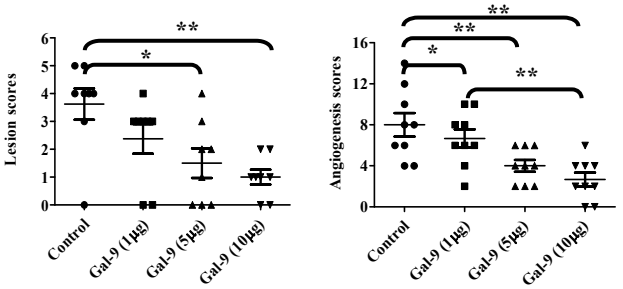
Figure 4. 3 Effect of galectin-9 treatment on the severity of SK.

C57B/6 animals (n=12) infected with $5x10^3$ PFU of HSV were treated with galectin-9 (10µg, 50µg and 100µg) daily starting from day 4 until day 14. A. Lesion and angiogenesis scores of control and galectin-9 treated animals at day 14 are shown. B. C57B/6 animals (n=9) infected with $5x10^3$ PFU of HSV were injected subconjuctivally with 1µg, 5µg or 10µg of galectin-9 daily starting from day 4 until day 13. Lesion and angiogenesis scores of control and treated animals at day 14 are shown. C. The comparative lesion scores of HSV infected animals at 14 dpi when treated with galectin-9 either 10µg sub-conjuctivally or 100µg i.p. is shown D. The comparative reduction in lesion severity and angiogenesis scores of HSV infected animals treated locally with 10µg of galectin-9 daily is shown. E. Therapeutic effect of galectin-9 (10µg) administration on SK in C57B/6 animals infected (n=8) with $5x10^3$ PFU of HSV. Kinetics of lesion and angiogenesis expression in control and galectin-9 treated animals (8-14 days pi) at 15 dpi is shown. All experiments were repeated at least three times. Data was analysed using one way ANOVA test with Dunnett's post settings except in E where Student 't' test was used.

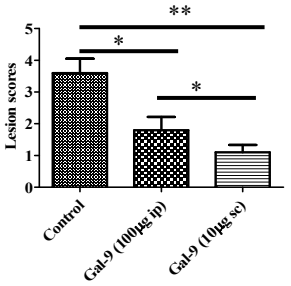
A


Lesion scores
Angiogesesis scores
Control
Gal-9 (10µg)
Gal-9 (50µg)
Gal-9 (100µg)

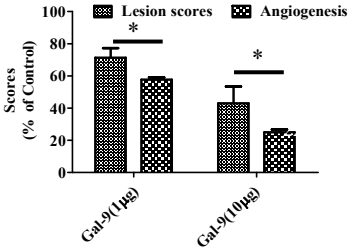
B


Lesion scores
Angiogenesis scores
Control
Gal-9 (1µg)
Gal-9 (5µg)
Gal-9 (10µg)

C


Lesion scores
Control
Gal-9 (100µg ip)
Gal-9 (10µg sc)

D


Lesion scores
Angiogenesis
Scores
(% of Control)
Gal-9(1µg)
Gal-9(10µg)

E

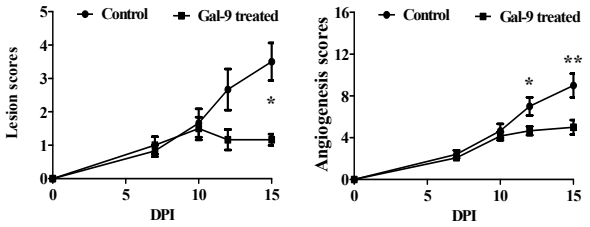

Control
Gal-9 treated
Lesion scores
Angiogenesis scores
DPI

Figure 4. 4 Effect of galectin-9 treatment on cellular infiltration and cytokine levels in corneas of HSV infected animals.

C57B/6 animals infected with $5x10^3$ PFU of HSV were given 10μg of galectin-9 subconjuctivally daily starting from day 4 until day 13 as described above in Fig 3B. A. Total numbers of $CD4^+$ and $CD4^+TIM\text{-}3^+$ T cells in the corneas of control and galectin-9 treated animals is shown at day 14. B-C. The frequencies and numbers of infiltrated PMNs ($CD11b^+Gr1^{hi}$) and a cell type akin to MSCs ($CD11b^+Gr1^{lo}$) is shown in control and galectin-9 treated animals. D. Levels of cytokines (IL-6, TGF-β and IL-12) and angiokine (VEGF-A) in pooled corneal samples each consisting of four cornea/group isolated from control and galectin-9 treated animals as analyzed by sandwich ELISA are shown. E. Levels of IL-6 and TGF-β in pooled corneal samples isolated from control and therapeutically treated HSV infected animals are shown. Experiments were repeated twice. Statistical levels of significance were estimated by Student's 't' test

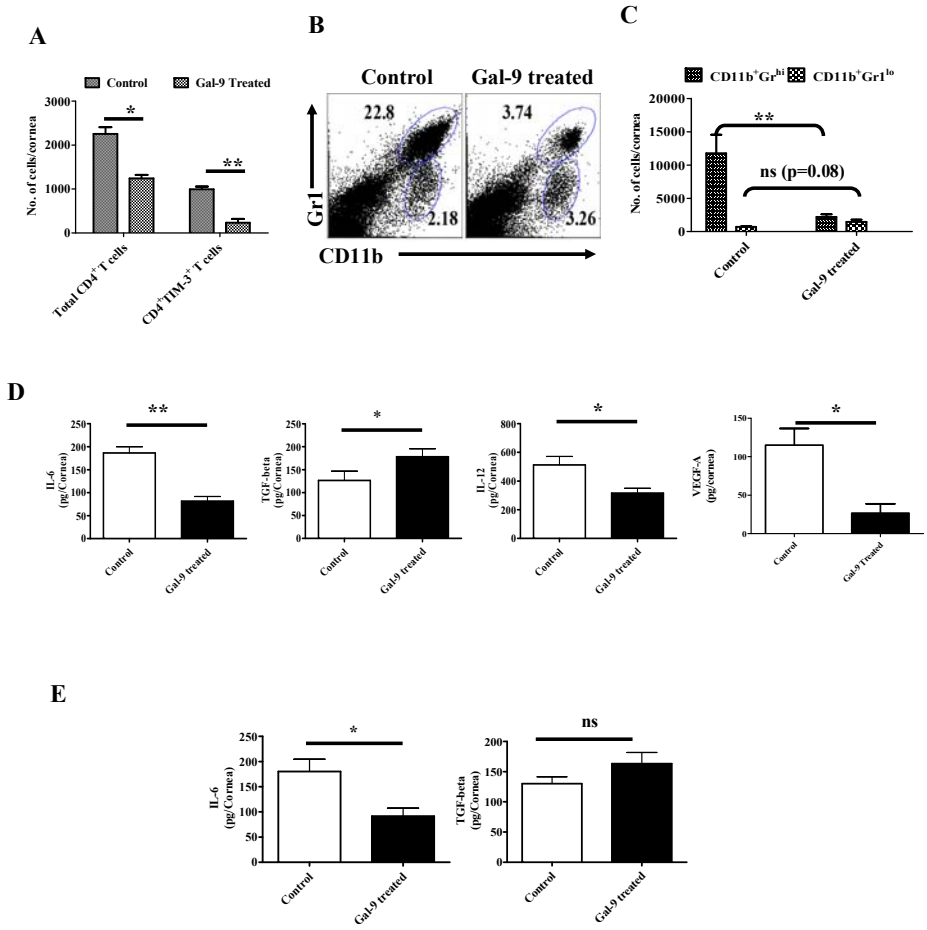

A
Control
Gal-9 Treated
No. of cells/cornea
Total CD4+ T cells
CD4+TIM-3+ T cells
B
Control
Gal-9 treated
22.8
2.18
3.74
3.26
Gr1
CD11b
C
CD11b+Gr1hi
CD11b+Gr1lo
No. of cells/cornea
ns (p=0.08)
Control
Gal-9 treated
D
IL-6 (pg/Cornea)
TGF-beta (pg/Cornea)
IL-12 (pg/Cornea)
VEGF-A (pg/cornea)
Control
Gal-9 treated
E
IL-6 (pg/Cornea)
TGF-beta (pg/Cornea)
ns
Control
Gal-9 treated

Figure 4. 5 Differential effect of galectin-9 therapy on regulatory T cells and effector $CD4^+$ T cells in HSV infected animals.

A. Total numbers of $CD4^+$ T cells and $CD4^+$IFN-γ^+ T cells in the DLNs and spleens of control and galectin-9 treated (systemic) animals in shown. B-D. Induction of apoptosis of $CD4^+$Foxp3$^-$ TIM-3^+ T cells by galectin-9. Cells isolated from cervical LNs of HSV infected Foxp3-GFP knock in animals (day 8) and uninfected animals were cultured in the presence of PBS, galectin-3 and galectin-9 for 8 hours and thereafter stained for annexin-V, B. Annexin-V^+CD4^+Foxp3$^-$ and Foxp3^+ T cells isolated from infected (upper panel) and uninfected (lower panel) animals incubated with PBS and galectin-9 animals is shown. C. The bar diagram showing the percentages of annexin-V^+ cells under indicated conditions is shown. D. Co-staining of $CD4^+$ T cells for annexin-V and TIM-3 is shown. E-G. Proliferative responses of Foxp3- and Foxp3^+CD4^+ T cells in HSV infected animals after galectin-9 therapy. Frequencies (E) and absolute numbers (F) of $CD4^+$Foxp3^+ T cells in the spleens (E and F) and DLNs (F) of control and galectin-9 treated animals are shown. G. DLN cells isolated from control and galectin-9 treated animals were labeled with CFSE and their proliferative response in the presence of anti-CD3 and anti-CD28 were analyzed after 3 days. $CD4^+$ gated population analyzed for the dilution of CFSE in Foxp3^+ and Foxp3^- $CD4^+$ T cells from control (thin line) and galectin-9 treated (thick line) animals is shown.

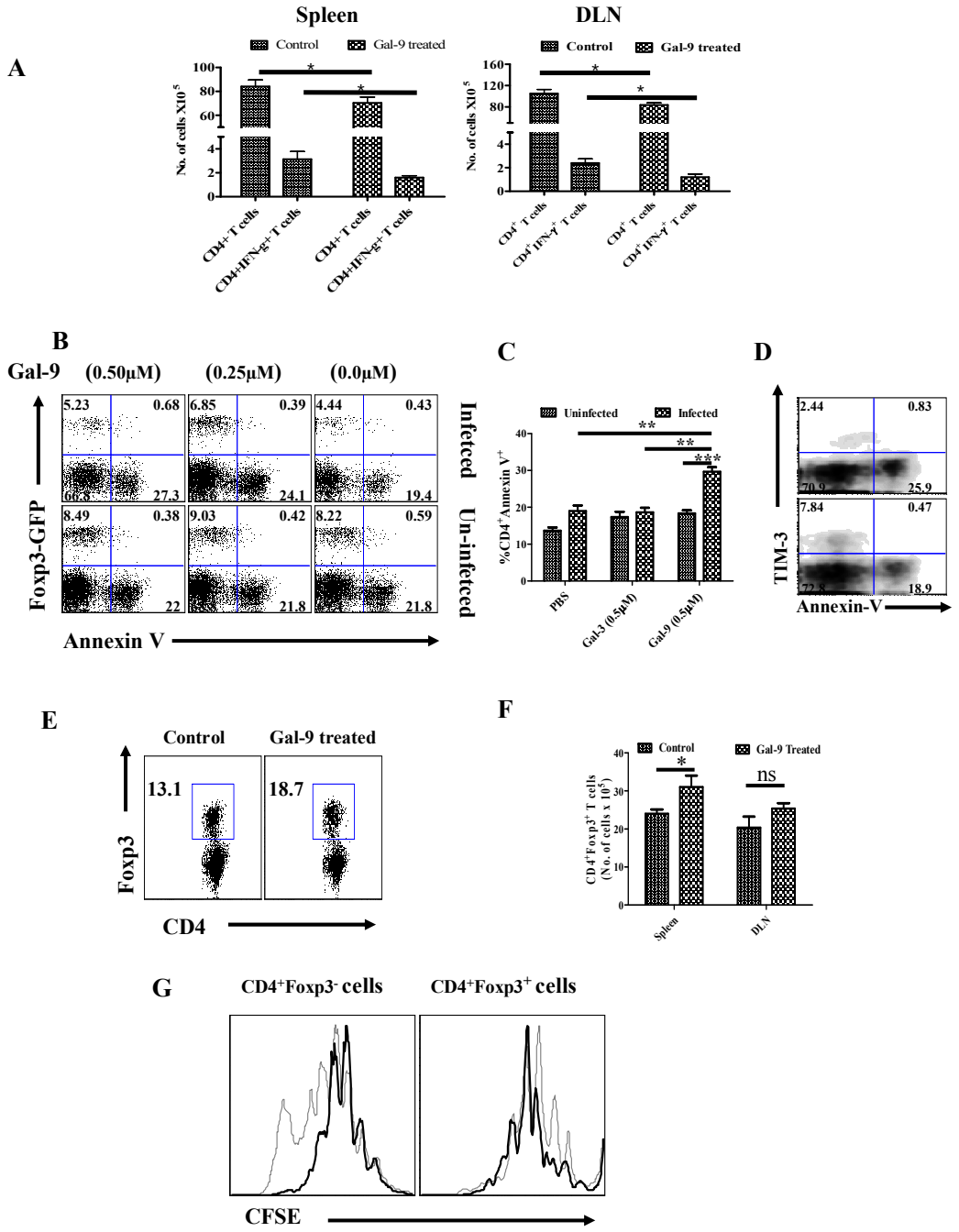
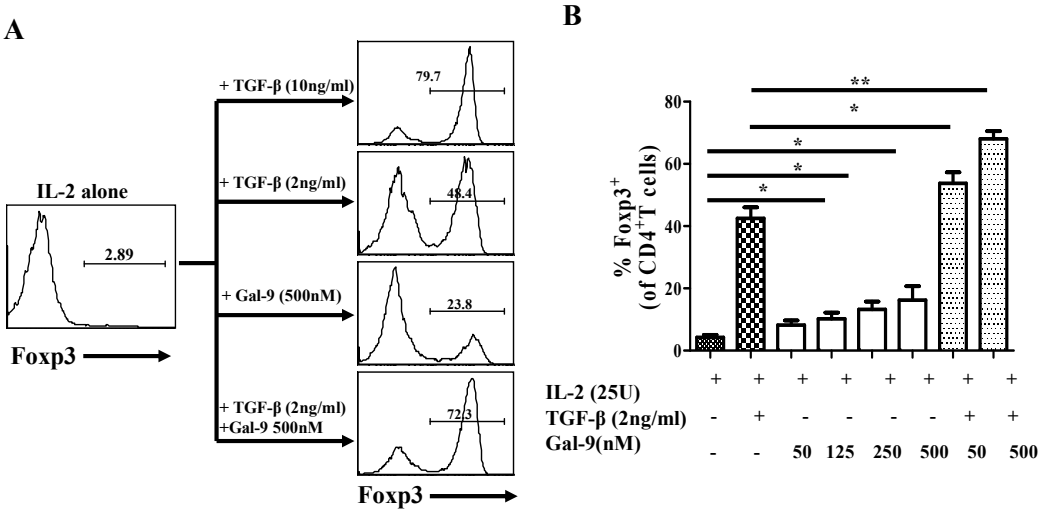
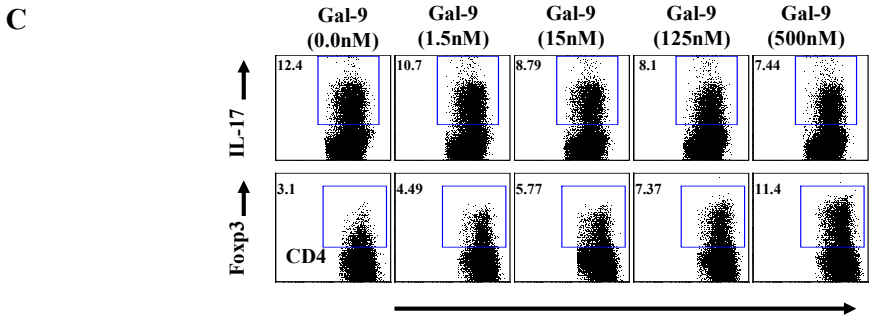

A
Spleen
DLN
Control
Gal-9 treated
No. of cells X10^5
CD4+ T cells
CD4+IFNg+ T cells
CD4+ IFN-γ+ T cells
B
Gal-9
(0.50µM)
(0.25µM)
(0.0µM)
Infeted
Un-infeted
Foxp3-GFP
Annexin V
C
Uninfected
Infected
%CD4+Annexin V+
PBS
Gal-3 (0.5µM)
Gal-9 (0.5µM)
D
TIM-3
Annexin-V
E
Control
Gal-9 treated
Foxp3
CD4
F
Control
Gal-9 Treated
ns
CD4+Foxp3+ T cells (No. of cells x 10^5)
Spleen
DLN
G
CD4+Foxp3- cells
CD4+Foxp3+ cells
CFSE

Figure 4. 6 Effects of galectin-9 treatment on the generation of $CD4^+Foxp3^+$ regulatory T cells and Th17 cells in vitro.

Splenocytes isolated from DO11.10RAG2-/- animals were cultured with IL-2 alone, IL-2+TGF-β 2ng/ml, IL-2+Galectin-9, IL-2+TGF-β+galectin-9, IL-2+TGF-β +IL-6 with or without galectin-9. A. Representative FACS plots from more than 6 experiments showing the Foxp3 induction in TCR stimulated $CD4^+$ T cells isolated from DO11.10RAG2-/- animals under indicated conditions. B. The bar diagram shows the percentage of $CD4^+$ T cells expressing Foxp3 under indicated conditions. The levels of significance was calculated by ANOVA test C. Cells were cultured under Th17 differentiating conditions (IL-2+TGF-β +IL-6) with or without galectin-9. The dot plots show the inhibitory effects on the generation of Th17 cells (upper panel) and the stimulatory effects on the generation of $Foxp3^+$ T cells (lower panel) of various doses of galectin-9 in in vitro cultures. D. The bar diagram shows the percentages of Th17 or $Foxp3^+$ Tregs induced under indicated conditions. The experiments were repeated at least four times and the level of significance was determined by Student 't' test.


A
IL-2 alone
2.89
Foxp3
+ TGF-β (10ng/ml)
79.7
+ TGF-β (2ng/ml)
48.4
+ Gal-9 (500nM)
23.8
+ TGF-β (2ng/ml)
+Gal-9 500nM
72.3
Foxp3
B
% Foxp3+
(of CD4+T cells)
IL-2 (25U)
TGF-β (2ng/ml)
Gal-9(nM)
50 125 250 500 50 500


C
Gal-9 (0.0nM)
Gal-9 (1.5nM)
Gal-9 (15nM)
Gal-9 (125nM)
Gal-9 (500nM)
IL-17
Foxp3
CD4
12.4
10.7
8.79
8.1
7.44
3.1
4.49
5.77
7.37
11.4

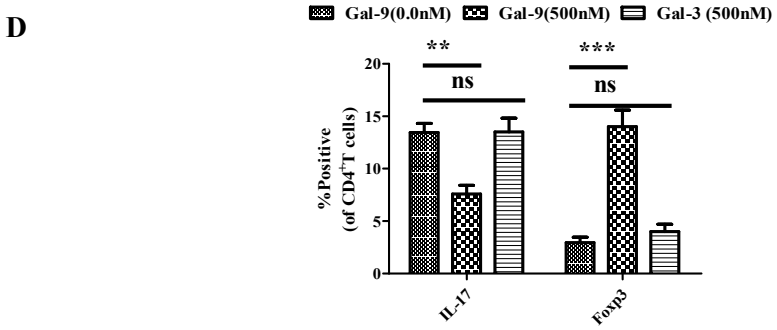

D
Gal-9(0.0nM)
Gal-9(500nM)
Gal-3 (500nM)
% Positive
(of CD4+T cells)
ns
ns
IL-17
Foxp3

Figure 4. 7 Effects of galectin-9 administration on $CD11b^+$ myeloid suppressor cells.

C57B/6 animals infected with $5x10^3$ PFU of HSV were treated i.p. with 100μg of galectin-9 daily starting from day 4 until day 14 as described in Fig 4.3A and $CD11b^+$ cells from control and galectin-9 treated animals were phenotypically characterized at day 15 post infection. A-B. FACS plots (A) and bar diagram (B) depicting the percentages of $CD11b^+$ cells in the spleens of control and galectin-9 treated animals at day 14 are shown. C-D. Phenotypic characterization of $CD11b^+$ cells from control and galectin-9 treated animals with respect to the expression of Gr1, F4/80, CD11c and Ly6C is shown. C. Bold lines in hitograms in D show the expression of the indicated marker on $CD11b^+Gr1^{lo}$ gated population while thin lines show the expression of indicated surface molecules on $CD11b^+Gr1^{hi}$ gated population from the spleens of galectin-9 treated animals. E. Total numbers of cells in gated $CD11b^+Gr1^{hi}$ and $CD11b^+Gr1^{lo}$ cells in the spleens of control and galectin-9 treated animals as shown in C. Student 't' test was used to calculate significance level between groups.

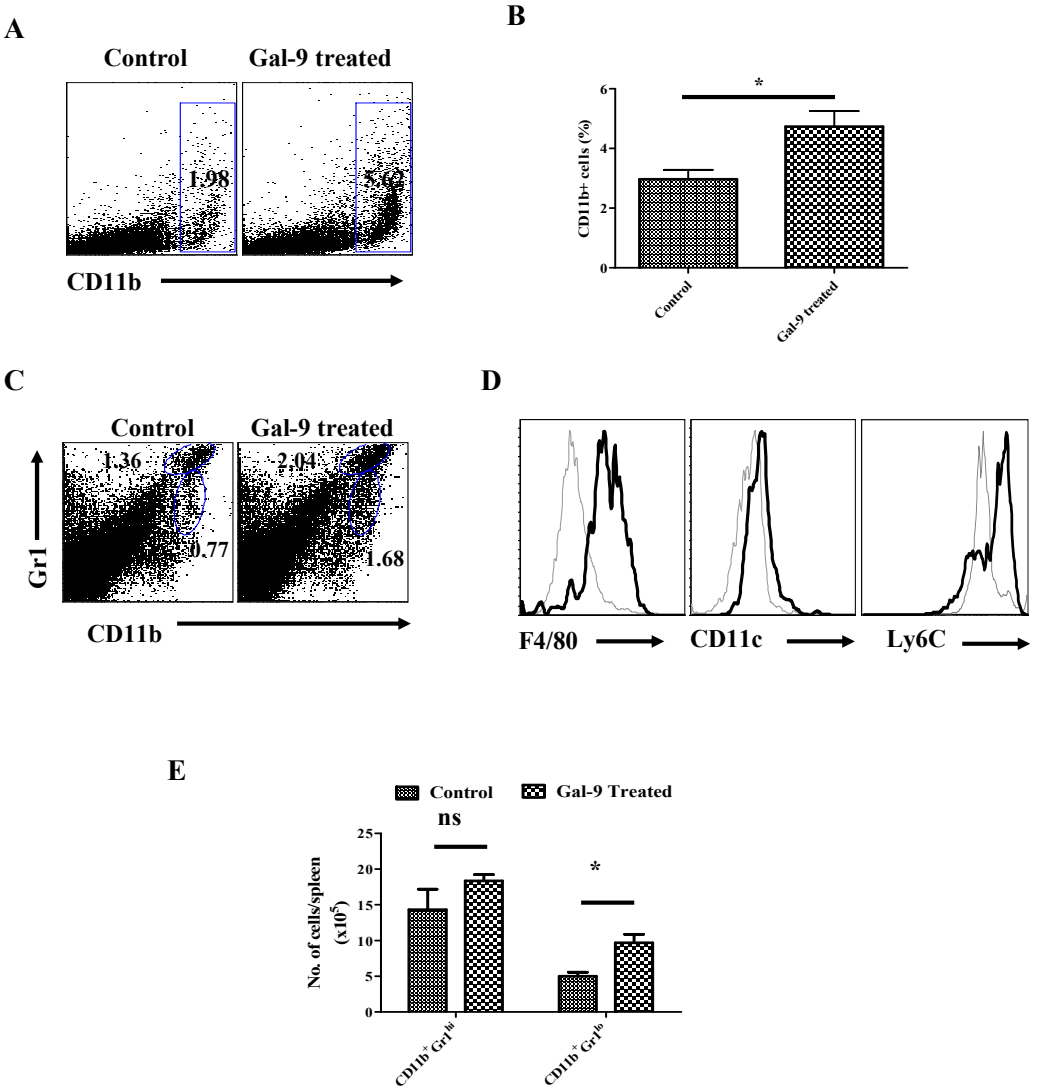
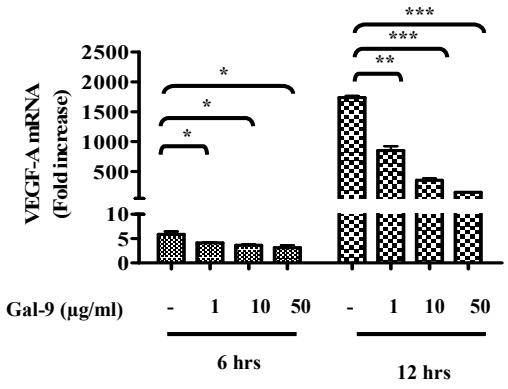

A
Control
Gal-9 treated
1.98
5.02
CD11b
B
CD11b+ cells (%)
*
Control
Gal-9 treated
C
Control
Gal-9 treated
1.36
2.04
0.77
1.68
Gr1
CD11b
D
F4/80
CD11c
Ly6C
E
Control
Gal-9 Treated
ns
*
No. of cells/spleen (x10^5)
CD11b+ Gr1hi
CD11b+ Gr1lo

Figure 4. 8 Effects of galectin-9 on VEGF-A production.

MKT (stromal fibroblast cell line) cells were used to observe the effect of galecetin-9 on VEGF production. The cells were infected with 5MOI of HSV KOS and were then treated with various doses of galectin-9. A VEGF-A mRNA expression was measured at 6 hours and 12 hours from control and galectin-9 (1μg, 10μg and 50μg/ml) treated cells as normalized with HPRT expression is shown. One was ANOVA was used to calculate the level of significance.

A


VEGF-A mRNA
(Fold increase)
2500
2000
1500
1000
500
10
5
0
*
*
*
***
***
**
Gal-9 (µg/ml)
-
1
10
50
-
1
10
50
6 hrs
12 hrs

VITA

Sharvan Sehrawat was born on January 05, 1978, in Haryana, India. After his primary education in a village school, he attended S.M. Hindu Sr. Secondary School, Sonepat (Haryana) and completed high school and seconday education from Board of School Edulation Haryana, Bhiwani, in 1993 and 1995 respecectively. He was then admitted to college of Veterinary Science, Chaudhary Charan Singh Haryana Agricultural University, Hisar in 1996 and received his Bachelor in Veterinary Science and Animal Husbandry (B.V. Sc. & A. H.) in 2001. He obtained his Master of Veterinary Science (M.V.Sc) in Immunology from the department of Veterinary Microbiology of the same institute in Feb 2004.

After graduation, he worked as a Senior Research Fellow until December 2004 in the department of Veterinary Parasitology, college of Vetrinary Science, CCS Haryana Agricultural University, Hisar. He worked as a Research Fellow in All India Institute of Medical Sciences, New Delhi from January 2005 through August 2005. In August 2005, he joined the University of Tennessee, Knoxville and received a Doctor of Philosophy (Ph.D) in Comparative and Experimental Medicine, from the department of Pathobiology, college of Veterinary Medicine in December 2008. He plans to pursue his post doctoral training.