MECHANISMS OF CD8+ T CELL PERIPHERAL TOLERANCE TO OUR OWN ANTIGENS

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1. ABSTRACT

CD8+ T cells represent a powerful arm of the adaptive immunity that is particularly effective against intracellular pathogens. These cells might also participate in the generation and sustenance of autoimmune responses. The escape of a certain number of CD8+ autoreactive T lymphocytes from thymic negative selection occurs normally in all individuals, but this event is rarely associated with the onset of autoimmune diseases. Several peripheral mechanisms have been evolved by the immune system to take control of these potentially harmful self-aggressive cytotoxic CD8+ T cells. In the present review, we will discuss the principal strategies by which the immune system is capable of maintaining CD8+ T cell tolerance to self, thus making them ineffective to harm their own host but still capable to defend the host against foreign invaders.

2. INTRODUCTION

The most important function of the immune system is to identify invading pathogens and to promptly react for their elimination. However, there are three general states of the immune system to a given antigen, namely immunity, tolerance and ignorance**.** In particular tolerance, conventionally defined as induced lack of T cell immune response to what is normally an immunogenic challenge in an immunocompetent individual, plays a pivotal role in maintaining T cell unresponsiveness when the potential antigen (Ag) is a component of the host itself (self-Ag). In fact, if uncontrolled, an immune reaction against self-Ags might easily lead to immunopathology and autoimmunity. Thymic clonal deletion of autoreactive T cells is certainly the primary mechanism that leads to self-tolerance but, interestingly, T cells recognizing self Ags are normally present in most individuals. These autoreactive T cells, which have escaped thymic negative selection, can be potentially activated in a relatively easy manner (1). However, autoimmunity is a rare event, suggesting that the operating peripheral tolerance mechanisms are a very effective system to keep autoreactive T cells under a strict control.

Among the whole T cell population, CD8+ T lymphocytes play a crucial role in the elimination of pathogens, and rejection of tumors and transplanted foreign tissues and organs. However, uncontrolled activation of self-reactive CD8+ T cells can trigger or amplify autoimmune reactions. CD8+ T cells recognize major histocompatibility complex (MHC) class I molecules bound to 8- to 10-residue peptides derived from proteins of different origin, including self-Ags. Induction of primary autoreactive CD8+ T cell responses requires that self peptides are presented by class I molecules on professional antigen-presenting cells and in particular dendritic cells (DCs), because only these cells can provide the costimulatory signals required to induce activation and proliferation of naïve T cells. Peptides recognized by CD8+ T lymphocytes can be generated from endogenous proteins synthesized by DCs (direct priming) or from proteins originally synthesized by "donor" cells (cross-priming) (2-4).

In the present review, we will discuss how the immune system can induce a state of tolerance of CD8+ T cells to self-antigens in periphery to prevent the induction of autoimmunity, and in particular we will focus the attention on the role played by DCs and the so called regulatory T cells (Treg).

3. THE DUAL FUNCTION OF DENDRITIC CELLS: ACTIVATION OR TOLERIZATION OF CD8+ T CELL RESPONSES

DCs belong to the professional antigen presenting cell (APC) family, and are typically capable to efficiently internalize extracellular Ags, process both exogenous and endogenous Ags into short peptides, load Ag-derived peptides on MHC molecules expressed on their surface and present peptide-MHC complexes to Ag-specific T cells. In addition, DCs exhibit several important properties, such as their unique ability to stimulate naïve T cells and transport Ags from peripheral tissues to T-cell areas of secondary lymphoid organs (5). Importantly, they are able to present

exogenous Ags to specific CD8+ T cells in the context of MHC class I molecules, a phenomenon defined as "cross-presentation". Although DCs are critical in activating specific effector T cells, they are also necessary to induce and maintain CD8+ T-cell tolerance to self (6-8).

To better understand this dual function of DCs, it must be considered that DCs in their immature form (iDCs) possess a highly efficient machinery to take up Ags. This is obtained by constitutive micropinocytosis, receptor-mediated endocytosis and phagocytosis. Whereas iDCs display the highest capacity to internalize Ags but are poor T cell activators, mature DCs (mDCs) down-regulate their endocytic activity and are excellent T cell stimulators. Importantly, in steady-state conditions, e.g., in the absence of inflammation, iDCs reside in peripheral tissues. During inflammation, DC maturation is triggered by different combinations of exogenous and/or endogenous mediators released by bystander cells within the inflammatory microenvironment. Maturation signals include pro-inflammatory cytokines, bacterial or viral components and ligation of molecules of the TNF receptor family on the DC surface during cognate interactions with T cells. After maturation, DCs (mDCs) display long-lasting peptide-MHC complexes on their surface and upregulate membrane levels of T-cell costimulatory molecules (CD80, CD86) chemokine receptor CCR7 and many different adhesion molecules. Furthermore, mDCs produce high levels of IL-12 and TNF-alpha, and traffic to the T-cell areas of secondary lymphoid tissues in response to CCR7 ligands to encounter Ag-specific T cells (9-12).

On the other hand, naïve CD8+ T cells including those specific for self-Ags constantly travel from the blood to secondary lymphoid organs. Here they may encounter DCs that have captured Ags in non-lymphoid peripheral tissues. After appropriate activation, antigen-primed CD8+ T cells undergo proliferation and differentiation. Their progeny includes effector T cells, which gain the ability to migrate to peripheral tissues and display immediate effector function. They also generate memory cells, which travel through secondary lymphoid organs and can differentiate into effector cells after re-encounter with self-antigen. At the site of immune attack, the functions exerted by effector CD8 T cells include the release of cytokines to mediate local inflammation and deposition of perforins at the vicinity of target-cell membranes to induce target-cell death by apoptosis.

The induction of naïve CD8+ T cells by cross-presentation (cross-priming) implies that soluble (exogenous) self antigens induce cytotoxic CD8+ T cells after uptake of these proteins by DCs and their subsequent processing through the class I pathway. The requirement of DCs for cross-priming is mandatory because other types of APCs are not able to deliver exogenous Ags to class-I presentation pathway. If cross-priming is the ability of DCs to induce CD8+ T cells specific to exogenous proteins, cross-tolerance is the capacity of the same cells to silence this response. This important aspect of the immune response similarly involves protein-antigen processing and presentation via DCs, but in this case the outcome is not

CD8+ cytotoxic T cell (CTL) response induction but rather response ablation. When a distinct subset of immature or partially mature DCs migrate in small numbers from the normal tissues into the lymph nodes they induce tolerance of naïve T cells specific for self-antigens, including those derived from apoptotic cells (13-15). We have demonstrated in previous studies that in some pathological conditions apoptotic cells can express CD40L. These cells can induce DC maturation that in turn activate by cross-priming CD8+ T cell specific to apoptotic cell-associated self-antigen, including cell cytoskeleton components. In conditions in which CD40L$^-$ apoptotic cells numerically prevail on their CD40L+ counterpart (as it occurs in normal non-inflammatory conditions), DCs do not undergo a proper maturation process and induce instead tolerance of self-reactive CD8+ T cells. Thus, the balance between CD40L- and CD40L+ apoptotic cells during cross-presentation may determine whether tolerance or induction of autoreactive CD8+ T lymphocytes will be the final outcome (16). These findings point out how DC stimulation via CD40/CD40L interaction as normally occurs after cognate interaction of DCs with CD4+ T cells is mandatory for proper cross-priming of effector CD8+ T cells, whereas maturation of DCs by other powerful stimuli such as TNF-alpha is not sufficient for T cell activation but rather promote cross-tolerance (17, 18). Therefore, CD4+ T cells can induce DCs to acquire their complete capability to activate the autoreactive CD8+ T cells, providing DCs with what has been expressively defined as the "license to kill" (19, 20). More recently, it has been shown that the quantity of Ag that has been uptaken by DCs and its presence in the form of proteasome substrates rather than digestion products decides whether DC will be able to alert CD8+ T cell precursors for cross-priming. The relevance of these findings for the induction of cross-tolerance deserves further investigation (21, 22). It has been also shown that stable DC-T cell interactions occur during the induction of priming, whereas brief contacts may contribute to the induction of T cell tolerance (23). It also appears that memory CD8+ T cells although less dependent on costimulatory stimuli are functionally incapacitated by cross-tolerance, although in contrast to naive or recently activated effector T cells, they are not irreversibly tolerized since they quickly regain effector function upon disappearance of the antigen or stimulation with cytokines including IL-2 (24).

In summary, fully activated DCs will induce self-reactive CD8+ T cells, whereas DCs not expressing all the required signals will induce tolerance (25, 26). Moreover, if self- antigen expression is below the detection threshold of autoreactive CD8+ T cells, a situation of "ignorance" will occur. Also in this case, CD8+ T cells will remain in a tolerance state. DCs with their array of antigen-ingestion and –processing mechanisms, their migratory properties and their versatile array of surface markers and soluble mediators, appear ideally equipped to induce prompt CTL responses or turn them off in the absence of exogenous or endogenous danger (1). It can be therefore hypothesized that cross-presentation *per se* is a system specifically directed at inducing tolerance, given the exceptional requirements by DCs for activation signals   (such as

CD40L) to induce T cell responses, the low efficiency of exogenous protein processing in the class-I pathway with consequent low number of peptide/class I complexes on the DC surface. Under these circumstances, DCs are unlikely to deliver a sufficiently strong and sustained stimulation to induce rapid proliferation of CD8+ T cells and their differentiation into effector cells (27). If we apply this model to autoimmunity, this poor capacity to mount efficient self-reactive CD8+ T cell responses may constitute a kind of protective mechanism against the onset of autoimmune diseases, and this could account for the relative rarity of autoimmune diseases in spite of self-reactive T cells being physiologically present in periphery.

4. ROLE OF REGULATORY T CELLS TO MAINTAIN CD8+ T CELL TOLERANCE

The hallmark feature of autoimmune diseases is the breakdown of peripheral immunoregulatory mechanisms assuring self-tolerance. The idea that specialized T cell population(s) could modulate (or suppress) the effector T cell immunity is not new (28, 29). However, only recently more stringent evidence points to regulatory T (Treg) cells as an active mechanism to suppress autoreactive T cells that have escaped central tolerance. It is now being increasingly accepted that Treg cells can modulate the immune responses to a variety of antigens, including self-antigens (30). In this regard, one of the most important discoveries has been the identification of a subpopulation of CD4+ T cells expressing IL-2 receptor α chain CD25. These cells constitute about 5% of the peripheral CD4+ T-cell repertoire in humans, and possess potent immunoregulatory functions (31-33). Indeed, these naturally occurring CD4+CD25+ Treg cells have been demonstrated in many experimental models to prevent the development of multi-organ-specific autoimmunity, and their removal from peripheral lymphoid compartments appears to significantly increase the occurrence of autoimmune reactions (34).

Since the initial discovery of these CD4+CD25+ T cells (Treg), research has centered on their phenotype, activation requirements and mechanism of suppression. It has been shown that freshly isolated Treg do not manifest suppressive activity, and T-cell receptor (TCR) stimulation is required to induce suppressor function. Once pre-stimulated with antigen and IL-2, they suppress CD8+ (and CD4+ as well) T cell responses in an antigen nonspecific (bystander) fashion. Most murine and human in vitro studies have concluded that Treg-mediated suppression adopts a complex mechanism involving either suppressive cytokines such as IL-4, IL-10 and TGF-beta1, but also direct cell contact, possibly mediated by a membrane-bound, latent (inactive) form of TGF-beta1 or the perforin/granzyme pathway (35-41).

By the use of peptide–MHC I tetramers, it has been reported that CD4+CD25+ T cells can suppress the proliferation and interferon-gamma production by CD8+ T cells in a T–T, APC-independent manner. These findings do not negate a role for APC, because the initial activation of Treg is APC dependent (42). In addition to constitutively expressing CD25, several genetic studies have tried to identify additional surface molecules that are unique to these lymphocytes when compared with CD4+CD25- T cells. Recent studies have shown that the transcription factor Foxp3 is a functional marker of Treg cells, playing a central role in their generation (43-45). Although primarily expressed in human CD4+CD25+ Treg cells, conventional CD4+CD25+ T cells can also upregulate expression of this molecule. However, Foxp3-overexpressing mice have more Treg cells, and can potently suppress the development of autoimmune disease. It remains to be seen which genes are being modulated by Foxp3 and the impact they have on Treg function. CD4+CD25+ Treg cells account for the vast majority of Foxp3 expression within murine lymphoid cells, and Foxp3-/- mice suffer from an autoimmune pathology secondary to a loss of CD4+CD25+ Treg cell function (46, 47). Collectively, these studies suggest that Foxp3 has a determining role in the generation of CD4+CD25+ Treg cells and illustrate how certain genetic pathways affect Treg function, the consequences of which is that autoreactive CD8+ T cells are left uncontrolled and allowed to induce autoimmunity (48, 49).

Studies on the thymic and peripheral signals required for Treg generation and maintenance (50) (51) have pointed out the importance of CD28 co-stimulation in thymic development and peripheral homeostasis of the Treg cells. CD28 engagement might be required to sustain a stable peripheral pool of Treg cells by promoting their survival and self-renewing potential, possibly through the expression of IL-2, anti-apoptotic molecules or through other cytokines that function as survival or suppressor activity maintenance factors (52-55). Interestingly, the functional disruption of B7–CD28 or CD40–CD40L co-stimulatory pathways results in quantitative or qualitative defects in CD4+CD25+ mediated functions in peripheral lymphoid compartments, and a consequential induction of autoimmunity (56, 57).

The antigen specificity directing thymic selection, differentiation and peripheral activation of Treg cells still remains uncertain (58). CD4+CD25+ Treg cells have a polyclonal TCR repertoire, based on diverse gene expression of various TCR α/β elements, and could potentially recognize a wide spectrum of self-antigens, although it is unclear whether they are biased towards recognition of a particular type or subset of self-antigens (59).

Other regulatory T cell populations have been recently described. These include T regulatory 1 (Tr1). These cells are specific for a variety of antigens arise *in vivo*, but may also differentiate from naive CD4+ T cells in the presence of IL-10 in vitro. Tr1 cells have a low proliferative capacity, which can be overcome by IL-15. Tr1 cells exert their suppressive activity via production of IL-10 and TGF-beta (60, 61). Moreover, T helper 3 has been also proposed to be an additional regulatory T cell population (62). However, the precise role of these and other regulatory T cells is still far from being fully elucidated (63).

5. CD8+ T CELLS CAN BE THEMSELVES EFFICIENT REGULATORS OF THE IMMUNE RESPONSE

More recently, increasing evidence has been provided that CD8+ T cells not only represent a powerful pro-inflammatory arm of the adaptive immune system, but, paradoxically, they can play a pivotal role also in suppression of T cell response (64-69). In this regard, we have recently reported evidence that hepatitis C virus (HCV)-specific CD8+ T cells producing IL-10 can be isolated from livers of patients with chronic hepatitis C. These cells appears to have a pivotal role in controlling the magnitude of immune responses sustaining both a long-lasting virus/host symbiosis and a constant pool of memory cells instrumental for providing concomitant immunity. These cells appear necessary to avoid self-destructive reactions, also considering that HCV is not a cytopathic virus, and liver damage during chronic infection is mostly immune-mediated. It can be proposed that effector CD8+ T cells undergo programmed contraction even if infection persists, in order to prevent the maintenance of high frequencies of non-protective CD8+ T cells, that may be detrimental for the host. CD8+ T cells with regulatory function may therefore intervene in the attempt to minimize pathological responses by the continuous waves of effector CD8+ T cells generated in response to persisting infection. This on the one hand allows the generation of stable number of memory cells, while controlling an excessive viral replication on the other hand (70). Similarly, in a murine model of EAE, tolerance was observed via the induction of Ag-specific CD8+ T regulatory cells capable of suppressing T cell immunity (66). Moreover, a new subset of natural CD8 regulatory T cells has been recently identified in normal healthy animals. This subset expresses low levels of CD45RC at its surface (CD45RC^{low}), produces mainly IL-4, IL-10, and IL-13 upon in vitro stimulation, expresses Foxp3 and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), and is not cytotoxic against allogeneic targets (71).

Finally, it must be emphasized that CD8+ T cell tolerance seems also to play an important protective role for survival of the fetus. In this regard, it has been shown that human leukocyte antigen (HLA)-G expressed on trophoblast cells during pregnancy protects cells from lysis by allo-cytotoxic T lymphocytes. It has been proposed that HLA-G molecule, in either a membrane-bound or soluble form might exert its immunosuppressive activity by inducing apoptosis of activated CD8+ T cells (72, 73).

6. CONCLUDING REMARKS

Peripheral CD8+ T cell tolerance is essential to avoid autoimmunity. This is accomplished by the immune system through different strategies, including cross-tolerance presentation of exogenous antigens to autoreactive T cells by improperly activated DCs, ignorance of non-accessible self proteins, impaired function of CD4+ T cells, presence of both CD4+ or CD8+ T cells with regulatory/suppressor activity. The failure or impairment of one or more of these mechanisms can lead to the onset of autoimmune diseases. The better understanding of the circumstances under which CD8+ T cells lose their tolerance to self-Ags is important for the development of safe intervention strategies to prevent these diseases. The possibility to modulate DC function or the expansion and activity of regulatory T cells may prove to be attractive strategies to regain tolerance to self (74-79). There are still however a lot of puzzles to solve in CD8+ T cell tolerance research, and further studies are necessary to translate our basic knowledge into disease treatment.

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