Review

Generation and Regulation of CD8$^+$ Regulatory T Cells

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Research into the suppressive activity of CD4$^+$FoxP3$^+$ T regulatory cells (Treg) has defined a sublineage of CD4$^+$ cells that contribute to self-tolerance and resistance to autoimmune disease. Much less attention has been given to the potential contribution of regulatory sublineages of CD8$^+$ cells. Analysis of a small fraction of CD8$^+$ cells that target autoreactive CD4$^+$ cells through recognition of the MHC class Ib molecule Qa-1 in mouse and HLA-E in human has revitalized interest in CD8$^+$ Treg. Here we summarize recent progress and future directions of research into the role of this CD8$^+$ sublineage in resistance to autoimmune disease. *Cellular & Molecular Immunology*. 2008; 5(6):401-406.

Key Words: CD8$^+$ regulatory T cell, self-tolerance, autoimmune disease, EAE, Qa-1, NKG2A

Introduction

The adaptive immune response that defends the body against invasion by pathogenic microorganisms, including bacteria, viruses, fungi, parasites and cancer cells, is initiated by T cells. Although T cell clones express receptors for foreign antigen, positive selection of these clones by self-antigens during development in the thymus means that each clone has a measurable affinity for self-antigens. Although negative selection deletes strongly self-reactive T cells (1), this process is incomplete. T cells carrying receptors with intermediate and low affinity for self-antigen are routinely released into peripheral lymphoid tissues, where they may become activated, expand and possibly initiate autoimmune disease (2). This process is held in check by several cellular mechanisms, including specialized regulatory T cells (Treg) that suppress immune reactions to self. Treg belonging to the CD4$^+$ T cell lineage have been extensively studied (3) and drawn tremendous attention in the past few years. Although a sublineage of CD8$^+$ Treg was initially noted to display immunoregulatory activity (4), early analysis was hindered by limitations in technology. Recently, advanced molecular approaches to this problem and increased understanding of the immune system as a whole have allowed substantial progress to be made in this area. Here we review research into CD8$^+$ Treg and its contribution to self-tolerance.

CD8$^+$ T cells as suppressor cells

CD8$^+$ Treg were first observed by Gershon and colleagues in the early 1970's (4). The ability to characterize T cell subsets using the CD8 surface molecule (termed Lyt2t) suggested that suppressive activity by a subpopulation of CD8$^+$ cells specifically inhibited T helper responses by CD8$^-$CD5$^+$ T cells (5). Subsequent studies determined that this inhibitory interaction reflected CD8$^+$ Treg recognition of Qa-1 on target CD4$^+$ cells (6).

Suppressive activity of CD8$^+$ T cells in autoimmune disease was first demonstrated in a murine model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE). Animals that were deficient in CD8$^+$ T cells secondary to genetic mutation or antibody-dependent depletion displayed a chronic and persistent form of EAE that was characterized by a high frequency of disease relapse. Early experiments in mice and humans also suggested that CD8$^+$ Treg lysed encephalitogenic CD4$^+$ T cells (7, 8). The regulatory role of CD8$^+$ T cells was also observed in other autoimmune disease models, including autoimmune Herpes Stromal Keratitis and myocarditis. For example, CD8$^+$ T cell deficient mice developed more severe autoimmune myocarditis than heterozygous CD8$^{+/-}$ mice or CD8 wild type controls (9). CD8-deficient mice were also more susceptible to relapse of autoimmune arthritis after immunization with self antigens (10), suggesting that regulatory activity of CD8$^+$ T cells might reside in the CD8$^+$ memory pool. CD8$^+$ Treg have also been implicated in the regulation of human

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Received Dec 10, 2008. Accepted Dec 22, 2008.

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autoimmune diseases, including inflammatory bowl disease and MS (11, 12).

Definition of CD8+ Treg

Although regulatory activity of CD8+ T cells has been implicated in diverse settings, detailed studies of CD8+ Treg have been hindered by the lack of a reliable surface marker to distinguish CD8+ Treg from conventional CD8+ cells. Several cell surface molecules have been associated with CD8+ Treg in different experimental systems. For example, CD8+CD28- cells behave as regulatory T cells, since transfer of these cells (but not CD28+ cells) into CD8-deficient mice significantly suppressed EAE (13). Moreover, CD8+CD28- but not CD8+CD28+ cells also inhibited IFN-γ production of myelin oligodendrocyte glycoprotein (MOG)-specific CD4+ T cells through an interaction that required cell-cell contact. Similarly, CD8+CD45R+ and CD8+CD122+ T cells have been implicated in regulatory activity (14, 15). CD8+ Treg may also be induced by plasmacytoid dendritic cells (pDC) from tumor ascites. These cells have an IL-10+ CCR7+ CD45RO+ CD8+ phenotype and can suppress tumor antigen-specific T cell effector function through IL-10 secretion (16). Similarly, immature conventional DC can induce CD8+ Treg (17) and FoxP3+ alloantigen-specific CD8+CD28- T suppressor cells can also be generated *in vitro* by tolerized endothelial cells (18). Primary suppressive CCR7+CD45RO+CD8+ T cells have been isolated from human tumors, suggesting that they may contribute to the tumor-induced immunosuppressive network (19).

Qa-1-restricted CD8+ Treg

The subset of Qa-1-restricted CD8+ Treg is perhaps the best-defined of the candidate CD8+ regulatory cells. This subpopulation of CD8+ cells recognizes the MHC class Ib product Qa-1 (HLA-E in human) and suppresses the development and relapse of EAE, (20). Generation and analysis of Qa-1-deficient mice several years ago has allowed further definition of the role of Qa-1 in the generation and mediation of suppressive activity by this CD8+ Treg subset. Mice lacking Qa-1-restricted CD8+ Treg develop exaggerated immune responses to self antigen (21). However, Qa-1-deficient mice have not been sufficient to fully define the contribution of Qa-1-restricted CD8+ T cells to immunological suppression, because of the bivalent interaction of Qa-1 with its receptors.

Presentation of peptide by the Qa-1 protein on APC activates antigen-specific CD8 cells. In addition, Qa-1 complexed to the Qdm peptide engages the CD94/NKG2 receptor expressed by CD8 cells and NK cells and attenuates the activities of these cells (22-24). As a result, the immune response phenotype of Qa-1-deficient mice reflects two opposing effects. Enhanced CD4-dependent immunity reflects loss of the Qa-1 target for CD8+ Treg activity (21), while reduced CD4-dependent immunity results from lysis of activated CD4+ cells by NK cells that are unencumbered by an inhibitory NKG2A-Qa-1/Qdm interaction (24).

To isolate and individually analyze these two Qa-1-dependent regulatory pathways, we exploited the fact that each Qa-1-dependent interaction utilizes distinct surface Qa-1 contact residues. Generation of a Qa-1 amino acid exchange mutation (Qa-1 D227K), which disrupts binding of Qa-1 to the CD8 co-receptor, prevents effective peptide presentation to CD8+ cells (25). Cells expressing the Qa-1 D227K mutation fail to present peptides to CD8+ cells, but retain the ability to bind to NKG2A receptors on CD8+ cells and NK cells. After targeting this Qa-1 point mutation into the Qa-1 locus and backcrossing to C57BL/6 mice, analysis of CD8+ Treg activity in this Qa-1 knock-in strain revealed that Qa-1 D227K knock-in mice fail to develop CD8+ Treg activity and display enhanced EAE by both PLP and MOG upon secondary immunization. This finding confirmed an important role for Qa-1 in generating and mediating CD8+ Treg activity and also revealed the suppressive interaction on a molecular level, indicating that an MHC-TCR interaction depends on the CD8 co-receptor.

A second Qa-1 amino acid mutant (Qa-1 R72A) knock-in mouse strain was also developed that disrupts Qa-1 binding to NKG2A expressed by NK cells and CD8+ cells, rendering CD4+ cells vulnerable to NK and CD8+ cells lysis, but sparing Qa-1-dependent peptide presentation to the TCR (24). Interestingly, genetic interruption of this interaction in Qa-1 R72A knock-in mice allows development of robust CD8+ Treg activity and complete resistance to the development of EAE.

These findings provide insight into the molecular constraints that dampen CD8+ Treg activity. Qa-1/Qdm engagement of CD94/NKG2A on CD8+ Treg transmits an inhibitory signal that attenuates CD8+ Treg suppressive activity. The CD94/NKG2A/C/E receptors belong to the inhibitory NK receptor family (iNKRs) and express two immunoreceptor tyrosine-based inhibition motifs (ITIMs) that recruit SHP-1, which dephosphorylates signaling molecules and inhibits cellular activation. Although the CD94 chain can pair with other members of the NKG2 family, including NKG2C or NKG2E [reviewed in (26)], relatively high levels of expression and stronger binding affinity of CD94/NKG2A for the Qa1-Qdm result in its dominance. As a result, Qa-1-dependent engagement of CD94/NKG2A receptors expressed by activated CD8+ cells during viral infection downregulates cytotoxicity and production of pro-inflammatory cytokines. Interestingly, although only a small proportion of CD8+ cells express NKG2A, according to immunofluorescence with available antibodies, the majority of CD8+ cells specifically bind Qa-1-Qdm tetramers, perhaps allowing a more substantial impact of NKG2A ligation (27).

These data also suggest that the relative ratio of the two classes of Qa-1 peptide ligand – Qdm *vs.* non-Qdm (e.g. HSP60) peptide – on the surface of activated CD4+ cells may determine susceptibility of these CD4+ T cells to suppression by CD8+ Treg. According to this view, engagement of NKG2A on CD8+ Treg by the Qa-1-Qdm ligand on target CD4+ cells inhibits Treg activity, while engagement of the

TCR expressed by $CD8^+$ Treg by Qa-1-non-Qdm peptide ligands, such as Hsp60 peptide, activates suppressive activity (28). Since the majority of murine $CD8^+$ T cells, including $CD8^+$ Treg, bind to Qa-1-Qdm tetramer and can express functional receptors for both ligands, differential expression of Qa-1-Qdm and Qa-1-non-Qdm peptide ligands on activated Qa-1 $CD4^+$ target cells may decide the cell's fate.

The tight regulatory control of $CD8^+$ Treg may be explained in part by their autoreactive recognition of Qa-1-restricted self antigens. Indeed, expansion of MHC class Ib-restricted CD8 cells in triple knock-out mice lacking Qdm ($K^{b-/-}D^{b-/-}$CIITA$^{-/-}$) induces autoimmune disease (29). Thus, regulation of Qa-1-restricted autoreactive cells through Qa-1-Qdm and CD94/NKG2A interaction may be essential to prevent $CD8^+$ autoimmunity.

Since autoreactive $CD4^+$ cells expressing the Qa-1 R72A point mutation fail to transmit an inhibitory signal *via* NKG2A to $CD8^+$ Treg, they were fully susceptible to inhibition and failed to induce EAE despite expression of the pathogenic $2D2^+$ TCR transgene. These findings suggest that releasing the brakes on $CD8^+$ Treg activity by blockade of Qa-1-NKG2A interaction may facilitate new approaches to mobilizing $CD8^+$ Treg in the context of autoimmune disease. Indeed, antibody-dependent blockade of this interaction during ongoing EAE unleashed robust CD8-dependent Treg activity that attenuated disease progression and induced complete remission of this murine model of MS.

HLA-E-restricted $CD8^+$ Treg in human and regulation by CD94/NKG2A

Recent reports have also demonstrated the participation of $CD8^+$ regulatory cells in controlling human MS (multiple sclerosis). Analysis of T cells from MS patients has shown that HLA-E restricted $CD8^+$ Treg can be cloned from both MS patients and healthy individuals. $CD8^+$ Treg isolated from MS patients lyse myelin-reactive $CD4^+$ T cells by a cytolytic process that is restricted by the non-classical MHC class Ib molecule HLA-E, the human homolog of Qa-1 in mouse (30).

Consistent with findings in the EAE model, HLA-E-restricted $CD8^+$ Treg cloned from patients during disease exacerbation express higher levels of CD94/NKG2A at their surface than CD8 cells cloned from MS patients in remission or CD8 cells from healthy controls, which correspond to their less cytotoxic activity against myelin-reactive $CD4^+$ T cells, indicating that CD94/NKG2A inhibitory receptors expressed by $CD8^+$ cells restrain their HLA-E-restricted suppressive activity. Moreover, antibody-dependent blockade of the HLA-E-CD94/NKG2A interaction enhanced lysis of auto-reactive $CD4^+$ T cell clones by HLA-E-restricted $CD8^+$ cells from MS patients (30). These findings also suggest that combination therapy with cloned HLA-E-restricted $CD8^+$ cells and anti-NKG2A antibody may result in long-term improvement and disease remission.

Glatiramer Acetate (GA; Copaxane), a synthetic copolymer of alanine, glutamic acid, lysine and tyrosine, was discovered during studies of EAE. Copaxane treatment can reduce exacerbations and suppress disability in relapsing-remitting MS (12). Although GA has received FDA approval as a treatment for MS, the mechanism of action of GA is unknown. Recent analyses have suggested that treatment of MS patients with GA results in up-regulation of regulatory/suppressive GA-reactive $CD8^+$ T cells. GA-induced $CD8^+$ Treg expressed high levels of perforin and directly lysed $CD4^+$ T cells that presented GA by HLA-E (12). In contrast, untreated patients showed an overall deficit in $CD8^+$ T cell-mediated suppression, compared with healthy subjects. Recent studies have begun to define the role of Qa-1-restricted Treg in GA-dependent inhibition of murine EAE.

Perspectives

Phenotypic characterization of Qa-1-restricted $CD8^+$ Treg

A major obstacle in the study of regulatory $CD8^+$ T cells has been the lack of a surface marker (like that for $CD4^+$ Treg) to identify and isolate this population. The use of polyclonal populations also hinders further study of the suppressive mechanism *in vitro*, due to the low frequency of $CD8^+$ Treg in this subpopulation.

Theoretically, the best potential marker for Qa-1-restricted $CD8^+$ Treg is the Qa-1 tetramer. However, there are obstacles to this approach that come from the peptide binding properties of Qa-1. The association between Qa-1 and its binding peptide is relatively weak, resulting in an unstable Qa-1/peptide complex, even with its dominant peptide Qdm. The dissociation rate of Qdm peptide from Qa-1 has a t½ of approximately 1.5 h at 37°C, compared with peptide (SIINFEKL) binding to the MHC class Ia molecule, which dissociates with a t½ of 11-31 h (31). Peptides other than Qdm bind with even lower affinity to Qa-1, further hindering formation of a stable Qa-1 peptide complex, which is crucial for tetramer production. The repertoire of peptides presented by Qa-1 is substantially smaller than the repertoire of classical MHC molecules. These include the dominant Qdm peptide (31) as well as peptides derived from proteins, including heat shock protein 60 (Hsp60), insulin, Salmonella, GroEL, the TCR and the varicellovirus (UL49.5) (32-36). Peptides derived from T-cell receptor have been shown to be able to induce Qa-1-restricted $CD8^+$ Treg and inhibit the development of autoimmune disease (35, 37). Moreover, a signal peptide derived from the leader sequence of Hsp60sp bound to Qa-1 targets Qa-1-restricted $CD8^+$ T cells (28). Vaccination with Hsp60sp-loaded dendritic cells (DCs) induces $CD8^+$ Treg that protect animals from subsequent induction of EAE. Whether other Hsp60sp-derived peptides function as targets for the Qa-1-restricted regulatory $CD8^+$ T cells is not known. A combination of surface markers and tetramer analysis may eventually allow full characterization of Qa-1-restricted $CD8^+$ Treg.

Recent studies have isolated a $CD8^+$ Treg clone derived from immunization of PLJ mice with a peptide derived from the T-cell receptor. This $CD8^+$ clone inhibits activation of $V\beta8.2^+CD4^+$ T cells in the context of EAE (37). The clone,

which expresses the CD8αα homodimer, recognizes a peptide derived from a conserved region of the TCR Vβ8.2 chain bound to Qa-1a. Since a single clone was analyzed in this study, whether all Qa-1-restricted CD8 Treg express CD8αα is unclear. Most $CD8\alpha\alpha^{+}TCR\alpha\beta^{+}$ T cells reside in IELs (CD8αα IELs) and are present in mice genetically deficient in MHC class Ia molecules (38, 39), consistent with restriction by MHC class Ib (40). Further studies are warranted to determine whether Qa-1-restricted Treg express $CD8\alpha\alpha^{+}$ and, if so, whether they represent circulating CD8αα IELs or a separate lineage.

Induction (priming) of $CD8^{+}$ Treg

Unlike $CD4^{+}FoxP3^{+}$ Treg which arise naturally in the thymus, CD8 Treg activity must be induced by a primary antigen stimulation (although this type of induction is inefficient, since $CD8^{+}$ Treg activity is not developed in any given antigen response). The most efficient system for generating $CD8^{+}$ Treg occurs during the induction of EAE, at which CD8 suppressive activity develops during the primary immunization and can prevent subsequent induction of disease in the same host (21). Other approaches include T-cell vaccination (TCV), immunization with Qa-1-restricted peptide or DC loaded with such peptides (28, 35). $CD8^{+}$ Treg can also be induced by introducing antigens into immune privileged sites like the anterior chamber (AC) (41). However, Qa-1 restriction of the $CD8^{+}$ Treg has not been confirmed, although they share many features with Qa-1-restricted $CD8^{+}$ Treg.

The process and mechanisms by which $CD8^{+}$ Treg are induced is poorly understood. It is likely that costimulation is critical for their development. For example, the co-stimulatory molecule CD137 (4-1BB), which is expressed on activated $CD8^{+}$ T cells and can interact with its ligand 4-1BBL on dendritic cells and tumor cells, is important for immunity against tumors and viruses as well as the generation of $CD8^{+}$ memory cells. More importantly, several reports have shown that immunization in combination with anti-4-1BB induced generation of $CD8^{+}$ Treg that profoundly inhibited $CD4^{+}$ T cell responses (42, 43). We have recently observed that administration of anti-4-1BB during both the induction and effector phase of $CD8^{+}$ Treg increased CD8 suppressive activity (Kim et al., unpublished data).

Cytokines have also been implicated in the generation of $CD8^{+}$ Treg. $CD8^{+}$ Treg activity cannot be induced in IFN-$\gamma R^{-/-}$ mice, indicating the essential role of IFN-γ in either generation or expression of $CD8^{+}$ Treg activity. On the other hand, $CD8^{+}$ Treg generated after immunization of IFN-$\gamma^{-/-}$ mice are fully capable of suppressing $CD4^{+}$ T cell responses, suggesting that IFN-γ secretion of $CD8^{+}$ Treg does not contribute to suppressive activity (Lu et al., unpublished data).

Signal transducers and activators of transcription (STAT) proteins are latent cytoplasmic transcription factors that are phosphorylated by Janus kinases in response to interferon. Phosphorylated STAT proteins translocate to the nucleus, where they transiently turn on specific sets of genes. STAT1 proteins can be activated by IFNγ treatment of CD8 T cells. Recent studies have shown that STAT1 signaling in CD8 T cells is required for their efficient expansion by promoting the survival of activated $CD8^{+}$ T cells upon vaccinia viral infection *in vivo*, suggesting that the direct effect of IFN-γ on $CD8^{+}$ T cells is mediated by STAT1. Since the IFNR expression on $CD8^{+}$ T cells is essential for the development of CD8 suppressive activity, the role of STAT1 in the generation of Qa-1-restricted $CD8^{+}$ suppressor cells will also be addressed.

Cytokines that stimulate $CD8^{+}$ T cells might also be important for the development of Qa-1-restricted $CD8^{+}$ Treg. One such example is IL-15. Qa-1-restricted CD8 as well as $CD8^{+}$ T cells restricted by other MHC class Ib molecules belong to a unique subpopulation of $CD8^{+}$ cells termed innate-CD8 with unique properties that include selection on hematopoietic cells, expression of memory markers, rapid production of cytokines, and dependence on IL-15 (44). The potential role of IL-15 in the development of Qa-1-restricted Treg requires further clarification.

How do $CD8^{+}$ Treg inhibit immune responses?

Several mechanisms have been implicated in the suppressive activity of $CD8^{+}$ Treg, including direct lysis of target cells and secretion of immunosuppressive cytokines. Possibly, different subsets of $CD8^{+}$ Treg utilize different inhibitory mechanisms, depending on the context of the immune reaction. Several early studies have described lysis of target cells by $CD8^{+}$ Treg. Further examination of the key molecules involved in $CD8^{+}$ Treg-mediated killing suggested that perforin-mediated cytolytic activity is necessary for suppression by $CD8^{+}$ Treg both *in vivo* and *in vitro* (45). Similar results obtained in studies of $CD8^{+}$ Treg isolated from human MS patients showed that perforin-mediated cytotoxicity is tightly constrained by the Qa1/NKG2A interaction (30).

Perforin-dependent lysis may not be the only mechanism utilized by Qa-1-restricted $CD8^{+}$ Treg. $CD8^{+}CD122^{+}$ Treg can secrete IL-10 to exert inhibitory activity (46). Although IFN-γ can inhibit immune responses, as discussed above, IFN-γ is more likely to play a role in priming Qa-1-restricted Treg rather than mediating suppressive function (Lu et al., unpublished data).

Acknowledgements

This work was supported in part by research grants from the NIH (AI037562) and National Multiple Sclerosis Society and a gift from the Schecter Family Research Foundation to HC; LL is a Claudia Adams Barr Investigator and NIH NRSA Fellow (T32 AI07386). We thank Alison Angel for manuscript preparation.

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