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Effector CD4 cell tolerization is mediated through functional inactivation and involves preferential impairment of TNF- α and IFN- γ expression potentials

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Abstract

It has recently been shown that effector/memory T cells can undergo peripheral tolerization in response to self-antigen. In the present study, we found that within 24 h self-antigen profoundly impairs the ability of CD4 effectors to express TNF- α (and to a lesser extent IFN- γ); however, several days of self-antigen exposure is required to impair non-effector functions such as IL-2 expression and proliferation. Since only half of the initial effector CD4 cell population expresses effector cytokines following brief antigenic stimulation, tolerization might have been mediated either through functional inactivation of effector-competent cells, or alternatively by the selective deletion of competent and expansion of non-competent cells. When briefly stimulated effectors were fractionated based on their expression of IFN- γ , the IFN- γ ⁻ sub-population was able to express IFN- γ following secondary stimulation, indicating that all effector CD4 cells are functionally competent. Furthermore, both IFN- γ ⁺ and IFN- γ ⁻ sub-populations underwent tolerization in response to self-HA (although the former was slightly more prone to deletion at later time points). Thus, effector CD4 cell tolerization is mediated primarily through the functional inactivation of effector-competent cells.

Keywords

Peripheral T cell tolerance; Effector CD4 cells; Cytokines; Transgenic mice

1. Introduction

Mature T cells specific for self-antigens expressed exclusively in non-lymphoid tissues usually undergo a process of peripheral tolerization. Although peripheral tolerization has been extensively studied with regard to naïve T cells that encounter cognate self-antigen, it has only recently been demonstrated that self-antigen can also induce effector/memory T cells to undergo peripheral tolerization [1,2]. The ability of the immune system to tolerize effector/memory T cells might limit the extent of autoimmune damage that ensues when self-reactive clones that initially avoided tolerization are primed to cross-reactive pathogen-derived epitopes (i.e., molecular mimicry [3–8]). Additionally, this pathway might also limit the potency of tumor-reactive effector T cells, since tumor-antigens can be presented in a tolerogenic manner [9–11].

To study the process of peripheral tolerization in effector CD4 cells, we used our previously established system in which naïve clonotypic T cell receptor (TCR) transgenic CD4 cells

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specific for influenza hemagglutinin (HA) are adoptively transferred into non-transgenic (NT) recipients and primed with a recombinant vaccinia virus expressing HA (vacc-HA) to differentiate into resting Th1 effectors, and are then induced to undergo tolerization following retransfer into secondary recipients that express HA as a parenchymal self-antigen [1]. Following only one day of self-HA exposure, effector CD4 cells exhibited a dramatic reduction in their ability to express the effector cytokine TNF- α (and to a lesser extent IFN- γ) in response to in vitro stimulation with HA peptide-pulsed APCs. Longer periods of self-HA exposure were required to impair IL-2 expression and proliferative potentials.

Since only half of the initial effector CD4 cell population expresses effector cytokines following brief antigenic stimulation, we wanted to determine whether tolerization was mediated by functional inactivation of effector-competent cells, or rather by the selective deletion and/or expansion of cells possessing different functional capacities. During the earliest stages of tolerization (24 h), effector CD4 cells have neither divided, nor changed in frequency, inferring that they have not undergone deletion. Furthermore, when effector CD4 cells were fractionated into sub-populations that either did or did not express the effector cytokine IFN- γ following in vitro stimulation, both sub-populations were found to behave similarly when retransferred into mice expressing either self-HA or viral-HA. Although this result confirmed that the loss of effector cytokine expression potential is the result of functional inactivation of effector-competent CD4 cells, additional evidence suggested that the later stages of tolerization also involve a non-selective deletional component.

2. Materials and methods

2.1. Mice

All adoptive transfer recipients were on the B10.D2 (H-2^d), Thy1.2⁺ background. C3-HA^{high} transgenic adoptive transfer recipients express influenza HA (A/PR/8/34 Mount Sinai Strain) under the control of the rat C3(1) promoter, which directs HA expression to a variety of non-lymphoid organs, and have been previously described [12,13]. Viral-HA adoptive transfer recipients are NT mice inoculated i.p. with 1×10^6 pfu of vacc-HA (a recombinant vaccinia virus that expresses the identical HA peptide as the C3-HA^{high} transgenics) one day prior to adoptive transfer as previously described [1,14]. 6.5 TCR transgenic mice (adoptive transfer donors) express a clonotypic TCR that recognizes an I-E^d-restricted HA epitope (¹¹⁰SFERFEIFPKE¹²⁰) [15], and were backcrossed to a RAG-sufficient B10.D2, Thy1.1⁺ congenic background.

2.2. Adoptive transfer and flow cytometry

Adoptive transfers of resting Th1 effector Thy1.1⁺ CFSE-labeled 6.5 clonotypic CD4 cells (depleted of CD8 cells) into Thy1.2⁺ recipients, vaccinia inoculations, and subsequent functional analyses were performed as previously described [1,14], with the following modifications. Double-cytokine intracellular staining was performed following in vitro stimulation of clonotypic CD4 cells with HA peptide (100 μ g/ml) for 6 h, and then staining using anti-Thy-1.1 PerCP (BD Pharmingen, San Diego, CA), fixation (2% formaldehyde (Sigma) in PBS) and permeabilization (0.25% saponin (Sigma)), followed by staining with either anti-IL-2 APC or anti-TNF- α APC and either anti-IFN- γ PE or anti-TNF- α PE (eBioscience, San Diego, CA) as indicated. All FACS samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

2.3. Isolation and retransfer of IFN- γ ⁺ and IFN- γ ⁻ effector CD4 cell sub-populations

IFN- γ ⁺ and IFN- γ ⁻ effector CD4 cell sub-populations were isolated by affinity matrix capture technology using the Mouse IFN- γ Secretion Assay and Cell Enrichment and Detection Kit (PE*) (Miltentyi Biotec, Auburn, CA) as per the manufacturer's instructions. In short, $1 \times$

10⁸ spleen cells containing day 6 vacc-HA-primed resting clonotypic Th1 effector CD4 cells were stimulated with HA peptide (50 µg/ml) for 3 h, washed in cold buffer, labeled with a bispecific anti-CD45-anti-IFN-γ capture Ab on ice, and then diluted into warm media (6 × 10⁴ clonotypic CD4 cells/ml) and incubated for 45 min at 37 °C with gentle mixing. After washing in cold buffer, the matrix-captured IFN-γ was detected with PE-conjugated anti-IFN-γ, and then labeled with anti-PE microbeads to allow separation of IFN-γ⁺ and IFN-γ⁻ cells by passage over a MACS column. Subsequently, equal numbers of both IFN-γ⁺ and IFN-γ⁻ cells were relabeled with CFSE, retransferred into the indicated secondary adoptive transfer recipients, and recovered for analysis 5 days later from both the spleen and lungs following perfusion as previously described [1].

3. Results

3.1. Tolerization of effector CD4 cells involves rapid loss of TNF-α expression potential

We have previously established an in vivo system to study the peripheral tolerization of Th1 effector CD4 cells in response to parenchymal self-antigen in which HA-specific naïve clonotypic TCR transgenic CD4 cells are adoptively transferred into NT mice that have been inoculated with a recombinant vaccinia virus that expresses HA (vacc-HA) and allowed to differentiate into resting Th1 effectors, and are subsequently recovered and retransferred into secondary recipients that express HA as a parenchymal self-antigen. Although these clonotypic effector CD4 cells initially undergo a strong proliferative response upon encountering self-HA, they ultimately lose the ability to undergo cell cycle progression as well as the potential to express IFN-γ and IL-2 [1]. To extend this analysis, we also examined the ability of effector CD4 cells to express the Th1 effector cytokine TNF-α at both early and later stages of this tolerization process (Fig. 1).

When day 6 resting clonotypic effector CD4 cells were relabeled with CFSE and retransferred into secondary hosts that express self-HA (C3-HA^{high} transgenics, S), and cytokine expression potential was assessed following recovery from spleens 24 h later by analyzing intracellular cytokine staining following in vitro restimulation with HA peptide-pulsed APCs, TNF-α expression potential was found to be 17-fold lower than in clonotypic effector CD4 cells retransferred into viral-HA (NT + vacc-HA, V) immunogenic controls, and 19-fold lower compared to day 7 primary resting clonotypic effectors (1°). IFN-γ expression potential increased several-fold following retransfer into viral-HA secondary recipients, while self-HA induced only a marginal decrease (both compared to day 7 primary resting effectors). Nonetheless, IFN-γ expression potential was 4.5-fold lower in self-HA compared to viral-HA exposed clonotypic effectors. IL-2 expression potential was not markedly effected by either self-HA or viral-HA. Following 8 days of self-HA exposure, IFN-γ expression potential was reduced to one-sixth the level of time-matched (day 14) primary resting effectors, although the ratio of viral-HA to self-HA IFN-γ expression potential remained similar to the earlier time point (~5-fold) due to the former returning to resting levels. Additionally, at the day-8 time point, self-HA induced a modest impairment in IL-2 expression potential (~3-fold), while TNF-α expression potential remained low.

3.2. The initial phase of effector CD4 cell tolerization is mediated through functional inactivation

Since approximately half of the clonotypic effector CD4 cells do not express cytokines following in vitro peptide restimulation directly prior to self-HA exposure, but that nearly all of them undergo robust proliferation thereafter [1], it was not clear whether tolerization is mediated through functional inactivation of effector-competent cells, or rather by selective deletion of competent, and expansion of non-competent cells. We did not think that the latter possibility was likely, in part because effector non-competent CD4 cells might not be expected

to have a high proliferative potential. Furthermore, we observed a substantial impairment in cytokine expression potential (particularly for TNF- α) only 24 h following exposure to self-HA (Fig. 1). At this early time point, the frequency of self-HA-exposed clonotypic CD4 cells was on slightly less than viral-HA-exposed counterparts, suggesting a lack of significant cell division or death. Nonetheless, although naïve clonotypic CD4 cells require at least 24 h of in vivo antigenic stimulation to initiate division (data not shown), it was possible that the effector clonotypic CD4 cells had undergone limited division within 24 h of tolerogen exposure since effector CD4 cells require a shorter period of antigenic stimulation to commit to cell division than do naïve counterparts [16]. To test this possibility, the clonotypic effectors recovered from viral-HA and self-HA secondary recipients in Fig. 1 were analyzed for their proliferative time course (Fig. 2). Twenty four hours post-retransfer into both viral-HA and self-HA secondary recipients, clonotypic effector CD4 cells were mostly blasting (i.e., they exhibited high forward light scatter (FSC)), but had not yet divided (i.e., undergone CFSE-dilution). Eight days post-retransfer, both recipient groups exhibited fully diluted CFSE intensity, but were mostly non-blasting. This result is consistent with our previous observation that following exposure to self-HA, clonotypic effector CD4 cells initially undergo a robust proliferative response, but eventually lose the ability to undergo cell cycle progression despite the continual presence of cognate antigen [1]. Taken together, these data indicate that at an early time point in the tolerization process when TNF- α expression potential has already become profoundly impaired (Fig. 1B), clonotypic effectors have neither undergone division (Fig. 2), nor has their frequency been significantly altered (Fig. 1B), inferring that they have not undergone deletion. Thus, the initial phase of tolerization appears to be mediated through functional inactivation.

3.3. The response of fractionated effector CD4 cell sub-populations during tolerization

Although the previous experiments indicated that the initial stage of effector CD4 cell tolerization results from functional inactivation rather than the selective deletion and/or expansion of cells with different functional capacities, the latter mechanism might have been operative during the later stages of the tolerization process. To investigate this possibility, we used affinity matrix capture technology [17] to fractionate the primary clonotypic resting effector CD4 cell population into sub-populations that appeared to be either effector-competent or non-competent (based on their ability to express IFN- γ following a short in vitro stimulation with HA peptide), in order to study their individual responses following encounter with either self-HA or viral-HA. We used IFN- γ expression as the criterion for fractionation in part because it differentiates the initial clonotypic resting effector CD4 cell population into roughly equal halves [1]. When day 6 resting clonotypic effector CD4 cells were restimulated with HA peptide-pulsed APCs and stained for extracellular IFN- γ using the affinity matrix capture technique, ~40% stained positively (roughly comparable to intracellular staining data [1]). Following passage over a MACS column, the IFN- γ^+ (i.e., column-bound, C) sub-population was purified to 98% homogeneity. There was a substantial amount of IFN- γ^+ cells contaminating the IFN- γ^- (i.e., flow-through, FT) fraction (~20%), however, they all stained weakly for IFN- γ (Figs. 3A and B), presumably explaining why they bound less well to the MACS column. Interestingly, when the IFN- γ^- sub-population was restimulated with HA peptide-pulsed APCs, ~80% became IFN- γ^+ as assessed by intracellular staining. Most of these cells also expressed TNF- α (Figs. 3A and C). Since naïve clonotypic CD4 cells do not efficiently express IFN- γ under similar stimulation conditions (data not shown), presumably because the chromatin region containing the IFN- γ gene only exists in a de-condensed (i.e., accessible) conformation in primed Th1 effectors [18–22], this result indicated that virtually all of the vacc-HA primed clonotypic CD4 cells had become competent to express effector cytokines, even though only half express them following brief antigenic stimulation.

To follow the individual fates of the IFN- γ^+ and IFN- γ^- clonotypic effector CD4 cell sub-populations during tolerization, they were re-labeled with CFSE, retransferred into self-HA

and viral-HA secondary recipients, and recovered for analysis from the spleen and lungs 5 days later (Fig. 4). Consistent with a previous report that IFN- γ^+ effector CD4 cells are short-lived *in vivo* [23], we found that this sub-population persisted less well than IFN- γ^- counterparts in both the spleen and lungs of both recipient groups. Even though both sub-populations underwent robust and comparable CFSE dilutions in all cases (Fig. 4A), we consistently recovered a lower frequency of clonotypic CD4 cells in IFN- γ^+ relative to IFN- γ^- recipients (Figs. 4A and B). Interesting, the frequency of both IFN- γ^+ and IFN- γ^- clonotypic effector CD4 cells was consistently lower in both organs of self-HA relative to viral-HA recipients (Figs. 4A and B), suggesting that deletion also plays a role in effector tolerization. Although the frequency of un-fractionated effectors was equivalent in self-HA vs. viral-HA recipients 8 days post-retransfer (Fig. 1C), it is worth noting that less than one-tenth the number of fractionated effectors were retransferred compared to un-fractionated effectors, suggesting that deletion might occur more readily as the frequency of effectors decreases. Overall, the ability of the IFN- γ^+ and IFN- γ^- sub-populations recovered from both viral-HA and self-HA secondary recipients to express IFN- γ and TNF- α following *in vitro* restimulation (Figs. 4C and D) were similar to that of retransferred un-fractionated effectors (Fig. 1). Thus, both the IFN- γ^+ and IFN- γ^- sub-populations underwent equivalent secondary immunogenic and tolerogenic responses following encounter with viral-HA and self-HA, respectively.

4. Discussion

It is well established that naïve T cells are readily tolerized following encounter with cognate peripherally-expressed self-antigen, however, it has only recently been demonstrated that effector/memory T cells can be tolerized under the same physiological conditions [1,2]. While tolerization of self-reactive naïve T cells functions to prevent the initiation of autoimmunity, the ability of the immune system to tolerize effector/memory T cells might help to limit the extent of autoimmune damage that ensues when self-reactive T cells that initially avoided tolerization are primed by pathogens that express cross-reactive epitopes (i.e., molecular mimicry [3–8]). Effector T cell tolerization might also negatively impact immune-based therapies to treat cancer. Since tumor-antigens can be presented in a tolerogenic manner [9–11], tumor-reactive effector T cells elicited by vaccination or introduced by adoptive transfer might also be subject to tolerization. We find that effector CD4 cell tolerization involves a differential loss of function, with effector functions such as TNF- α expression (and to a lesser extent IFN- γ) being lost more rapidly and profoundly than non-effector functions such as IL-2 expression and proliferation. This observation may have physiological significance, since both IFN- γ and TNF- α play critical roles in various models of autoimmunity and tumor immunity [24–33]. Thus, if effectors selectively lose their ability to produce TNF- α and IFN- γ , but not their ability to produce IL-2 and proliferate, they would likely be less effective in mediating autoimmune pathology or tumor neutralization than if the opposite functional loss occurred. From a mechanistic perspective, the observation that TNF- α , IFN- γ and IL-2 expression potentials are lost at different rates and magnitudes during tolerization suggests that there are unique elements in the signal transduction pathways leading to the expression of these three cytokines that are differentially sensitive to tolerogen.

Prior to tolerization, the resting Th1 effector CD4 cell population is heterogeneous with regard to the ability to express effector cytokines following brief *in vitro* antigenic stimulation, suggesting that tolerization is either mediated through functional inactivation of effector competent cells, or alternatively by the selective deletion of the most competent, and expansion of the least competent cells. Following only 24 h of tolerogen exposure, effector CD4 cells exhibit a profound impairment in TNF- α expression potential, but have neither divided, nor significantly changed in frequency, inferring that they have not undergone deletion. Thus, functional inactivation appears to operate during the initial phase of tolerization. When the heterogeneous effector CD4 cell population is fractionated into IFN- γ^+ and IFN- γ^- sub-

populations, the latter is revealed to be highly competent to express both IFN- γ and TNF- α following secondary stimulation, suggesting that the heterogeneity in cytokine expression following the initial antigenic stimulation is not reflective of an inherent functional heterogeneity. Although unequal stimulation of T cells during the in vitro culture might account for the heterogeneity in cytokine expression, a recent study suggests that a stochastic process that is cell intrinsic might also contribute to this effect [34]. Nonetheless, the ability of IFN- γ ⁻ effectors to express IFN- γ and TNF- α following a secondary in vitro stimulation, in conjunction with the observation that both IFN- γ ⁺ and IFN- γ ⁻ sub-populations undergo comparable tolerogenic and immunogenic responses following retransfer into self-HA and viral-HA secondary recipients, respectively, provides additional evidence that tolerization is not the result of a selective deletion and/or expansion of CD4 cells possessing different functional capacities. Since both sub-populations were found to be present at lower frequencies in self-HA compared to viral-HA secondary recipients 5 days post-retransfer, tolerization might also involve a non-selective deletional component that follows the initial phase of functional inactivation and cell division.

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Fig. 1.

Cytokine expression potentials of effector CD4 cells tolerized in response to self-antigen. Resting Th1 effector clonotypic CD4 cells (identified as Thy1.1⁺) were recovered from vacc-HA-infected NT primary adoptive transfer recipients, relabeled with CFSE, retransferred into secondary recipients expressing either viral-HA (NT + vacc-HA, V) or self-HA (C3-HA^{high} transgenics, S), and recovered from spleens either one or eight days later for analysis by performing intracellular staining for IFN- γ , TNF- α and IL-2 following in vitro restimulation with HA peptide. (A) Representative dot plots are shown for the day 8 analysis, with the percentages of cytokine-expressing clonotypic CD4 cells (and levels of IFN- γ and TNF- α expression (mean fluorescence intensity, MFI) in parentheses) shown for each quadrant (which were set based on staining with isotype control Abs, data not shown). Note that cytokine staining was never detected in the absence of peptide stimulation (data not shown). (B) Quantitative analysis of the day 1 time point. Total cytokine expression is calculated as the product of the % of cytokine positive clonotypic CD4 cells $\times$ the MFI per positively expressing cell, and expressed in arbitrary units as previously described [14]. For comparison, cytokine expression is also shown for time-matched primary resting clonotypic effector CD4 cells that had not been re-exposed to HA (1°). Additionally, the ratios of total cytokine expression for viral-HA vs. self-HA and 1° effectors vs. self-HA are shown, as well as the frequencies of recovered clonotypic CD4 cells. $N = 3$ for both viral-HA and self-HA groups, and $n = 6$ for 1° effectors (collected from two separate experiments). (C) Quantitative analysis of the day 8 time point was performed as in panel B. $N = 9$ for viral-HA, and 10 for self-HA (each collected from

three separate experiments), and $n = 3$ for 1° effectors. Data is expressed as the mean $\pm$ the standard error of the mean (SEM).

Fig. 2.

Resting effector CD4 cells require greater than 24 h to undergo division following exposure to tolerogen. Day 6 resting effector clonotypic CD4 cells were recovered from spleens of vacc-HA-infected NT primary adoptive transfer recipients, relabeled with CFSE, and their time course of blastogenesis (FSC) and division (CFSE-dilution) assessed following retransfer into vacc-HA and self-HA recipients and recovery from spleens. The plots shown are representative of 3–10 replicates for each group.

Fig. 3.

Separation and restimulation of IFN- γ^+ and IFN- γ^- effector CD4 cell sub-populations. (A) Day 6 resting effector clonotypic CD4 cells were restimulated in vitro with HA peptide, stained and fractionated for IFN- γ expression using affinity matrix capture technology (the percentage and level of IFN- γ expression (MFI) are shown, with the cutoff for IFN- γ expression determined by a parallel staining reaction that lacked capture antibody (data not shown)). The IFN- γ^- fraction (flow-through, FT) was subsequently restimulated with HA peptide and stained for intracellular IFN- γ vs. TNF- α . Data shown is representative of three separate experiments. Quantitative analyses ($n = 3$) of affinity matrix capture staining and separation (B), and IFN- γ and TNF- α intracellular staining of the IFN- γ^- fraction following restimulation with HA peptide (C), are presented as in previous figures.

Fig. 4. Response of retransferred IFN- γ ⁺ and IFN- γ ⁻ effector CD4 cell sub-populations. (A) Representative experiment (from a total of 3) in which IFN- γ ⁺ and IFN- γ ⁻ effector CD4 cell sub-populations fractionated in Fig. 3 were relabeled with CFSE, retransferred in equal numbers into C3-HA^{high} (self-HA, S) or NT + vacc-HA (viral-HA, V) secondary recipients, and recovered from the spleen and lungs following perfusion 5 days later. Histogram plots show the percentage of clonotypic CD4 cells (Thy1.1⁺) within total lymphocytes, as well as CFSE fluorescence of gated Thy1.1⁺ cells (a dashed line is placed directly to the left of the location of undivided cells). (B) Table showing the frequency of IFN- γ ⁺ and IFN- γ ⁻ clonotypic CD4 cells recovered from the spleen and lungs of self-HA and viral-HA secondary recipients for three separate experiments. (C) IFN- γ vs. TNF- α intracellular staining dot plots of recovered IFN- γ ⁺ and IFN- γ ⁻ clonotypic effector CD4 cells corresponding to panel A following in vitro restimulation with HA peptide. (D) Quantitative analysis of total intracellular IFN- γ and TNF- α staining following in vitro restimulation with HA peptide is presented as in previous figures ($n = 3$ for each group).