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ORIGINAL

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# Non-antigen specific CD8+ T suppressor lymphocytes

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**Abstract** The homeostasis of peripheral immune system function is maintained by the activity of regulatory lymphocytes. Among these cells, a subset of CD8+CD28- T suppressor lymphocytes has recently been characterized for the capacity to mediate their effects without antigen restriction. These non-antigen-specific CD8+ T suppressor lymphocytes originate from circulating CD8+CD28- T lymphocytes after stimulation with interleukin-2 and interleukin-10. CD8+ suppressor cells inhibit both antigen-specific CD4+ T cell proliferation and cellular cytoxicity through secretion of cytokines such as interferon-γ, interleukin-6, and interleukin-10. The function of CD8+ suppressor cells is impaired in patients with systemic lupus erythematosus in relapse as well as in patients with systemic sclerosis with disease progression, suggesting the involvement of CD8+ suppressor cells in the pathogenesis of autoimmune diseases. Interestingly, CD8+ suppressor cells have been found among tumor-infiltrating lymphocytes, which could be related to tumor-induced-immunosuppression. Failure to generate CD8+ suppressor cells from the peripheral blood is frequently observed in HIV-infected patients. It remains to be clarified whether this phenomenon is due to depletion and/or functional impairment of this cell subset or to their compartmentalization in peripheral tissues and immunocompetent organs where they could contribute to the induction of immunodeficiency.

**Key word** CD8+ T suppressor lymphocytes • Non-antigen-specific • Cytokines • Immunosuppression

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## Introduction

Autoreactive lymphocytes are a normal part of the immune system [1–3]. Studies in the last decade of thymocyte selection have emphasized that the T lymphocyte repertoire is constituted by cells that are positively selected on the basis of a certain level of reactivity with autoantigens [2]. Furthermore, the high degree of degeneracy of T cell receptor (TCR) antigen recognition implies that T cells have an elevated probability of cross-reacting with autoantigens [3]. The function of autoreactive lymphocytes needs to be strictly controlled at the periphery to avoid the onset of autoimmune responses. Among the several mechanisms used by the immune system to regulate its own activity, the function of regulatory T cell clones is considered to be of great importance. The immunoregulatory cellular network appears to be quite complex. Different subsets of T lymphocytes with regulatory/suppressor activities have been identified in both the CD4+

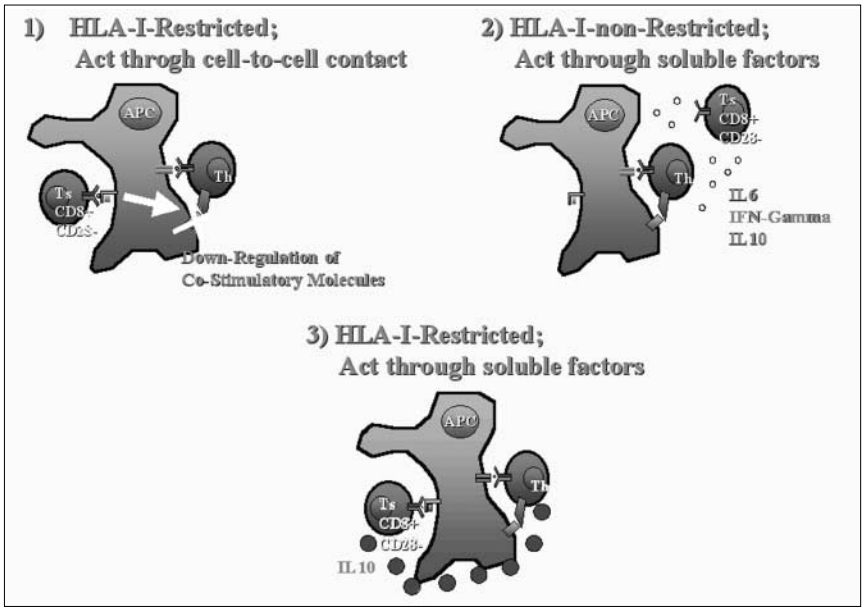


**Fig. 1** Different types of CD8+ T suppressor (*Ts*) cells and their relative mechanisms of action (*Th* T helper cells, *APC* antigen-presentig cell, *IL6* interleukin-6, *IL10* interleukin-10, *IFN-Gamma* interferon-γ)

and the CD8+ T cell compartments. The existence of CD8+ T suppressor cells (CD8+ Ts), was initially proposed in the early 1970s by Gershon and Kondo [4]. Unfortunately, their experiments did not fully convince the immunological community, so that for years several immunologists questioned their existence [5]. It required a decade before CD8+ Ts were considered important for immune regulation, following the isolation of CD8+ T cell clones able to inhibit antigen-specific CD4+ T cell proliferation [6]. When the transfer of resistance to experimental autoimmune disease (EAE) was associated with CD8+ T cell subpopulations [7], efforts for the characterization of CD8+ Ts in humans acquired new energy.

In the following years, two subsets of CD8+ Ts were identified in healthy subjects. The first type acts in an antigen-dependent manner via the transfer of inhibitory signals to antigen-presenting cells (APC) by direct cell-to-cell contact [8–10]. The second does not require antigen recognition and works via cytokine secretion [11, 12]. More recently, a third subset of immunoregulatory CD8+ T lymphocytes has been identified as being antigen specific and acting through interleukin (IL)-10 secretion [13]. Thus, immune regulation by the CD8+ T subsets is intricate involving cells that require antigen recognition and act differently (by cell-to-cell contact with APC, type 1, or by IL-10 secretion, type 3) as well as cells that are antigen independent (type 2) (Fig. 1). The reasons for the existence of different immunoregulatory CD8+ T cell subsets, their reciprocal interactions, their functional overlapping and specificities are unknown, and further work is needed to shed light on the complexity of this immunoregulatory CD8+-dependent network. The present review will focus specifically on the knowledge and recent discoveries relative to the non-antigen specific CD8+ Ts cells.

## Phenotype

Non-antigen-specific CD8+ Ts, as well as type 1 CD8+ Ts, are phenotypically characterized by the lack of expression of the CD28 co-stimulatory molecule [8, 11, 12]. The concentration of circulating CD8+CD28- T cells varies in the blood of healthy subjects [14]. These cells derive from prolonged stimulation of CD8+CD28+ cells [15, 16]. Thus, CD8+ Ts may be considered as T lymphocytes that have already undergone repeated activation, likely due to antigen stimulation. Our recent results confirm this assessment. We found that CD8+ Ts express the CD45RA but not the CD27 and the CCR7 antigens [17], a phenotype characterizing repeatedly activated effector CD8+ T cells [14, 18]. In accordance with this hypothesis, we found that the repertoire of TCR Vβ chain expressed by CD8+ Ts largely overlaps that of peripheral blood CD8+ T lymphocytes from which CD8+ Ts are generated [17].

## Precursors

Generation of non-antigen-specific CD8+ Ts is only possible from CD8+CD28- T cells. Massive cell death is observed when CD8+CD28+ T lymphocytes are cultured under conditions necessary for the generation of CD8+ Ts. Thus, peripheral blood CD8+CD28- T lymphocytes must be considered the circulating precursors of non-antigen-specific CD8+ Ts [17]. In particular, this suggests that the maturation to suppressor cells is not directly related to the down-modulation of the CD28 molecule from the cell membrane. Circulating CD8+CD28- T lymphocytes from healthy subjects do not

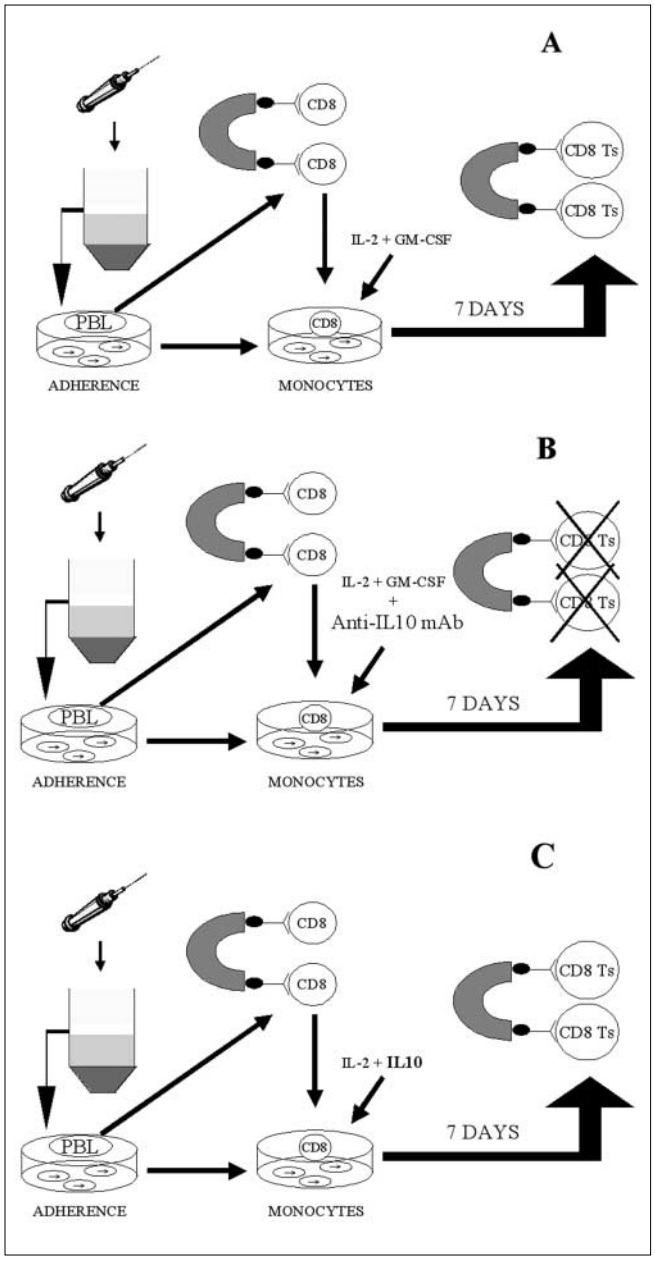


**Fig. 2** The generation of non-antigen-specific CD8+ Ts is IL-10 dependent. **A** Basic procedure for the in vitro generation of CD8+ Ts. After separation by centrifugation of peripheral blood on a Ficoll gradient, peripheral blood leukocytes (*PBL*) are incubated in culture plates for 1–2 h. Adherent cells are transferred to another culture plate; CD8+ T cells are purified from PBL through magnetic beads coated with an anti-CD8+ monoclonal antibody (*mAb*). Generation of CD8+ Ts occurs after 7 days of co-incubation in 96-well plates of purified CD8+ T cells ($1x10^5$ cells/well) and monocytes ($2.5x10^4$ cells/well) in the presence of IL-2 (20 U/ml) and granulocyte macrophage colony stimulating factor (*GM-CSF*) (10 ng/ml). **B** The generation of non-antigen-specific CD8+ Ts is abolished if an anti-IL10 mAb is added to the culture. **C** The generation of non-antigen-specific CD8+ Ts is also feasible by incubating purified CD8+ T lymphocytes without monocytes in the presence of IL-2 (20 U/ml) and IL-10 (40 ng/ml)

show suppressive activity and need a particular culture milieu to generate CD8+ Ts. Hence, we speculate that non-antigen-specific CD8+ Ts come from cells that have been already stimulated, losing the CD28 expression, and that are further induced to differentiate in to suppressor cells under specific culture conditions. On the basis of these considerations, we hypothesize that non-antigen-specific CD8+ Ts are effector lymphocytes specifically devoted to regulatory/suppressive functions instead of cytotoxic activity. Study of the pattern of gene expression of CD8+ Ts will likely clarify the metabolic adjustments needed by CD8+CD28- T lymphocytes to acquire suppressive functions.

The cytokine directly responsible for the commitment of CD8+CD28- T cells to suppressor cells is IL-10. Experiments performed in our laboratory clearly demonstrated that blocking IL-10 by a neutralizing antibody inhibits the generation of non-antigen-specific CD8+ Ts; furthermore, it was possible to generate these cells by simply incubating purified CD8+CD28- T lymphocytes with IL-10 in the absence of macrophages (Fig. 2) [17]. Thus a scenario is depicted in which danger signals activate a complex series of pro-inflammatory events, including secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF). However, this cytokine on the one hand supports inflammatory mechanisms [19, 20] and on the other induces the secretion of IL-10 by macrophages [21]. IL-10 acts on neighboring CD8+CD28- T lymphocytes, attracted to the tissue site by chemokines or present in the lymph nodes, inducing metabolic rearrangements that allow the expression of suppressive functions. Thus, the same cytokine (GM-CSF) appears to be involved in pro- and anti-inflammatory circuits, sustaining a functional balance that contributes to the establishment and maintenance of immunological homeostasis at the periphery.

## Activity

Antigen recognition and cell-to-cell contact are not required by CD8+ Ts to mediate their function [11, 12]. These cells induce suppression of antigen-specific as well as mitogen-dependent T cell proliferation. Their action targets equally well autologous and allogeneic T cells, an observation that indicates that HLA recognition does not occur [17]. Accordingly, when CD8+ Ts were incubated with anti-CD3-stimulated autologous T lymphocytes in wells in which the two T cell subpopulations were separated by a porous membrane, suppressive effects were maintained. Thus, soluble factors have to be invoked to explain the mechanism of action of non-antigen-specific CD8+ Ts. The study of intracytoplasmic cytokines of these cells showed the presence of interferon-γ (IFN-γ), IL-6, and IL-10 [12, 17]. Inhibitors specific for each of these cytokines were able to counteract the suppressive functions of CD8+

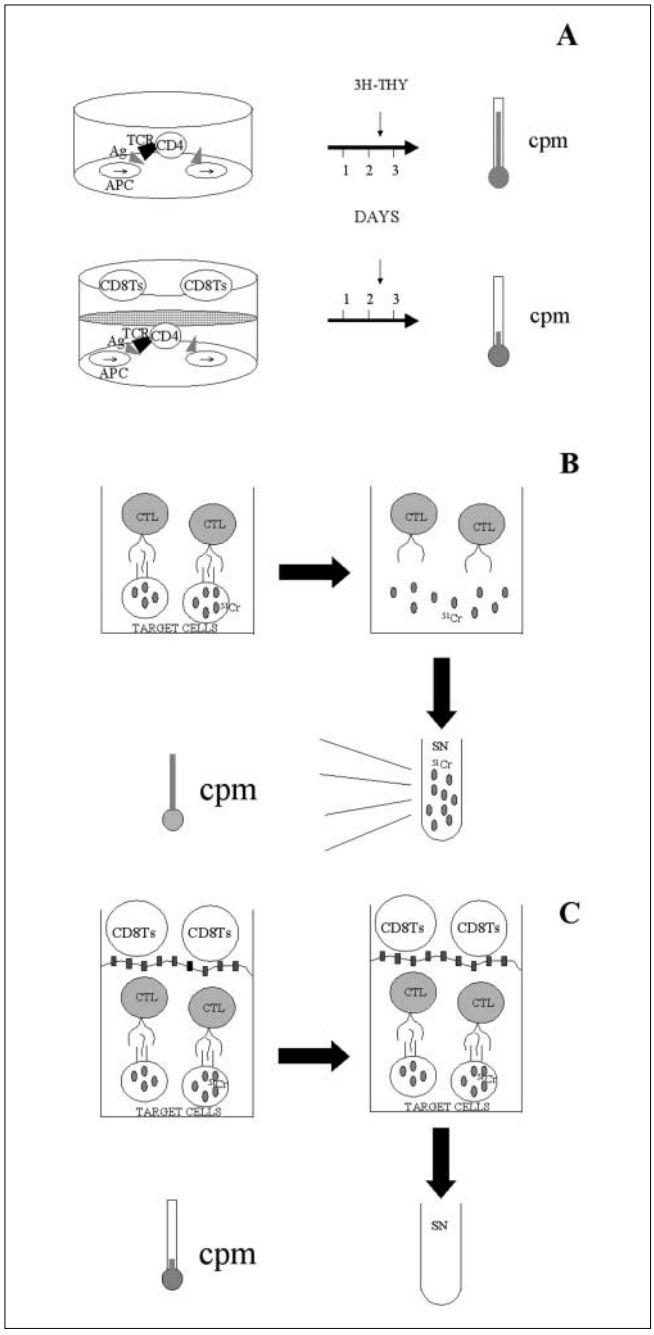


**Fig. 3** Non-antigen-specific CD8+ Ts inhibit both the antigen-specific proliferation of CD4+ T lymphocytes (**A**) and the antigen-specific cytotoxic activity of cytotoxic T lymphocytes (CTL) (**B** and **C**). **A** Schematic representation of an antigen-dependent CD4+ T cell proliferation assay based on the incorporation of 3H-thymidine by proliferating cells. **B** Schematic representation of a standard cytotoxic assay based on the labeling of target cells with $Na_2\,^{51}CrO_4$ and on the release of the radioactive tracer after cell killing by CTL. **C** Non-antigen-specific CD8+ Ts inhibit the antigen-dependent CTL activity through soluble factors, as demonstrated by the fact that the phenomenon occurs also when suppressor cells are separated from the CTL-target cell culture by a pored membrane (*Ag* antigen, *TCR* T cell receptor, *SN* supernatant after 4 h of culture)

Ts. Thus, suppression of T cell proliferation due to non-antigen-specific CD8+ Ts seems to be related to a complex of cytokines likely acting in concert.

Non-antigen-specific CD8+ Ts also antagonize the cytotoxic function of antigen-specific cytotoxic T lymphocytes (CTL). This effect does not require cell-to-cell contact and is mediated by IL-10 [17]. Hence, both CD4+ and CD8+ T cell subpopulations, and the relative functions, are inhibited by the effects of CD8+ Ts (Fig. 3). This suggests that in the presence of non-antigen-specific CD8+ Ts the immune response is profoundly suppressed, and that this effect is not restricted to a specific T cell clone. Thus, non-antigen-specific CD8+ Ts are potent regulators of the immune response and likely themselves need a strict control to avoid a complete block of the immune system function.

## CD8+ Ts and diseases

CD8+ Ts seem to be involved in the pathogenic mechanisms of diseases. Failure to generate non-antigen-specific CD8+ Ts is associated with the development of relapses in patients whith multiple sclerosis and systemic lupus erythematosus [11, 12]. Recently, we studied a series of 11 patients with diffuse systemic sclerosis (Table 1). We took into consideration clinical parameters relative to skin, microvascular, esophageal, pulmonary and renal localization as tools to monitor the progression of the disease. Seven patients had disease progression due to worsening of clinical parameters in the preceding 6 months, while no signs of progression were detected in the other four patients. The two groups of patients did not differ in age, gender, disease duration, and severity. In all patients in progression the generation of non-antigen-specific CD8+ Ts was impaired; this was not the case in the four patients with stabilized disease (Fig. 4). Thus, impairment of generation/function of non-antigen-specific CD8+ Ts is a common pattern in the autoimmune diseases studied to date. Studies are in progress to define whether these alterations result from an in situ compartmentalization of the CD8+ Ts with alienation from the circulation, or from physical and/or functional depletion.

From the immunological point of view, neoplasias constitute “the other site of the coin” with respect to autoimmune diseases. Generation of regulatory/suppressor cells may be helpful for the tumor to induce and sustain immunological tolerance against the tumor itself. Interestingly, we found that the supernatant of a GM-CSF-producing melanoma cell line was able to support the generation of CD8+ Ts lymphocytes starting from peripheral blood CD8+ T cells. This prompted studies to search for infiltrating CD8+ Ts at the tumor sites. Preliminary results from our laboratory in a series of 16 cancer patients with different histological types of disease showed the presence

**Table 1** Scleroderma patients

| Patient no. | Gender | Age (years) | Main clinical manifestations | Treatment | Disease progression |
|---|---|---|---|---|---|
| 1 | F | 68 | Skin, lung | *N*-Acetylcysteine | Yes |
| 2 | F | 51 | Skin | Cyclosporin A | Yes |
| 3 | F | 60 | Skin, lung, esophagus | Cyclosporin A | No |
| 4 | F | 38 | Skin | Cyclosporin A | Yes |
| 5 | M | 60 | Lung | *N*-Acetylcysteine | Yes |
| 6 | F | 53 | Heart | Cyclosporin A | No |
| 7 | F | 48 | Skin | Iloprost | No |
| 8 | F | 60 | Skin, lung, esophagus | Iloprost | Yes |
| 9 | M | 57 | Skin, esophagus | Iloprost | Yes |
| 10 | F | 34 | Skin, lung, esophagus | Cyclosporin A | Yes |
| 11 | F | 55 | Skin, esophagous | Iloprost | No |

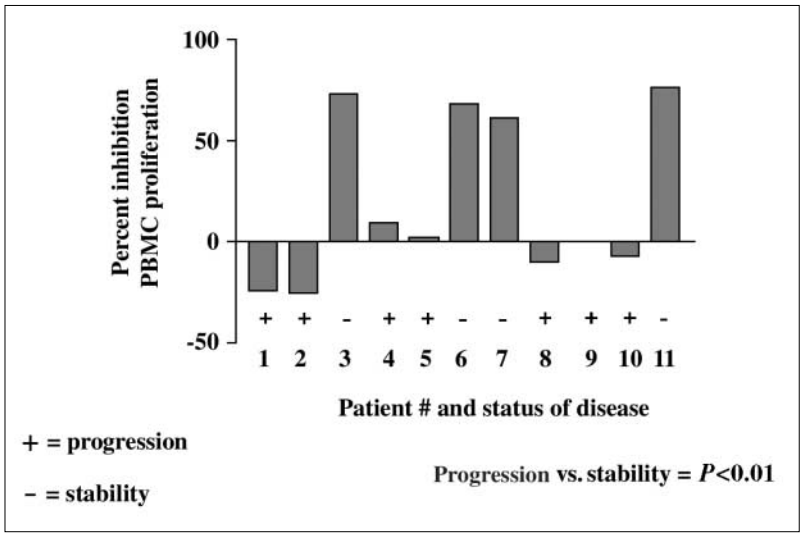


**Fig. 4** Impaired function of non-antigen-specific CD8+ Ts in patients with systemic sclerosis and disease in progression. A statistically significant difference ($P<0.01$) was detected by Mann-Whitney test between the percentage suppressor activity of cells from patients with stabile disease and those patients with progression

**Table 2** CD8+ T suppressor lymphocytes (Ts) infiltrates tumor lesions (*NA* not available)

| Patient no. | Cancer | CD8+Ts in: | | | |
|---|---|---|---|---|---|
| | | Primitive mass | Metastatic lymph nodes | Non metastatic lymph nodes | Peripheral blood |
| 1 | Kidney | NA | Yes | No | Yes |
| 2 | Kidney | Yes | Yes | NA | No |
| 3 | Thyroid | Yes | Yes | NA | Yes |
| 4 | Head-neck | NA | Yes | NA | Yes |
| 5 | Colon | NA | Yes | NA | Yes |
| 6 | Gastric | NA | Yes | NA | Yes |
| 7 | Pancreas | NA | Yes | NA | No |
| 8 | Colon | NA | Yes | NA | Yes |
| 9 | Sigma | Yes | Yes | NA | Yes |
| 10 | Colon | NA | Yes | NA | Yes |
| 11 | Breast | Yes | Yes | NA | Yes |
| 12 | Gastric | Yes | Yes | NA | Yes |
| 13 | Liver | Yes | Yes | NA | Yes |
| 14 | Liver | Yes | NA | No | Yes |
| 15 | Melanoma | NA | Yes | NA | NA |
| 16 | Colon | Yes | Yes | No | Yes |

of non-antigen-specific CD8+ Ts in metastatic satellite lymph nodes and/or in the primitive tumor mass but not in non-metastatic lymph nodes (when available) (Table 2). Taken together, these results suggest that tumors can take advantage of the in situ generation of CD8+ Ts due to the potent suppressor activity of these lymphocytes. Indeed, the blockage of both the T helper/inducer and the cytotoxic/effector arms of the immune system potentially mediated by these cells may cause a profound impairment of antitumor immune responses.

It is likely that regulatory lymphocytes, including non-antigen-specific CD8+ Ts, are involved in pathogenic mechanisms of other diseases with immunological involvement. We are conducting studies to verify the possibility of generating non-antigen-specific CD8+ Ts from HIV-infected patients. The results so far obtained in a series of 20 HIV-infected patients demonstrate that generation of non-antigen-specific CD8+ Ts from peripheral blood is impaired in the great majority (16 of 20) independent of the stage of disease and the type of HAART therapy. It remains to be clarified whether this phenomenon corresponds to a physical/functional deletion of suppressor lymphocytes or to the migration of these cells to the immune-competent sites where they could contribute to the onset and maintenance of the immunodeficiency.

## Conclusions

Technical and speculative reasons for a long time halted the study of regulatory lymphocytes and in particular on CD8+ T suppressor cells. However, experiments in the last 10 years have highlighted both the existence and the functional importance of these cell subpopulation for the maintenance of immune homeostasis. Several regulatory/suppressor lymphocyte subsets have been identified to date, possessing some overlapping functional features but also differences. Both the mechanisms regulating activation and suppression of their cell function and the interconnections among the different subsets are still poorly understood.

Studies of the non-antigen-specific CD8+ Ts, a peculiar subpopulation of CD8+ T suppressor cells, have highlighted their pleiotropic function. These cells are able to strongly inhibit the antigen-specific proliferation of CD4+ T lymphocytes as well as the cytotoxic activity of antigen-specific CTL. Furthermore, the activity of these cells is neither antigen induced nor antigen specific. For this reasons these cells represent a potent effector cell type committed to immune suppression. Data relative to the analysis of their phenotype seem to support the concept that they may be considered as effector cells mediating suppression. These cells may be involved in the pathogenic processes of chronic inflammatory diseases with immunological involvement, either due to an impairment of their function (autoimmune diseases) or due to an unregulated function (tumors, HIV infection).

The last aspect that must be emphasized is that, based on the biological characteristics of these cells, their use in protocols of cell-mediated immunotherapy in autoimmune diseases can be envisaged. Ex vivo expansion of autologous non-antigen-specific CD8+ Ts followed by re-infusion during relapses might be useful to lower the level of the autoimmune reaction and to facilitate the return to immune homeostasis.

In conclusion, the study of non-antigen-specific CD8+ Ts is clarifying aspects of immunoregulation and posing new questions on the cellular/functional network devoted to the maintenance of immune homeostasis. Moreover, it is opening up possibilities for the establishment of new protocols of immunotherapy of autoimmune diseases.

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