ASBMT
American Society for Blood
and Marrow Transplantation

Immune Tolerance to Self-Major Histocompatability Complex Class II Antigens after Bone Marrow Transplantation: Role of Regulatory T Cells

Allan D. Hess and Christopher J. Thoburn

The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University, Baltimore, Maryland

Correspondence and reprint requests: Allan D. Hess, PhD, The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University, 1650 Orleans St, Baltimore, MD 21231 (e-mail: adhess@jhmi.edu).

ABSTRACT

The immune system undergoes rapid reconstitution after autologous or syngeneic bone marrow transplantation with the re-establishment of tolerance to self-antigens. Administration of drugs such as cyclosporine that inhibit thymic-dependent clonal deletion disrupts the reconstitution of the immune system. In the absence of a peripheral regulatory T cells eliminated by the preparative regimen, systemic autoimmunity with pathology similar to graft-versus-host disease often develops. Moreover, the resolution of autoaggression is dependent on the reconstitution of CD4$^+$ regulatory T cells. This study examined the specificity and function of this regulatory population assessed ex vivo that plays a critical role in down-regulating the autoreactive T lymphocyte response in cyclosporine-induced syngeneic graft-versus-host disease. The results suggest that both the antigen-specific regulatory and pathogenic effector T cells recognize a common peptide antigen framework (CLIP, a peptide derived from the invariant chain) presented by major histocompatibility complex class II molecules. Analysis of the CD4$^+$ T-cell compartment revealed two subsets of CLIP-reactive T cells that differentially require the N- and C-terminal flanking domain of this peptide. Regulatory function is associated with the cells that require the C-terminal flanking domain. This population expresses the Foxp3 nuclear transcription factor and plays a critical role in re-establishing tolerance to self-major histocompatibility complex class II antigens. In addition to suppressing the production of type 1 cytokines, these regulatory T cells can direct the apoptotic death of the pathogenic autoreactive lymphocytes. This study also suggests that the development of functional regulatory activity is an active response initiated by the presence of autoreactive lymphocytes that can present the target antigen (major histocompatibility complex class II CLIP) to the regulatory T cells. Moreover, this process can be mimicked by peptide antigen in the absence of the pathogenic effector lymphocytes leading to the development of functional regulatory T-cell activity.
© 2006 American Society for Blood and Marrow Transplantation

INTRODUCTION

Reconstitution of the immune system occurs quite rapidly after syngeneic or autologous bone marrow transplantation (BMT). In addition to the generation of a competent T-cell repertoire, immunologic tolerance to self-major histocompatibility complex (MHC) antigens is re-established. Disrupting thymic-dependent negative selection during immune reconstitution, however, can lead to the development of an MHC class II-dependent autoimmune syndrome with pathology remarkably similar to graft-versus-host dis-

ease (GVHD) [1-7]. Interestingly, administration of immunosuppressive drugs such as cyclosporine (CsA) that directly impair thymic-dependent clonal deletion can also elicit a systemic autoaggression syndrome. Termed "syngeneic GVHD," this autoimmune syndrome is primarily mediated by a highly restricted repertoire of autoreactive T cells that recognize the MHC class II invariant chain peptide (CLIP) complex [1-5].

The failure to delete autoreactive T cells because of the impairment of negative selection, by itself, is insufficient to allow for the development of syngeneic

GVHD. A peripheral immunoregulatory compartment must be inactivated or eliminated by the BMT preparative regimen to provide a permissive environment for the autoreactive cells to manifest autoimmunity [8-11]. Mature T cells in the periphery can prevent both the induction and the adoptive transfer of this autoaggression syndrome [8-11]. In naïve animals, effective suppression of syngeneic GVHD required both CD4$^+$ and CD8$^+$ T cells. Interestingly, the presence of autoreactive T cells induces dynamic changes in the regulatory compartment including the activation of CD4$^+$ T cells that are capable of suppressing the adoptive transfer of syngeneic GVHD [9-11]. Although antigen-specific CD8$^+$ T cells may have regulatory potential, the dominance of CD4$^+$ T cells may arise from their selection on and response to MHC class II antigens. Furthermore, CD4$^+$ regulatory T cells may also benefit from cytokines produced by mature T cells recognizing cognate antigen (CLIP). In addition, the resolution of syngeneic GVHD and re-establishment of active dominant tolerance are critically dependent on the reconstitution of this regulatory subset after cessation of CsA treatment [11,12].

This study examines the specificity and function of CLIP-reactive T-cell subsets within the CD4$^+$ T-cell compartment. Changes in the functional behavior within antigen-specific subpopulations over time appear to play a critical role in down-regulating the autoreactive T-cell response in syngeneic GVHD. The results also suggest that both the antigen-specific regulatory and pathogenic effector T cells recognize a common peptide antigen framework presented by MHC class II molecules. The regulatory T cells express the Foxp3 nuclear transcription factor and play a critical role in re-establishing tolerance to self-MHC class II antigens. In addition to suppressing the production of type 1 cytokines, some CLIP-reactive regulatory T cells can direct the apoptotic death of the pathogenic autoreactive lymphocytes. This study also suggests that an active response to the autoreactive lymphocytes promotes the development of functional regulatory T-cell activity. Moreover, this process can be mimicked by peptide antigen in the absence of the pathogenic effector lymphocytes leading to functional regulatory T-cell activity.

METHODS

Induction of Syngeneic GVHD

Syngeneic GVHD was induced in female Lewis rats 4 to 6 weeks of age (Charles River Breeding Laboratories, Wilmington, Mass) using the standard protocol as previously described [1-5]. After total-body irradiation (9.5 Gy) and reconstitution with syngeneic marrow, recipient animals were treated daily with 10 mg/kg CsA (dissolved in 0.02% ethanol and 5% emulphor in water) administered subcutaneously for 30 days. The onset of syngeneic GVHD occurred within 7 to 14 days after discontinuation of CsA treatment or the adoptive transfer of effector T cells as evidenced by the development of erythema, ruffled fur, and progressive weight loss. Syngeneic GVHD was confirmed by histologic analysis of target tissues [1,5,10].

Isolation of Effector and Regulatory T Cells

Spleens were harvested within 7 to 10 days after the initial onset of syngeneic GVHD (effector cells) and within 10 to 14 days after resolution of disease (regulatory cells). Splenic T cells were enriched by nylon wool fractionation. Purity assessed flow cytometric analysis revealed that greater than 95% of the cells expressed CD3. In addition, splenic T cells enriched by nylon wool fractionation were harvested form normal animals 2 weeks after an intravenous challenge with 60×10^6 irradiated (2000R) effector T cells harvested from animals with early onset (day 7-10) syngeneic GVHD [8-10]. Previous studies reveal that priming naïve animals with the syngeneic GVHD effector lymphocytes activates the regulatory population with the acquisition of in vitro regulatory function resistant to radiation whereas challenge with irradiated T cells from normal animals had no effect [10].

Normal Lewis rats were also immunized intradermally with antigen-presenting cells (APCs) (isolated by plastic differential adherence; 5×10^4 cells/site at 4 sites) pulsed with truncated variants of CLIP (see below) as previously described [13,14]. Splenic T cells were harvested 2 weeks after intradermal immunization with the peptide-loaded APCs. The spleens were pushed through a wire mesh screen to obtain a single cell suspension and the mononuclear cells isolated by ficol-hypaque density centrifugation [5,10]. The cells were further fractionated depleting specific subsets based on expression of the CD4, CD8, and CD25 cell surface markers with immunomagnetic beads as previously described [5,8,10]. Depletion rather than positive selection was used in the in vivo adoptive transfer studies to avoid the potential confounding effects of antibody to these markers on the cell surface because previous studies indicate that antibody to these markers on the cell surface inhibits their in vivo regulatory function [10]. Efficiency of depletion was confirmed flow cytometrically. On average, more than 95% of the cells expressing a specific cell surface determinant were removed from the splenic T-cell population after depletion.

Adoptive Transfer

Lethally irradiated (9.5 Gy) recipients were grafted with syngeneic bone marrow (40×10^6) together with the intravenous infusion of syngeneic GVHD effector

lymphocytes (30×10^6) and graded numbers (10,15, 30×10^6) of regulatory T cells as previously described [10]. An ex vivo/in vitro model was used to evaluate the specificity of the regulatory population as previously described [10]. The effector and regulatory T cells (irradiated 1250R) were incubated in vitro for 18 hours prior. Before this coculture period, the regulatory and effector T cells were pretreated with either antibodies to FasL (CD95 Ligand, Pharmingen, San Diego, Calif), MHC class I, MHC class II (Pharmingen), or CLIP [5] at concentrations of 1 µg/mL for 1 hour at 4°C and washed 3 times in RPMI 1640 before coculture. Controls included effector and regulatory cells pretreated with normal IgG. After the incubation period, the cells were extensively washed and adoptively transferred into the secondary recipients. The animals were examined daily (for 6 weeks) and assessed for the development of syngeneic GVHD as described above.

Isolation of MHC Class II CLIP-Specific T Cells

A soluble rat (Lewis) MHC class II mouse immunoglobulin fusion product (sMHC class II-Ig) loaded with peptide was used to isolate antigen-specific effector T cells as previously described [5,14]. The sMHC class II-Ig fusion product, purified by size exclusion on a Sephacryl 16/60 G-200 column (Pharmacia, Piscataway, NJ) was labeled with the fluorochrome Alexaflour 488 or 546 (Molecular Probes, Eugene, Ore) or biotinylated (counter staining with cychrome streptavidin) as previously described [5,14]. Briefly, splenic T cells ($1-5 \times 10^6$) were stained with conjugated sMHC class II-Ig (1 hour, 4°C) and washed 3 times in RPMI 1640. The cells were sorted on a Becton-Dickinson FACSVantage (San Jose, CA). Multicolor flow cytometry was used to isolate subsets of antigen-specific T cells based on expression of the CD4, CD8, and CD25 cell surface markers (phycoerythrin [PE]- or fluorescein isothiocyanate [FITC]-conjugated mouse antirat CD8, anti-CD4, and CD25 monoclonal antibodies, Serotec, Harlan Bioproducts for Science, Indianapolis, Ind). Specificity of the isolated T-cell subsets was confirmed as previously described with the CLIP-reactive T lymphocytes unable to kill target cells loaded with unrelated peptides (c-neu peptide, allopeptide, CLIP-inverted flanking domains) [5,14]. In addition, cells sorted based on reactivity to control peptides (ie, c-neu) did not recognize CLIP-loaded target cells.

CLIP Peptides and Loading

Truncated variants of rat CLIP containing just the MHC class II binding region or this region with either the N- or C-terminal flanking domains (sequences: C-terminal, MRMATPLLMRPLSMD, designated CLIP-C; N-terminal, KPVSPMRMATPLLMR, designated N-CLIP; MHC class II binding domain, designated BD-CLIP; MRMATPLLMR) were chemically synthesized and purified by high-pressure liquid chromatography (Quality Controlled Biochemicals, Hopkinton, Mass) [4,5,13,14]. Control peptides included MHC class II binding peptides from the c-neu oncogene, an allopeptide (from Brown Norway strain rats) or a CLIP variant with the flanking domains inverted as previously reported [4,5,13,14]. The peptides (>92% purity) were diluted to 10 µmol/L in RPMI 1640 before loading the soluble MHC class II-Ig construct. The sMHC class II-Ig construct was loaded in molar excess incubating 1 µg of the construct in 10 µmol/L of the peptides for 2 hours at 4°C. Unbound peptide was removed by size exclusion chromatography.

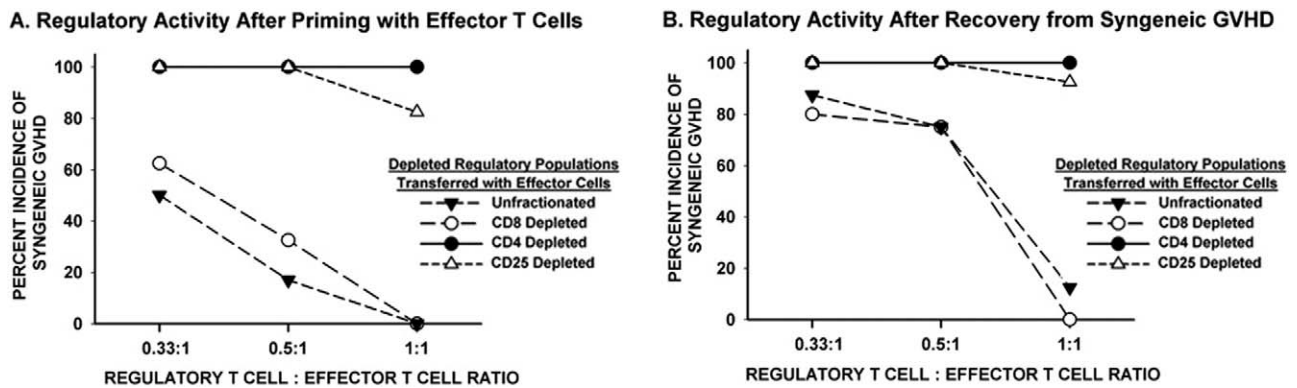### Quantitative Reverse Transcriptase Polymerase Chain Reaction

Quantitative reverse transcriptase polymerase chain reaction (PCR) (Taqman, Applied Biosystems, ABI, Foster City, Calif) was used to assess levels of cytokine and cell surface accessory molecule messenger RNA (mRNA) as previously described [4,14]. RNA was extracted and purified from the lysate of 1 to 5×10^5 cells with 500 µL Trizol (Invitrogen, Carlsbad, Calif) reagent [4,14] and complementary DNA prepared with Ready-to-Go You Prime First Strand (Pharmacia). The primers and probes for the cytokine genes (IL-2, IL-4, IFNγ, IL-10, and Foxp3) are commercially available from ABI [4,5,13,14]. Samples were analyzed by multiplexed real-time quantitative PCR [4,14]. Data were analyzed with software (Sequencer Detection, Version 1.6 ABI) with the results normalized against mRNA transcripts for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase [4,14]. For statistical comparison (t test), the fold increase or decrease in mRNA transcript levels of a select population relative to another population was calculated.

In Vitro Culture

Regulatory and effector T cells were suspended in complete tissue culture medium consisting of RPMI 1640 supplemented with glutamine and 5% heat-inactivated normal rat serum or fetal calf serum as previously described [3-5]. The cells (1×10^5) were cultured separately or in combination in flat bottom microtiter wells at 37°C and stimulated with peptide-loaded syngeneic APCs (1×10^4). After 48 hours of incubation, the cells were harvested and assessed for mRNA transcripts by quantitative PCR.

RESULTS

Initial studies assessed the phenotype of the regulatory T cells after an intravenous challenge (priming)

A. Regulatory Activity After Priming with Effector T Cells

B. Regulatory Activity After Recovery from Syngeneic GVHD

Figure 1. Suppression of adoptive transfer of syngeneic GVHD. Splenic T cells were harvested from animals after resolution of syngeneic GVHD or from normal animals after intravenous challenge with 60×10^6 effector T cells. Regulatory population was depleted of CD4$^+$, CD8$^+$, or CD25$^+$ cells with immunomagnetic beads. Graded numbers of regulatory cells were adoptively transferred into secondary recipients (n = 6-8) along with 30×10^6 effector T cells harvested from animals with acute syngeneic GVHD (7-10 days after initial onset). Recipients were examined daily for 6 weeks for development of syngeneic GVHD. Presence or absence of syngeneic GVHD was confirmed by biopsy.

of naïve animals with irradiated (2000R) syngeneic GVHD effector T lymphocytes (60×10^6). Regulatory function was assessed 14 days after priming. Graded numbers of splenic T cells (10, 15, 30×10^6) from the animals primed with the autoreactive lymphocytes were adoptively transferred with 30×10^6 effector spleen cells (harvested from animals 7-10 days after the initial development of syngeneic GVHD) into secondary, bone marrow–reconstituted syngeneic rats. As demonstrated in Figure 1A, unfractionated primed regulatory T cells effectively suppressed the adoptive transfer of syngeneic GVHD. Depletion of cells expressing either the CD4 or CD25 cell surface markers eliminated the ability to suppress the adoptive transfer of syngeneic GHVD. On the other hand, depletion of CD8$^+$ T cells failed to remove the regulatory activity. The results indicate that cells capable of regulating the activity of the syngeneic GVHD autoreactive lymphocytes reside within the CD4$^+$ T-cell subset after priming with the autoreactive lymphocytes. Similar results were obtained evaluating regulatory T-cell activity in the spleens of animals harvested within 10 to 14 days after resolution of syngeneic GVHD (Figure 1B). Previous studies indicate that CD4$^+$ T cells from naïve (unprimed) animals do not suppress the adoptive transfer of syngeneic GVHD unless accompanied by CD8$^+$ T lymphocytes and only at high (≥ 2) regulatory:effector cell ratios [8,10]. These data suggest that active recognition of the autoreactive lymphocytes or experimental challenge with the effector cells appears to be a necessary requirement to induce functional regulatory activity.

Regulatory T-cell function was also assessed in vitro. Primed regulatory T cells (1×10^5, irradiated 12.5 Gy) were cocultured with an equal number of syngeneic GVHD effector lymphocytes and stimulated with 1×10^4 syngeneic APCs loaded with CLIP. After 48 hours of culture, the cells were harvested and

assessed for cytokine transcripts by quantitative PCR. Production of IFNγ and IL-2 mRNA transcripts was significantly ($P < .01$) reduced (>20-fold) when the regulatory T cells were cocultured with the autoreactive lymphocytes compared with cultures that did not receive the regulatory population (Figure 2). The fold reduction of mRNA transcripts in the presence of the regulatory T cells in 3 separate experiments averaged 25.6 ± 3.8 (SD). Depletion of CD4$^+$ T cells or cells expressing the CD25 cell surface marker eliminated the regulatory T-cell activity. Identical results were obtained in two additional experiments demonstrating that depleting either CD4$^+$ or CD25$^+$ T cells eliminated regulatory function. Comparatively, addition of normal spleen cells did not significantly inhibit the in vitro cytokine response of the autoreactive lymphocytes (see below).

The specificity of the regulatory T cells was evaluated using an ex vivo/in vivo adoptive transfer model. This model is based on the findings that the in vitro activity of the primed regulatory T cells is radio resistant [10]. Irradiated regulatory lymphocytes can inactivate the effector T cells in vitro, although they cannot suppress the adoptive transfer of syngeneic GVHD when transferred separately. Primed regulatory cells (30×10^6; irradiated 1250R) were cocultured for 18 hours with an equal number of syngeneic GVHD effector lymphocytes before adoptive transfer into secondary recipients. As summarized in Table 1, effector T cells cocultured with the primed regulatory cells failed to adoptively transfer syngeneic GVHD. Treatment of the effector lymphocytes with antibodies to either MHC class II or to CLIP before coculture prevented their inactivation by the regulatory cells allowing the transfer of syngeneic GVHD into the secondary recipients. On the other hand, anti-MHC class I antibody treatment did not prevent the inactivation of the effector cells. Treatment of the regula-

Suppression of the *In Vitro* Cytokine Response of Autoreactive Effector Cells to CLIP
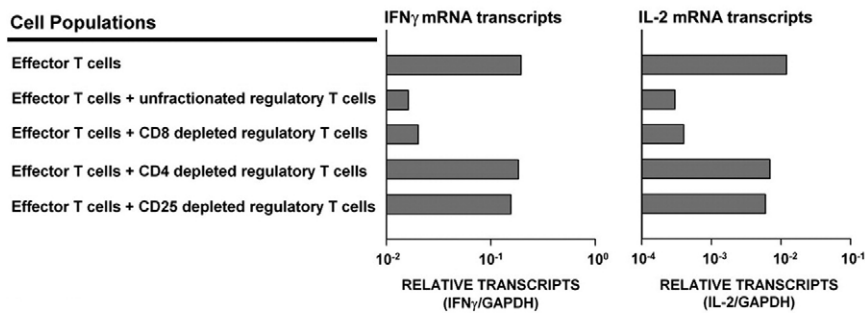
Figure 2. Suppression of in vitro cytokine response of autoreactive effector cells to CLIP. Splenic T cells were harvested from normal animals after intravenous challenge with 60×10^6 effector T cells. Regulatory population was depleted of $CD4^+$, $CD8^+$, or $CD25^+$ cells with immunomagnetic beads. Regulatory T cells (1×10^5) were irradiated (1250R) and incubated with equal number of autoreactive T cells harvested from animals with syngeneic GVHD. Cells were stimulated with syngeneic APCs (1×10^4) loaded with CLIP. After 48 hours of incubation, cells were harvested and assessed for levels of IL-2 and IFNγ mRNA transcripts by quantitative PCR. IFNγ and IL-2 mRNA transcripts were not detected in control cultures of irradiated regulatory cells cultured with peptide-loaded APCs. Data were standardized against mRNA transcript levels for housekeeping gene, GAPDH.

tory T cells with any of these antibodies did not inhibit their functional activity. Taken together, the results indicate that the regulatory T cells recognize the MHC class II CLIP complex expressed by the syngeneic GVHD effector lymphocytes. Moreover, the results of the depletion studies place these antigen-specific regulatory T cells within the CD4 and not the CD8 compartment. The depletion of regulatory activity by antibody to CD25 suggests that either these cells were recently activated in vivo or alternatively belong to the population that bears CD4 and CD25 on emerging from the thymus. Similarly, the possibility that effective regulation in vivo requires the synergistic activity of a $CD4^-CD8^-CD25^+$ subset cannot be excluded.

Previous studies evaluating the specificity of the autoreactive T cells in syngeneic GVHD revealed two functionally distinct subsets of CLIP-reactive T cells that have divergent cytokine profiles [4]. These two subpopulations can be separated based on their preferential requirement for the N- or C-terminal flanking domain of CLIP (designated N-CLIP and CLIP-C, respectively). Because anti-CLIP antibody could block the regulatory activity, it was important to determine whether N-CLIP- and CLIP-C-reactive lymphocytes had regulatory activity. A soluble MHC class II-Ig construct (loaded with truncated peptide variants of CLIP) was used to identify and isolate the subsets of CLIP-reactive T cells' flow cytometrically. In vitro studies were undertaken to evaluate the $CD4^+CD25^+$ T-cell subpopulation (a subpopulation that includes regulatory T cells) for N-CLIP- and CLIP-C-reactive T cells as previously described [4]. Analysis of spleen cells from normal animals revealed that approximately 4% of the total lymphocyte population consisted of $CD4^+ CD25^+$ T cells (Table 2). Although both sub-

Table 1. *Specificity of Primed Regulatory T Cells*

Coculture Before Adoptive Transfer		Incidence of Syngeneic GVHD
Effector Population	**Regulatory Population**	
Syngeneic GVHD effector T cells	**None**	**6/6**
Syngeneic GVHD effector T cells	**Unprimed regulatory cells (naïve)**	**6/6**
Syngeneic GVHD effector T cells	**Primed regulatory cells**	**0/6**
Syngeneic GVHD effector T cells + anti-MHC class I	**Primed regulatory cells**	**0/6**
Syngeneic GVHD effector T cells	**Primed regulatory cells + anti-MHC class I**	**0/6**
Syngeneic GVHD effector T cells + anti-MHC class II	**Primed regulatory cells**	**6/6**
Syngeneic GVHD effector T cells	**Primed regulatory cells + anti-MHC class II**	**0/6**
Syngeneic GVHD effector T cells + anti-CLIP	**Primed regulatory cells**	**6/6**
Syngeneic GVHD effector T cells	**Primed regulatory cells + anti-CLIP**	**0/6**

Syngeneic GVHD effector T cells (30×10^6) were cocultured with an equal number of primed $CD4^+$ regulatory lymphocytes (irradiated–1250R) for 18 hours at 37°C before adoptive transfer into secondary bone marrow reconstituted recipients. Before coculture, the effector and regulatory cells were separately treated with antibodies (1μg/mL) to MHC class I, MHC class II, or CLIP for 1 hour at 4°C. Treatment of the effector and regulatory T cells with normal IgG did not inhibit their functional activity. The cells were washed 3 times to remove excess antibody. Irradiated regulatory T cells are active *in vitro* but when transferred separately fail to suppress the adoptive transfer of syngeneic GVHD.

Table 2. *CLIP-specific T Cells in the CD4⁺CD25⁺ Lymphocyte Subset*

Subset	Percent CD4⁺CD25⁺ T Cells	Percent Total Lymphocytes
Normal Lewis rats		
CD4⁺CD25⁺		3.9
CLIP-C⁺	3.2	0.12
N-CLIP⁺	0.5	0.02
Active syngeneic GVHD		
CD4⁺CD25⁺		7.9
CLIP-C⁺	1.1	0.09
N-CLIP⁺	6.3	0.49
After resolution of disease		
CD4⁺CD25⁺		4.2
CLIP-C⁺	3.6	0.15
N-CLIP⁺	0.3	0.01

Splenic lymphocytes were harvested from normal animals and from animals with active syngeneic GVHD (day 7 after onset) and within 10 days after resolution of active disease. CLIP-C⁻ and N-CLIP⁻ reactive T cells in the CD4⁺CD25⁺ subset (determined flow cytometrically by staining with FITC-anti CD4 and PE-anti CD25) were identified by staining with the biotinylated soluble MHC class II-Ig loaded with the truncated variants of CLIP and counterstaining with streptavidin-cychrome.

sets were detected within this compartment, the majority of cells were CLIP-C reactive. In animals with syngeneic GVHD, the percent of CD4⁺ CD25⁺ T cells was modestly increased (approximately 2-fold). In addition, there were changes in the relative proportion of the CLIP-reactive T-cell subsets. During active disease, the majority of the cells were N-CLIP reactive. This increased percentage of CD4⁺ T cells expressing CD25 in animals with syngeneic GVHD may reflect the activation of autoreactive effector T cells rather than the expansion of the regulatory T-cell subset. Interestingly, the percentages of CD4⁺CD25⁺ T cells and the relative proportions of N-CLIP- and CLIP-C-reactive lymphocytes returned to levels detected in normal animals after disease resolution. Ex vivo assessment of their functional behavior, however, revealed striking differences between the N-CLIP- and CLIP-C-reactive CD4⁺CD25⁺ T-cell subsets.

The N-CLIP- and CLIP-C-reactive CD4⁺CD25⁺ T-cell subsets were assessed ex vivo for levels of type 1 and 2 cytokine mRNA transcripts by quantitative PCR and a representative experiment (1 of 4) is illustrated in Figure 3. Interestingly, IFNγ mRNA transcript levels were substantially elevated (>1000-fold increase on average; $P < .001$, n = 4) in both the N-CLIP- and CLIP-C-reactive CD4⁺CD25⁺ subsets isolated from animals with syngeneic GVHD compared with these two subsets isolated from normal animals. High levels of IL-2 mRNA transcripts were also detected in these subsets. Comparatively, IL-2 transcripts were not detected in the CD4⁺CD25⁺ N-CLIP⁺-reactive T cells whereas only low levels of transcripts for this cytokine were expressed by the CD4⁺CD25⁺ CLIP-C⁺-reactive cells isolated from

normal animals. On the other hand, elevated levels of type 2 cytokine (IL-4, IL-10) mRNA transcripts were also detected in the N-CLIP- and CLIP-C-reactive T-cell subsets harvested from animals with active GVHD. Although levels of IL-4 and IL-10 mRNA transcript levels were modestly increased (5- to 10-fold) in N-CLIP-reactive T cells harvested from animals with syngeneic GVHD, there was nearly a 100-fold increase in IL-4 mRNA transcripts in the CD4⁺CD25⁺ CLIP-C-reactive T-cell subset. The up-regulation of type 1 cytokine mRNA transcripts during syngeneic GVHD probably reflects the activation of the autoreactive effector lymphocytes because antigen stimulation induces CD25 expression in T cells. On the other hand, the up-regulation of IL-4 mRNA transcript expression in the CLIP-C-reactive subpopulation may reflect the activation of an autoreactive T cell that allows for progression of disease [14]. Analysis of the CLIP-reactive CD4⁺CD25⁺ subsets after resolution of syngeneic GVHD, however, revealed a marked increase (approximately 100- to 150-fold) in levels of IL-10 mRNA transcripts within the CLIP-C-reactive subpopulation. Levels of IL-10 mRNA were found to be consistently elevated (mean ± SD; 117 ± 9, 163 ± 21) in this population harvested from animals that recover from syngeneic GVHD compared with the levels detected in this subset harvested from normal animals or from animals with active disease, respectively. In contrast, levels of mRNA transcripts for IL-2, IL-4, and IFNγ were similar to the levels detected in the CLIP-specific subsets isolated from normal animals.

The N-CLIP- and CLIP-C-reactive CD4⁺CD25⁺ T cells were also evaluated for Foxp3 mRNA transcript levels, a nuclear transcription factor commonly expressed in regulatory T cells [15,16]. A representative experiment illustrated in Figure 4 reveals that Foxp3 mRNA transcripts were predominantly expressed in the CLIP-C-reactive CD4⁺CD25⁺ T-cell subset isolated from naïve animals. Levels of Foxp3 mRNA transcript levels were markedly reduced (approximately 20-fold) in CLIP-C-reactive CD4⁺CD25⁺ T cells isolated from animals with syngeneic GVHD. In contrast, the up-regulation of Foxp3 gene expression was concordant with the resolution of syngeneic GVHD. Levels of Foxp3 mRNA transcripts were markedly increased in the CLIP-C-reactive CD4⁺CD25⁺ T-cell subset. Levels of Foxp3 mRNA transcripts were found to be consistently elevated in this subset of CLIP-reactive CD4⁺CD25⁺ T cells isolated from animals that recover from syngeneic GVHD compared with levels detected in this subset of cells isolated from normal animals and animals with syngeneic GVHD (mean fold increases of 62 ± 8.6 and 104 ± 12.7, respectively; $P < .01$). Interestingly, Foxp3 transcript levels in the N-CLIP-reactive CD4⁺CD25⁺ T-cell subset were much lower (approximately 30-fold)

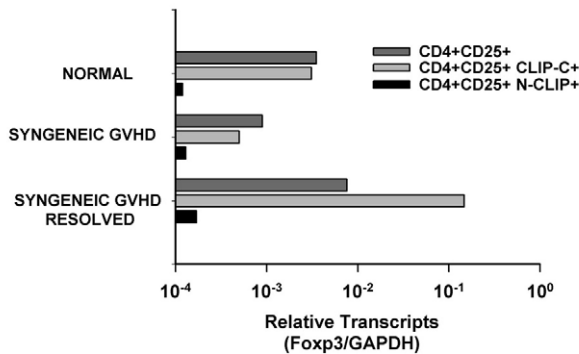## *Ex Vivo* Analysis of Cytokine mRNA Transcripts in CLIP Reactive CD4+CD25+ T Cell Subsets
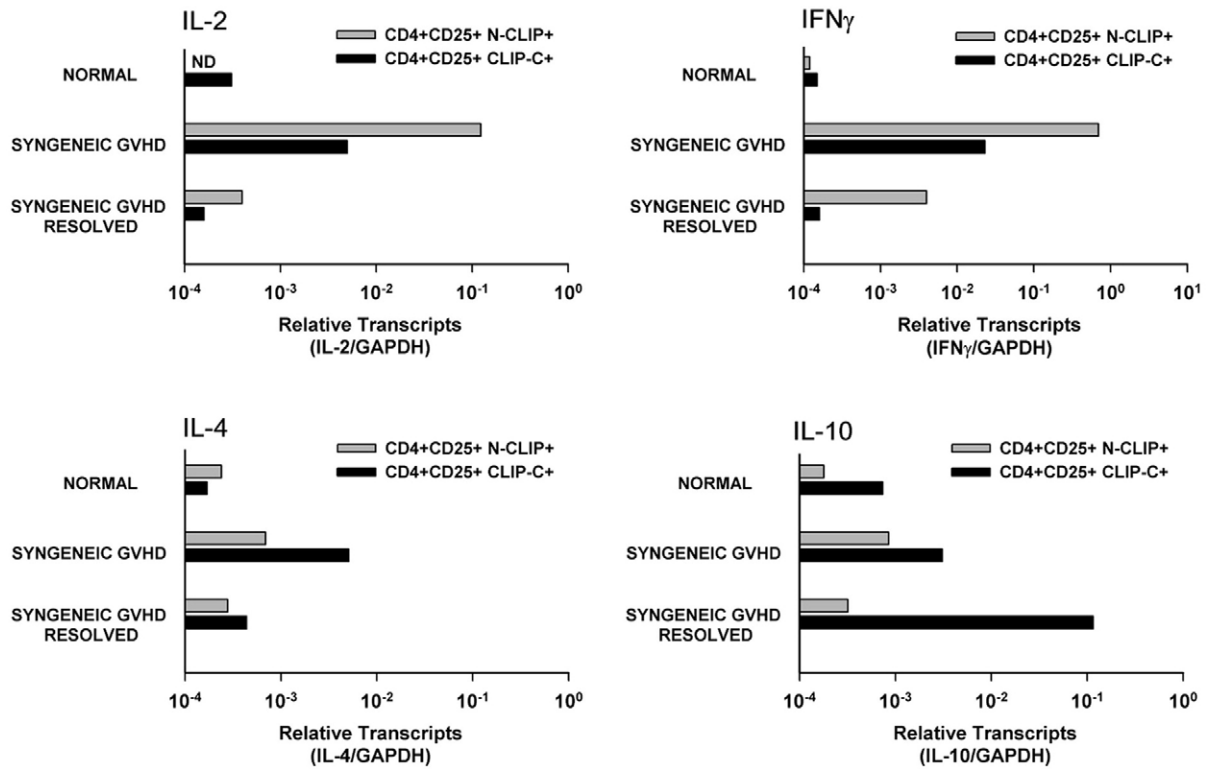
Figure 3. Ex vivo analysis of cytokine mRNA transcripts in CLIP-reactive CD4$^+$CD25$^+$ T-cell subsets. N-CLIP- and CLIP-C-reactive CD4$^+$CD25$^+$ T cells were isolated flow cytometrically from spleens of normal animals, animals with syngeneic GVHD, and animals after resolution of disease. Cells were assessed for cytokine (IL-2, IL-4, IL-10, and IFNγ) mRNA transcripts by quantitative PCR. Data were standardized against mRNA transcript levels for housekeeping gene, GAPDH. ND = Not detected.

overall with only minimal changes detected over the course of the disease.

Additional studies assessed whether the CLIP-reactive CD4$^+$ T-cell subsets had functional regulatory activity. N-CLIP- and CLIP-C-reactive T cells iso-


Figure 4. Foxp3 Expression in CLIP-reactive CD4$^+$CD25$^+$ T-cell subsets. N-CLIP- and CLIP-C-reactive CD4$^+$CD25$^+$ T cells were isolated flow cytometrically from normal animals, animals with syngeneic GVHD, and animals after resolution of disease. Foxp3 mRNA transcript levels were assessed by quantitative PCR. Data were standardized against GAPD mRNA transcript levels.

lated ex vivo from animals after resolution of syngeneic GVHD were assessed for their ability to suppress the in vitro response of the autoreactive effector T cells. Production of mRNA transcripts for both IFNγ and IL-2 in response to stimulation with the target antigen (CLIP presented by syngeneic APCs) was suppressed when CLIP-C-reactive T cells were cocultured with the syngeneic GVHD effector T cells (Figure 5). Two additional experiments yielded remarkably similar results. On average (n = 3), there was more than a 40-fold reduction in the production of type 1 cytokine mRNA transcripts when the CLIP-C-reactive subset was cocultured with the autoreactive T effector T cells. Depletion of CD4$^+$ T cells eliminated the regulatory activity from the CLIP-C-reactive subpopulation. On the other hand, the in vitro response of the autoreactive effector T cells was marginally altered by any of the N-CLIP-reactive T-cell subsets. Regulatory activity was primarily contained within the CLIP-C-reactive CD4$^+$ T-cell subset.

Previous studies suggest that the development of functional regulatory activity in normal animals is primarily initiated by a response to the autoreactive T cells [10]. Studies were undertaken to determine

**Regulation of the *In Vitro* Cytokine Response
by N-CLIP and CLIP-C Reactive T Cell Subsets**

Regulatory Cells Added

**Response of Effector T Cells Stimulated
with CLIP-loaded APC's**
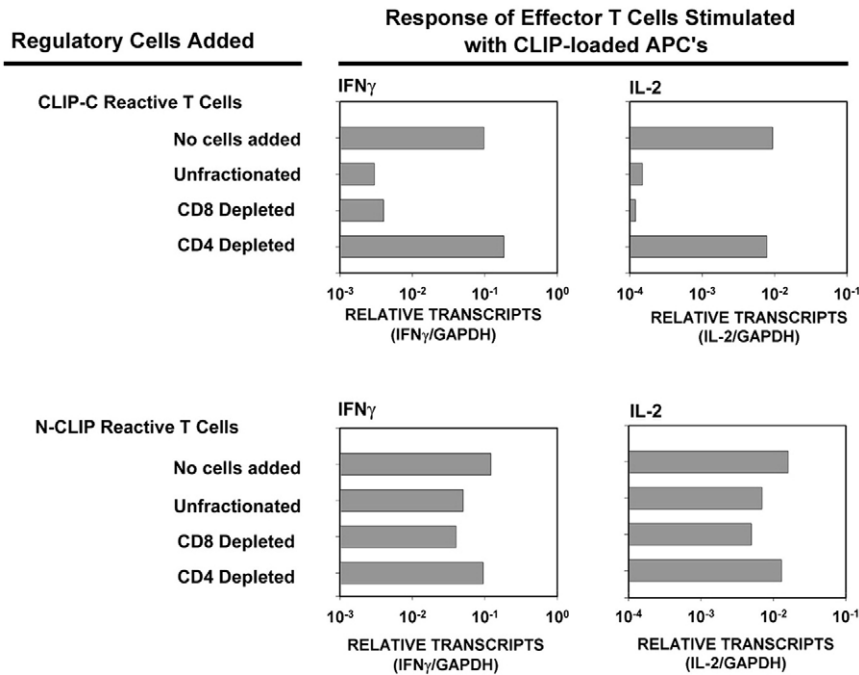
Figure 5. Regulation of in vitro tesponse by N-CLIP- and CLIP-C-reactive T-cell subsets. N-CLIP- and CLIP-C-reactive T cells were isolated flow cytometrically from animals after resolution of syngeneic GVHD. In addition, CLIP-reactive T-cell subsets were depleted of cells expressing CD4, CD8, or CD25 with immunomagnetic beads. Cells (1×10^5) were extensively washed and assessed for their ability to suppress in vitro response of syngeneic GVHD effector lymphocytes (1×10^5) stimulated with CLIP presented by syngeneic APCs (1×10^4). After 48 hours of culture, cells were harvested assessed for levels of IL-2 and IFNγ mRNA transcripts by quantitative PCR. IFNγ and IL-2 mRNA transcripts were not detected in control cultures of irradiated regulatory cells and peptide-loaded APCs. Data were standardized against mRNA transcript levels for housekeeping gene, GAPDH.

whether in vivo functional regulatory population could be activated by experimental challenge with the CLIP-C peptide and a representative experiment (1 of 3) is illustrated in Figure 6. Normal Lewis rats were immunized intradermally with APCs (4 sites, 5×10^4 cells/site)

loaded with the CLIP-C peptide. Fourteen days later, the spleen cells were harvested and assessed for their ability to suppress the response of the syngeneic GVHD effector T cells. Splenic lymphocytes from animals immunized with the CLIP-C peptide inhib-

Regulatory Activity Following Peptide Immunization

Populations Co-Cultured
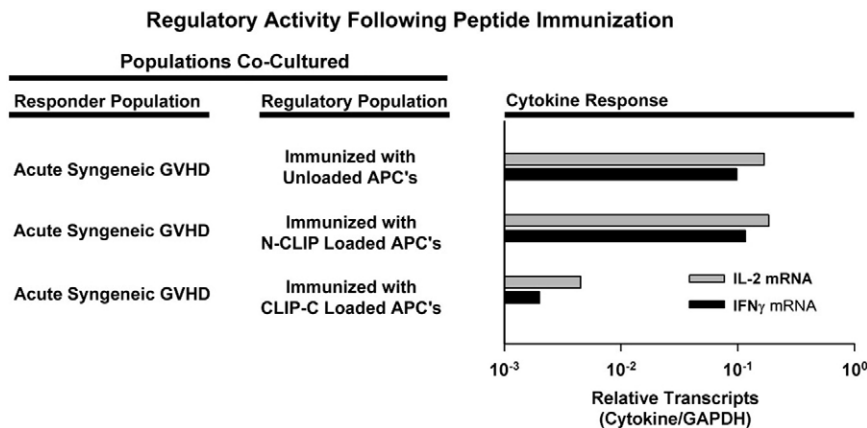
Figure 6. Regulatory activity after peptide immunization. Normal Lewis rats were immunized intradermally with syngeneic APCs loaded with either N-CLIP or CLIP-C. Fourteen days later, splenic T cells (1×10^5) were harvested and cocultured with equal number of syngeneic GVHD effector T cells. Spleen cells were stimulated with syngeneic APCs (1×10^4) loaded with CLIP. After 48 hours of culture, cells were harvested assessed for levels of IL-2 and IFNγ mRNA transcripts by quantitative PCR. Data were standardized against mRNA transcript levels for housekeeping gene, GAPDH.

ited the in vitro response of the autoreactive lymphocytes to stimulation with the autoantigen (MHC class II CLIP presented by APCs). Levels of mRNA transcripts for both IL-2 and IFNγ were markedly reduced in the presence of the regulatory cells. There was a 35 ± 6.3- and a 83 ± 9.4-fold reduction in the levels of mRNA transcripts for IL-2 and IFNγ when the spleen cells from the animals immunized with the CLIP-C peptide were cocultured with the autoreactive effector cells (n = 3; $P < .05$, $P < .01$, respectively). Interestingly, Foxp3 mRNA transcript expression in CD4+CD25+ T cells was increased 30- to 50-fold after immunization with the CLIP-C peptide whereas stimulation with N-CLIP was ineffective (data not shown).

Subsequent studies further evaluated the specificity of the response elicited by immunization with the N-CLIP and CLIP-C peptides. As summarized in Figure 7, CD4+CD25+ T cells from CLIP-C-immunized animals preferentially responded to this peptide variant with increased levels of mRNA transcripts for both type 2 cytokines (particularly IL-10) and Foxp3. On the other hand, levels of mRNA transcripts for type 1 cytokines were either modestly reduced or remained unchanged. Comparatively, CD4+CD25+
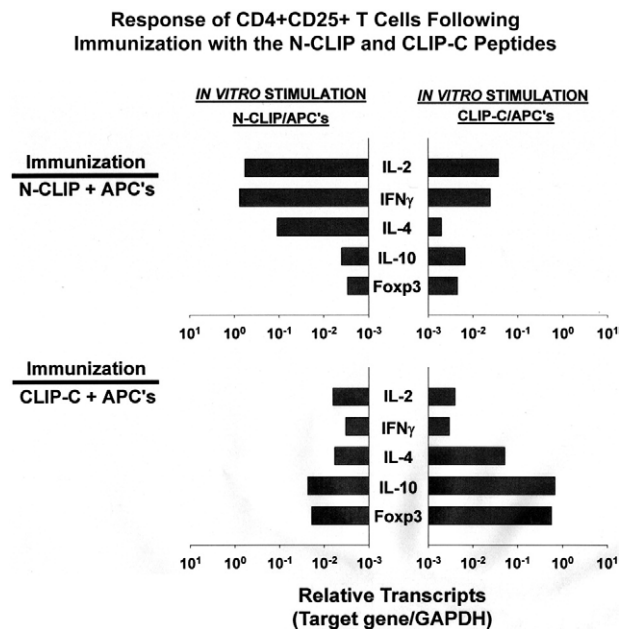
Figure 7. Response of CD4+CD25+ T cells after immunization with N-CLIP and CLIP-C peptides. Normal Lewis rats were immunized intradermally with N-CLIP and CLIP-C peptides presented by syngeneic APCs. Fourteen days later, CD4+CD25+ T cells (1 × 10^5) were stimulated in vitro with syngeneic APCs (1 × 10^4) loaded with either N-CLIP or CLIP-C peptide variant. After 48 hours of culture, the cells were harvested assessed for cytokine and Foxp3 mRNA transcript levels by quantitative PCR. Data were standardized against mRNA transcript levels for housekeeping gene, GAPDH. These results are representative of 3 separate experiments.

T cells from N-CLIP-immunized animals preferentially responded to the N-CLIP variant with heightened production of mRNA transcripts for type 1 cytokines.

Because CD4+ T cell–dependent killing is often caused by the induction of apoptosis, the ex vivo/in vivo adoptive transfer model was used to examine Fas/FasL interaction as a potential mechanism of regulation. In this setting, induction of apoptotic death of the autoreactive T cells would prevent the adoptive transfer of syngeneic GVHD. The results in Table 3 demonstrate that treatment of the primed regulatory T cells with antibody to FasL before coculture prevented the inactivation of the effector T cells and allowed syngeneic GVHD to be successfully transferred into the secondary recipients. Treatment of the effector T lymphocytes with the antibody to FasL was ineffective.

DISCUSSION

During the past several years, a number of studies have clearly indicated that CD4+CD25+ regulatory T cells play a central role in maintaining peripheral tolerance to self-antigens and can facilitate the development of transplantation tolerance [17-20,24]. The major subset of CD4+ regulatory T cells that occurs naturally expresses CD25 and is functionally active as it emigrates from the thymus. In addition, an inducible subset of CD4+ regulatory T cells appears to require an activation signal before the acquisition of functional activity. A breakdown in these regulatory mechanisms often precedes the onset of autoaggression.

Peripheral regulatory mechanisms are critical in preventing the development of syngeneic GVHD after autologous or syngeneic BMT [8,10,11]. Characterization of this peripheral regulatory system has provided some insight into the dynamic nature of the mechanisms that underlie the restoration and maintenance of immune tolerance to self-antigens after BMT [8-11]. The results of the current studies provide evidence that a subset of CD4+ T cells can effectively suppress both the adoptive transfer of syngeneic GVHD and the in vitro cytokine response of the pathogenic autoreactive lymphocytes. This subset of regulatory T cells may also coexpress CD25. Because negative depletion techniques were used for the adoptive transfer studies, the possibility that effective regulation in vivo requires a subpopulation of CD25+ (CD8−CD4−) cells that acts synergistically cannot be excluded. Of importance, functional regulatory activity was only detected after the resolution of syngeneic GVHD or after experimental challenge of normal animals with the autoreactive T-cell population but not in naïve animals. These results suggest that the

Table 3. *Primed CD4⁺ Regulatory T Cells Inactivate Syngeneic GVHD Effector Lymphocytes Through a Fas/FasL⁻ Dependent Mechanism*

Coculture Before Adoptive Transfer		Incidence of Syngeneic GVHD in Secondary Recipients
Effector Population	Regulatory Population	
Syngeneic GVHD effector T cells	None	6/6
Syngeneic GVHD effector T cells	Primed regulatory cells	0/6
Syngeneic GVHD effector T cells + anti-FasL	Primed regulatory cells	0/6
Syngeneic GVHD effector T cells	Primed regulatory cells + anti-FasL	5/6

Primed CD4⁺ regulatory cells (30×10^6) were irradiated (1250R) and cocultured with syngeneic GVHD effector cells (30×10^6) for 18 hours before the adoptive transfer into secondary Lewis recipients. The regulatory and effector T cells were treated with antibody to FasL before coculture. Treatment of the effector and regulatory T cells with normal mouse IgG had no effect on their functional activity. Irradiated primed regulatory cells are active *in vitro* but when transferred separately fail to suppress the adoptive transfer of syngeneic GVHD.

development of functional regulatory activity within the CD4⁺ T-cell compartment is caused by an active response to the pathogenic autoreactive lymphocytes. Encounter with the autoreactive T cells leads to changes in both the function and phenotype of the regulatory system. Interestingly, previous studies in other animal models of autoimmunity suggest that regulatory T cells can be primed or activated by challenge with autoreactive lymphocytes [21-24].

Efforts to determine the specificity of the regulatory T cells revealed that these cells through their clonotypic receptor for antigen recognize the MHC class II CLIP complex expressed on the activated pathogenic effector lymphocytes [10]. Because the pathogenic autoreactive T cells also recognize the MHC class II CLIP complex [2-5], the results from the current study suggest that both the regulatory and pathogenic effector T cells in syngeneic GVHD recognize a common peptide antigen framework presented by MHC class II molecules. Surprisingly, these functionally distinct subsets of cells can be separated based on their differential affinities for variants of CLIP containing either the N- or C-terminal flanking domains of this peptide [14,25]. Analysis of the CD4⁺CD25⁺ lymphocyte compartment reveals two subpopulations that can be defined by antigen specificity into N-CLIP- and CLIP-C-reactive T cells. The CLIP-C-reactive subset includes cells that have phenotypic and functional properties consistent with regulatory T cells whereas the N-CLIP-reactive cells do not. The CD4⁺CD25⁺ CLIP-C-reactive T-cell subset expresses elevated levels of IL-10 and Foxp3 mRNA transcripts, the nuclear transcription factor preferentially associated with regulatory T cells [15,16,26]. Interestingly, the development of functional regulatory activity correlated with increased expression levels of Foxp3 mRNA transcripts in the CLIP-C-reactive T-cell subset. Comparatively, the N-CLIP-reactive CD4⁺CD25⁺ T cells did not have significant regulatory activity and expressed low levels of Foxp3 mRNA transcripts, suggesting that CD25 expression is not a unique marker for regulatory T cells.

Temporal analysis of the CLIP-reactive T-cell subsets revealed remarkable changes in both the function and the relative proportion of N-CLIP- and CLIP-C-reactive cells within the CD4⁺CD25⁺ T lymphocyte compartment providing unique insights into the maturation of the autoreactive/autoregulatory response associated with the development and resolution of syngeneic GVHD. The CD4⁺CD25⁺ T-cell compartment was expanded during active disease largely because of an increase in the number of N-CLIP T cells with autoreactive effector function. Although the relative percentage of CLIP-C-specific CD4⁺CD25⁺ T cells did not appear to change in animals with syngeneic GVHD, there were significant changes in the functional phenotype of these cells with increased levels of type 1 and 2 cytokine mRNA transcripts compared with CD4⁺CD25⁺ CLIP-C-reactive T cells isolated from naïve animals. These cells did not have any demonstrable regulatory activity. The absence of regulatory function in the CLIP-reactive T-cell subsets harvested during active disease correlated with minimal levels of Foxp3 mRNA transcripts. These data are consistent with the expansion of autoreactive effector T helper cells (that express CD25 on activation). It also appears that the relative number of regulatory lymphocytes is decreased in animals with syngeneic GVHD. However, it is unclear whether these cells are functionally active but at a frequency too low to effectively inhibit the autoreactive T cells.

Although regulatory T-cell populations have been isolated from and found to develop in multiple lymphoid compartments (lymph node, peripheral blood, spleen, and bone marrow), the adoptive transfer studies that identified and characterized the promiscuous autoregulatory compartment in syngeneic GVHD used T cells derived from the spleen. The apparent decrease in this regulatory T-cell population in animals with syngeneic GVHD is most likely a result of a delay in the reconstitution of this subset as a result of CsA treatment [5,8,11]. Previous studies indicate that CsA treatment not only inhibits the deletion of autoreactive T cells but also retards the thymic-dependent

development of regulatory lymphocytes [2,11,27]. Reconstitution of the CD4$^+$ regulatory T-cell compartment only occurs after the discontinuation of CsA treatment [11,12]. Of interest, recent studies also suggest that the regulatory T cells that develop after cessation of CsA treatment are recent thymic emigrants because T-cell recombinant excision circles can be detected in this subset of lymphocytes [11,12]. The failure to reconstitute this subset in thymectomized hosts also suggests that reconstitution of the regulatory compartment can occur de novo in the thymus [28].

Interestingly, the number of CD4$^+$CD25$^+$ T cells and the relative proportion of CLIP-C-reactive lymphocytes within this subset after resolution of syngeneic GVHD were comparable with the levels detected in normal animals. However, the CD4$^+$CD25$^+$ T-cell subset was now functionally active and capable of suppressing in vitro responses of the autoreactive T cells whereas depletion of cells expressing either the CD4 or CD25 cell surface marker eliminated in vivo regulatory activity. CLIP-C-reactive T cells that have in vitro regulatory function can also be detected within the CD4$^+$CD25$^+$ subset after resolution of active disease. Acquisition of functional activity was not associated with an increase in the relative percentage of reactive T cells but rather correlated with the up-regulation of IL-10 and Foxp3 mRNA transcripts. This is somewhat surprising given the relative comparability of the total number of antigen-reactive lymphocytes within the CD4$^+$CD25$^+$ CLIP-C-reactive population. The development of regulatory function may be a result of either activation of the regulatory cells or the expansion of a minor population of cells within this compartment. The antigen-reactive regulatory T cells may be quiescent unless these cells receive an activation signal that promotes the development of functional regulatory activity. In many systems, activation of regulatory T cells including CD4$^+$CD25$^+$ lymphocytes by antigen must occur before the development of functional regulatory activity [17-19,29-31]. The presence of activated autoreactive T cells may also lead to the activation of the regulatory T cell as established in several experimental models of autoimmunity [21-24]. Alternatively, the results from the current study may also be explained by an expansion of a minor subpopulation of CD4$^+$CD25$^+$ Foxp3$^+$ CLIP-C-reactive T cells within the regulatory compartment to sufficient levels that can modify the activity of the autoreactive lymphocytes. Because Foxp3 is a nuclear transcription factor, enumeration of CD4$^+$CD25$^+$ Foxp3$^+$ CLIP-C-reactive T cells by flow cytometry would require intracellular staining and, therefore, preclude functional assessment of the cells. Nevertheless, the development of functional activity was associated with increased expression of Foxp3 mRNA transcripts within the CD4$^+$CD25$^+$ CLIP-C-reactive T cells.

Functionally active regulatory cells were also detected after challenge of normal animals with the syngeneic GVHD effector T cells or after specific immunization with the CLIP-C peptide antigen presented by APCs, the apparent target antigen of the regulatory T cells. It is important to note that both CLIP and MHC class II are presented on the activated autoreactive T cells in the rat [3,5]. Challenge with the autoreactive T cells could represent specific antigen challenge to the regulatory compartment leading to activation and expansion. Because activation of any T cell can in principle lead to expression of the MHC class II CLIP complex, CLIP-C-reactive regulatory T cells could be activated. This would effectively set the stage for local regulation of the immune response. Indeed, evidence exists that nonconventional CLIP fragments are presented by MHC class II on dendritic cells. MHC class II CLIP complexes isolated from mature dendritic cells were found to contain "short" CLIP peptides that lack the N-terminal domain but have the C-terminal flanking region [32]. These activated APCs are thought to promote skewing of an autoreactive T-cell response to a type 2 cytokine phenotype. Short CLIPs are also differentially expressed on thymic epithelial cells and thought to play a role in selection of T cells [33]. In settings where the responses are to very broad and widely distributed antigens such as allogeneic BMT, endogenous signaling of these autoreactive/regulatory T cells would have a profound effect. Interestingly, the resolution of acute allogeneic GVHD in the rat is associated with broadly specific regulatory T cells that can dampen the immune response to many antigens [7,34-36]. Moreover, the finding that a regulatory target antigen can be expressed on many T cells after activation implies a potential mechanism for the generation and maintenance of the CD4$^+$ regulatory T-cell compartment.

REFERENCES

1. Glazier A, Tutschka PJ, Farmer ER, et al. GVHD in CsA treated rats after syngeneic and autologous bone marrow reconstitution. *J Exp Med*. 1983;158:1-12.
2. Hess AD, Thoburn CJ. Immunobiology and immunotherapeutic implications of syngeneic/autologous graft-vs-host disease. *Immunol Rev*. 1997;157:111-123.
3. Chen W, Thoburn C, Hess AD. Characterization of the pathogenic autoreactive T cells in cyclosporine-induced syngeneic graft-vs-host disease. *J Immunol*. 1998;161:7040-7046.

4. Thoburn CJ, Miura Y, Bright EC, et al. Functional divergence of antigen-specific T-lymphocyte responses in syngeneic graft-versus-host disease. *Biol Blood Marrow Transplant*. 2004;10:591-603.

5. Hess AD, Thoburn CJ, Horwitz L. Promiscuous recognition of major histocompatibility complex class II determinants in cyclosporine-induced syngeneic graft-vs-host disease. *Transplantation*. 1998;65:785-792.

6. Teshima T, Reddy P, Liu C, et al. Impaired negative selection causes autoimmune graft-versus-host disease. *Blood*. 2003;102:429-435.

7. Tivol E, Komorowkski R, Drobyski WR. Emergent autoimmunity in graft-versus-host disease. *Blood*. 2005;105:4885-4891.

8. Fischer AC, Beschorner WE, Hess AD. Requirements for the induction and adoptive transfer of syngeneic GVHD. *J Exp Med*. 1989;169:1031-1038.

9. Hequet O, Vocanson M, Saint-Mezard P, et al. CD4$^+$ T cells prevent skin autoimmunity during chronic autologous graft-versus-host disease. *Int J Transplant*. 2004;4:872-878.

10. Hess AD, Fischer AC, Horwitz L, et al. Characterization of peripheral autoregulatory mechanisms that prevent development of cyclosporine-induced syngeneic graft-vs-host disease. *J Immunol*. 1994;153:400-411.

11. Wu DY, Goldschneider I. Cyclosporin-induced autologous graft-vs-host disease: a prototypical model of autoimmunity and active (dominant) tolerance coordinately expressed induced by recent thymic emigrants. *J Immunol*. 1999;162:6926-6933.

12. Miura Y, Thoburn CJ, Bright EC, et al. Association of Foxp3 gene expression with graft-versus-host disease. *Blood*. 2004;104:2187-2193.

13. Hess AD, Thoburn C, Chen W, et al. The N-terminal flanking region of the invariant chain peptide augments the immunogenicity of a cryptic "self" epitope from a tumor-associated antigen. *Clin Immunol*. 2001;101:67-76.

14. Hess AD, Thoburn CJ, Miura Y, et al. Functionally divergent T lymphocyte responses induced by modification of a self-peptide from a tumor-associated antigen. *Clin Immunol*. 2005;114:307-319.

15. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299:1057-1061.

16. Khattri R, Cox T, Yasayko SA, et al. An essential role for Scurfin in CD4$^+$CD25$^+$ T regulatory cells. *Nat Immunol*. 2003;4:337-342.

17. Shevach E. Regulatory T cells in autoimmunity. *Ann Rev Immunol*. 2000;18:423-449.

18. Read S, Powrie F. CD4$^+$ regulatory T cells. *Curr Opin Immunol*. 2001;13:644-649.

19. Sakaguchi S. Naturally arising CD4$^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Ann Rev Immunol*. 2004;22:531-562.

20. Kronenberg M, Rudensky A. Regulation of immunity by self-reactive T cells. *Nature*. 2005;435:598-604.

21. Ben-Nunn AH, Wekerle H, Cohen H. Vaccination against autoimmune encephalomyelitis with a T lymphocyte line reactive against myelin basic protein. *Nature*. 1981;292:60-64.

22. Acha-Orbea H, Mitchell TJ, Timmerman L. Limited heterogeneity of the T cell receptors from lymphocytes mediating autoimmune encephalomyelitis allows specific immune intervention. *Cell*. 1988;54:263-271.

23. Gutierrez-Ramos JC, Andrew JL, Moreno Alboran I. Insights into autoimmunity; from classical models to current perspectives. *Immunol Rev*. 1990;118:73-84.

24. Piccirillo CA, Shevach EM. Naturally-occurring CD4$^+$CD25$^+$ immunoregulatory T cells: central players in the arena of peripheral tolerance. *Semin Immunol*. 2004;16:81-88.

25. Hess AD, Thoburn CJ, Chen W, et al. Complexity of effector mechanisms in syngeneic graft-vs-host disease. *Biol Blood Marrow Transplant*. 2000;6:13-24.

26. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead transcription factor Foxp3. *Nat Immunol*. 2005;6:331-337.

27. Fischer AC, Hess AD. Age related factors in cyclosporine-induced syngeneic graft-versus-host disease: regulatory role of marrow-derived lymphocytes. *J Exp Med*. 1990;172:85-94.

28. Beschorner WE, Ren H, Phillips J, et al. Recovery of thymic microenvironment after cyclosporine prevents syngeneic graft-vs-host disease. *Transplantation*. 1991;52:668-674.

29. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev*. 2003;3:199-210.

30. Graca L, Chen T-C, Le Moine A, et al. Dominant tolerance: activation thresholds for peripheral generation of regulatory T cells. *Trends Immunol*. 2005;26:130-135.

31. Hsueh C-S, Liang Y, Tyznik AJ, et al. Recognition of the peripheral self by naturally arising CD25$^+$CD4$^+$ T cell receptors. *Immunity*. 2004;21:267-277.

32. Rohn TA, Boes M, Wolters D, et al. Upregulation of the CLIP self-peptide on mature dendritic cells antagonizes T helper type 1 polarization. *Nat Immunol*. 2004;5:909-918.

33. Kasai M, Kropshofer H, Vogt A, et al. CLIP-derived self-peptides bound to MHC class II molecules of medullary thymic epithelial cells differ from those of cortical thymic epithelial cells in their diversity, length and C-terminal processing. *Eur J Immunol*. 2000;30:3542-3551.

34. Tutschka PJ, Ki P, Beschorner WE, et al. Suppressor cells in transplantation tolerance, II: maturation of suppressor cells in the bone marrow chimera. *Transplantation*. 1985;32:321-329.

35. Kupiec-Weglinski JW, Filho Strom TB, Tilney NL. Sparing of suppressor cells; a critical action of cyclosporine. *Transplantation*. 1983;38:97-104.

36. Hess AD. Chronic graft-versus-host disease: a breakdown of self-tolerance? *Blood*. 2005;105:4548-4549.