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Induction of Peripheral T Cell Tolerance by Antigen-Presenting B Cells. I. Relevance of Antigen Presentation Persistence

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Various mechanisms of peripheral T cell tolerization have evolved to avoid responses mediated by autoreactive T cells that have not been eliminated in the thymus. In this study, we investigated the peripheral conditions of Ag presentation required to induce T cell tolerance when the predominant APCs are B cells. We show that transient Ag presentation, in absence of inflammation and in a self-context, induces CD4+ T cell activation and memory formation. In contrast, chronic Ag presentation leads to CD4+ T cell tolerance. The importance of long-lasting Ag presentation in inducing tolerance was also confirmed in the herpes stromal keratitis autoimmune disease model. Keratogenic T cells could be activated or tolerized depending on the APC short or long persistence. Thus, when APCs are B cells, the persistence of the Ag presentation itself is one of the main conditions to have peripheral T cell tolerance. The Journal of Immunology, 2006, 176: 4012–4020.

The immune system of vertebrate animals has evolved to protect against perturbations provoked by incoming pathogens. Given the variety of pathogens it is necessary that the T cell repertoire be extremely diverse to allow elimination of all possible invading microorganisms. However, the generation of an immense T cell repertoire increases the possibility of developing autoreactive T cells. To limit self-tissue damages while maintaining T cell diversity, the immune system has therefore developed mechanisms for eliminating or rendering nonfunctional autoreactive T cells. Tolerance to tissue Ags is achieved through a combination of thymic and peripheral events that eliminate or inactivate potentially dangerous T cells (1). The thymus provides a very important initial step to eliminate potentially dangerous self-specific T cells (2). Nevertheless, many tissue proteins are not expressed in the thymus at a sufficient level to induce clonal deletion or tolerization (3). For this reason, several mechanisms of peripheral T cell tolerization have evolved and it has been shown that autoreactive T cells that actually recognize the Ag in the periphery can undergo anergy (4), deletion (5), or down-regulation of TCR (6) or coreceptors (7). Identifying inductive processes of tolerization is particularly relevant to reach a univocal definition of self and nonself and to define new therapeutic strategies for autoimmune disease, tumor, and transplant acceptance.

To investigate the peripheral conditions of Ag presentation that could lead to T cell tolerance in a model in which APCs are B cells, we took advantage of the IgG2aβ model (8). We have previously shown that T cells expressing at high density a TCR specific for the 435–451 peptide (Bpep) in the CH3 region of the IgG2aβ are not deleted in the thymus of Ighb animals (which produce IgG2aβ) and reach peripheral lymphoid organs (9). Nevertheless, there are some evidences that peripheral anti-IgG2aβ T cells can become tolerant. An example is given by herpes stromal keratitis (HSK), a HSV-1-induced autoimmune disease of the eye mediated by CD4+ T cells (3), to which Ighb animals are resistant. The CD4+ keratogenic T cell cross-recognize a corneal Ag and the Bpep (10), and the HSK resistance of Ighb animals has been unequivocally correlated with the tolerant state of their peripheral anti-IgG2aβ T cells. Thus, we investigated the mechanisms and conditions responsible for anti-IgG2aβ T cell tolerization. We set up an experimental model in which naive CD4+ T cells specific for the Bpep were transferred in mice showing persistent or transitory Bpep presentation. Because the Bpep is exclusively presented by IgG2aβ B cells in vivo (11), this system allowed us to investigate the conditions of in vivo T cell tolerization when T cells could only interact with B cells.

We show that, when the main APCs are B cells, persistence of Ag presentation could represent a major mechanism responsible for peripheral T cell tolerization.

Materials and Methods

Mice and experimental model

We have previously generated mice transgenic (Tg)3 for a TCR that recognize the 435–451 peptide (Bpep) in the CH3 region of IgG2aβ in association with I-Aβ (8). The Tg animals were obtained in different backgrounds: BALB/c, expressing the a and not the b allotype of IgG2aα; CB-17 expressing the IgG2aα; and TCR α-chain-deficient knockout (KO) BALB/c (αKOTgβb) and CB-17 (αKOTgβb+), to exclude endogenous α rearrangements (9). TCR α-chain-deficient BALB/c mice were the source of naive 2a T cells used in this work. To study the fate in the periphery of

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5 Abbreviations used in this paper: Tg, transgenic; KO, knockout; Bpep, 435–451 peptide of IgG2aβ; KLH, keyhole limpet hemocyanin; HSK, herpes stromal keratitis.
Ag-specific T cells once they encountered the Ag in absence of inflammation and without the continuous intervention of new thymic emigrants, we performed the adoptive transfer experiments described in the present work.

BALB/c, CB-17, and SCID mice were purchased from Harlan. C.AL-20 mice were from The Jackson Laboratory. αKOBALB/c mice (αKO-b\(^{-}\)) were provided by Dr. M. J. Owen (Imperial College, London, U.K.). They were backcrossed on CB-17 background to obtain αKO CB-17 mice (αKO-b\(^{-}\)). αKO-b\(^{-}\) mice were bred with αKOh\(^{-}\) mice to obtain (αKOh\(^{-}\) CB-17×BALT/c) F1 mice. All animals were kept in specific pathogen-free conditions.

**ELISPOT**

ELISPOT assay was performed as described (8).

**Cell preparations**

Naive TCR Tg anti-IgG2\(^{a}\) T cells (2a T cells) were purified from spleen and lymph nodes of αKOTg\(^{-}\) b\(^{-}\) mice. A total of 10\(^{6}\) cells/ml was stained with biotinylated anti-B220, anti-CD8, anti-CD11c, anti-CD11b, and anti-GR1 Abs (20 μg/ml), and washed and incubated with streptavidin MicroBeads (Miltenyi Biotec). Cells were then negatively selected on MS MACS separation columns according to Miltenyi Biotec instructions.

Small resting B cells were isolated from spleen of BALB/c mice. Splenic uncellular cell suspensions were incubated with anti-B220 (20 μg/ml) and anti-CD11c (20 μg/ml), and Abs, and cells were sorted by gating the small B220\(^{+}\) and CD11c\(^{+}\) population (purity 99%). T and B cells from keyhole limpet hemocyanin (KLH)-immunized CB-17 mice were purified from draining lymph nodes. Unicellular cell suspensions were stained with biotinylated anti-CD8, anti-CD11c, anti-CD11b, and anti-GR1 Abs (20 μg/ml), and washed and incubated with streptavidin MicroBeads (Miltenyi Biotec). Cells were then negatively selected on MS MACS separation columns according to Miltenyi Biotec recommendations.

**IgG2a\(^{b}\) serum levels**

To assess the amount of circulating IgG2a\(^{b}\) protein, blood samples were taken from the animals, and classic sandwich ELISA tests were performed. In brief, 96-well MaxiSorp immunoplates (Nunc) were coated overnight at 4°C with purified anti-mouse-IgG2a\(^{b}\) Ab, then blocked with PBS/BSA 3% for at least 2 h at room temperature. Thereafter, serial dilution of the samples (in PBS/BSA 0.5%) were transferred into the wells, along with dilution of purified IgG2a\(^{b}\) protein, and incubated overnight at 4°C. Biotinylated anti-mouse IgG2a\(^{b}\) Ab was then added and incubated for 2 h at 37°C and followed with 20 min incubation with streptavidin-HRP conjugate (room temperature). Developing substrate (tetramethylbenzidine; Sigma-Aldrich) was finally added for 20 min (room temperature) and reaction stopped with 2 N H\(_2\)SO\(_4\). Plates were read using a Hewlett-Packard reader.

All steps were interpolated with appropriate washing using tap water.

ELISA tests to assess circulating anti-KLH IgG2a\(^{b}\) were performed in the same way using different preliminary reagents: purified KLH in the coating phase, PBS plus 2% FCS to block wells, PBS 2% FCS to dilute samples and Abs, and PBS/Tween 20 0.05% in all washing steps.

**Adoptive transfer**

Naive 2a T cells were purified from lymph nodes and spleen of αKOTg\(^{-}\) b\(^{-}\) mice by magnetic negative selection, washed extensively in PBS, and then injected (1–2×10\(^{6}\) cells in 200 μl PBS/mouse) into recipient mice via the lateral tail vein. T cell response was followed over time from blood samples (taken on days 3, 5, 7, 15, 21, 28, and 35 after transfer) or lymph nodes.

**Flow cytometry**

Blood samples (50 μl) and single-cell suspensions of 1×10\(^{6}\) splenocytes or lymph node cells were pelleted and resuspended with the appropriate amount of Ab in 200 μl of PBS, and incubated for 30 min on ice in the dark. The cells were then washed once with 1 ml of PBS. Eventually, secondary reagent incubation in 100 μl of Quantum Red-conjugated streptavidin (diluted 1/100; Sigma-Aldrich) was performed for 20 min on ice in the dark. For FACS analysis, all Abs were purchased from BD Pharmingen.

The first-step Abs were: FITC- or PE-coupled anti-mouse CD4; FITC- or biotinylated-coupled anti-mouse V\(_{J}\)B14; FITC- or PE-coupled anti-mouse CD25; FITC- or biotinylated-coupled anti-mouse CD69; and FITC- or biotinylated-coupled anti-mouse CD44.

Analysis was performed using a BD FACScan and CellQuest software (BD Biosciences). Cell sorting was performed using a MoFlo FACS.

**In vitro T cell responsiveness**

2a T cells were purified, as previously described, from spleen and lymph nodes of αKOTg\(^{-}\) b\(^{-}\) mice or αKOb\(^{-}\) mice transferred 1 mo earlier with naive 2a T cells.

Purified 2a T cells (1×10\(^{6}\) cells/well) and small resting B cells (1×10\(^{6}\) cells/well) were resuspended in complete IMDM, plated in 24-well plate, and Bpep was added at different concentrations. After 4 h of coculture, V\(_{J}\)B14 down-regulation on 2a T cells was analyzed by FACSScan. After 24 h, clarified supernatants were tested for IFN-γ production, using IFN-γ Duo Elisa kit (R&D Systems).

**Anti-KLH IgG2a\(^{b}\)**

CB-17 mice were immunized s.c. with 200 μg of KLH (Sigma-Aldrich) in CFA (Sigma-Aldrich). Two weeks later, B and T cells from draining lymph node nodes were purified as earlier described and injected (4×10\(^{6}\) cells/mouse) i.p. in SCID mice together with 2a T cells (1.5×10\(^{6}\) cells/mouse) derived from αKOb\(^{-}\) mice transferred 1 mo earlier with naive 2a T cells. Transferred SCID mice were then i.p. injected with 200 μg of KLH in PBS, and 10 days later serum levels of anti-KLH IgG2a\(^{b}\) were measured by ELISA as previously described.

**Ocular infection and scoring of HSK**

Corneas of mice were scarified using a sterile gauge needle before infection with HSV-1 (5×10\(^{6}\) PFU) in the right eye and disease severity was scored on different days after infection as described (12) based on the degree of corneal opacity: 1, ≤25% of cornea; 2, ≤50%; 3, ≤75%; and 4, ≤100%. Incidence of disease is measured as the percentage of mice with a severity score ≥1.

**Results**

**Transitoriness of Ag presentation in steady state conditions induces T cell activation**

The fate of peripheral Ag-specific T cells after exposure to the cognate Ag at the steady state, which is absence of inflammation in a self-context, was followed in vivo. Naive CD4\(^{+}\) 2a T cells, specific for the Bpep of the IgG2a\(^{b}\), were purified by negative selection from TCR Tg α-chain-deficient (αKO) BALB/c (γ2a\(^{+}\)) animals (αKOTg\(^{-}\) b\(^{-}\)) and transferred into congenic αKO-CB-17 (IgG2a\(^{b}\)) mice (designated αKO-b\(^{-}\)), showing an average IgG2a\(^{b}\) serum level of 4–10 μg/ml. As control, 2a T cells were also transferred into αKOBALB/c (αKO-b\(^{-}\)) mice (γ2a\(^{+}\) mouse) (Fig. 1A).

As the Bpep is exclusively presented by IgG2a\(^{b}\), B cells in vivo even in mice that show very high Ig level in the serum (11), this system allowed us to measure a direct T cell-B cell interaction. As already observed in previous studies (13), T and B cells efficiently find each other in lymph nodes and spleen in absence of inflammation.

In αKOTg\(^{-}\) b\(^{-}\) mice around 34% of total CD4\(^{+}\) cells in blood, lymph nodes, and spleen expressed the Tg TCR (9). The remaining CD4\(^{+}\) population was TCR-negative or expressed endogenous β-chains. Moreover, some TCR-negative CD4\(^{+}\) cells could be detected also in blood, lymph nodes, and spleen of αKO recipient mice before transferring (14). As we transferred the total CD4\(^{+}\) population from αKOTg\(^{-}\) b\(^{-}\) in recipient mice having a TCR-negative CD4\(^{+}\) population, a large proportion of CD4\(^{+}\) cells detected after transfer were not Tg TCR-positive. Thus, CD4\(^{+}\) cells expressing the Tg TCR were unequivocally identified as CD4\(^{+}\) V\(_{J}\)B14\(^{+}\), and they were represented as percentage of the total CD4\(^{+}\) population in recipient mice.

During the first 3 wk after transfer, clonal expansion of 2a T cell population was observed in the blood and lymph nodes of αKO-b\(^{-}\) mice followed by a slight decrease reaching a plateau (Fig. 1B) that persisted for over 9 mo (data not shown). In contrast, in αKO-b\(^{-}\) recipients, 2a T cells underwent a measurable but extremely reduced expansion and persisted over time in a very small percentage (Fig. 1B). The in vivo activation of the transferred cells was
FIGURE 1. Activation kinetics of 2α T cells in adoptive hosts. A, Scheme of the T cell transferring protocol. Equal numbers of naive 2α T cells from αKOTgαβ−/− mice (1 × 10⁶) were transferred into αKO-b−/− and αKO-b− control recipients. B, The percentage of CD4αβ−/− T cells with respect to the total CD4− population was determined at the indicated time points. Activation marker expression by 2α T cells in adoptive hosts. Data represent the percentage of CD4αβ−/− T cells that were CD44high (C), CD25− (D), and CD69− (E) calculated on the basis of the geometric mean fluorescence values. Error bars represent the SD from the mean of three mice studied. The experiment was repeated three times with similar results.

Transitoriness of Ag presentation in steady state conditions leads to formation of memory T cells

In vitro analysis of transferred T cell responsiveness. Previous experimental systems have clearly shown that T cells exposed to Ags presented in a self-context, at the steady state, undergo an abortive activation (18–20). Thus the observed expansion and activation of transferred Tg 2α T cells could simply represent the first step of the tolerization process. To test whether 2α T cells were functional 1 mo after transfer, cells were recovered from lymph nodes and rechallenged in vitro with Bpep-loaded B cells. We observed that recovered 2α T cells strongly down-regulated the TCR once in contact with even very low amounts of peptide in vitro. We, thus, took this parameter as an index of 2α T cell activation. Other parameters, such as CD69 and CD25 up-regulation, could not be taken into account because the TCR down-regulation hampered a proper identification of the 2α T cells (using anti-Vβ14 Ab). The second parameter we analyzed to verify in vitro 2α T cell functionality was the production of effector cytokines, such as IFN-γ. Surprisingly, transferred 2α T cells responded to low peptide doses much more efficiently than naive T cells (Fig. 2A) and behaved like memory cells.

In vivo analysis of transferred T cell functionality. Anti-IgG2αβ−/− T cells show a killing activity vs IgG2αβ−/− B cells (21). Taking advantage of this behavior, the functionality of 2α T cells, 1 mo after transfer, was also investigated in vivo by analyzing their ability to suppress IgG2αβ−/− B cells. To perform this analysis, normal CB-17 (α2a−/−) mice were immunized with KLH. Two weeks after immunization, KLH-sensitized T and B cells were isolated and transferred into SCID recipient mice together with CD4+ T cells recovered from adoptively transferred αKO-b−/− hosts. SCID recipient mice were then challenged with KLH and serum levels of KLH-specific IgG2α measured 10 days later (Fig. 2B). The assumption was that if 2α T cells recovered from host mice were functional, once transferred into SCID mice they should be able to suppress IgG2αβ−/− B cells derived from KLH immunized CB-17 mice, and no KLH-specific IgG2α should be measured in the serum (although other anti-KLH isotypes should still be present). As shown in Fig. 2C, the KLH-specific IgG2α response was completely suppressed in mice that received Tg T cells from αKO-b−/− hosts. The specificity of this effect was demonstrated by comparing total anti-KLH IgG levels between control and test SCID animals (data not shown). From all of these experiments, we could conclude that transferred Tg T cells did not undergo a tolerization process when transferred in hosts mice presenting the Ag in a self-context, but became functional memory cells.

A possible explanation for this unexpected behavior was that in this particular system, Ag presentation, at the steady state in absence of inflammation, was not sufficient to induce tolerance and that the reason for the failure of tolerization could reside in a non-chronic exposure to the Ag (22). In fact, if IgG2αβ−/− B cells are killed after CD4+ anti-IgG2αβ−/− T cell encounter (21), Bpep-presenting cells (IgG2αβ−/− B cells) could be quickly cleared after T cell interaction.

In accordance with this hypothesis, serum IgG2αβ−/− disappeared around 20 days after T cell transfer (Fig. 3A), and no IgG2αβ−/− secreting B cells were measurable by ELISPOT (data not shown).

To confirm that Bpep-presenting cells did not persist in 2α T cell transferred animals, naive CFSE-labeled 2α T cells were transferred into αKO-b−/− mice 2 wk after the first transfer and their profile of proliferation analyzed 1 wk later (Fig. 3B). If Bpep-presenting APCs were completely eliminated following the first 2α...
T cell transfer, the transferred CFSE-labeled 2a T cells should not proliferate. As shown in Fig. 3C, 2a T cells were not able to proliferate in mice that had already received a first 2a T cell transfer (host 1) and in control mice not expressing the cognate Ag (control). In contrast, naive 2a T cells could be transferred into SCID mice together with 2a T cells from αKO-b+ host. Immediately after cell transfer SCID mice were challenged with KLH. IgG2a+ anti-KLH response was measured 10 days later. Control recipients and host 2 mice were first reconstituted with polyclonal T lymphocytes deprived of Vβ14+ cells to exclude the possibility that the 2a T cell proliferation observed in host 2 mice was due to space availability. This result indicates that cells able to present the Bpep did not persist in transferred animals.

**Persistent Ag exposure induces peripheral T cell tolerance**

If the peripheral T cell tolerization failure was due to the transitoriness of Ag presentation even though the Ag was presented in a self-context and in absence of inflammation, an increased number of Bpep-presenting B cells should lead to persistent Ag presentation and, thus, to peripheral Ag-specific T cell tolerization. To test this hypothesis, we investigated the fate of 2a T cells in conditions in which the Ag had already been transferred. We followed two strategies: 1) we took advantage of αKO-b+ mice naturally showing a higher frequency of Bpep-presenting B cells and 2) we performed repetitive injections of Bpep-presenting small resting B cells to increase their peripheral persistence.

**Fate of 2a T cells in mice naturally showing a high frequency of Bpep-presenting B cells**

Among αKO-b+ mice maintained in pathogen-free conditions some naturally show high levels of IgG2a+ in the serum, reaching a peak of 120 μg/ml (data not shown). First, we tested whether the Ig levels correlated with the frequencies of IgG2a+ secreting B
cells. By ELISPOT assay we verified that low IgG2a<sup>b</sup> levels corresponded to low IgG2a<sup>b+</sup> B cell frequency and vice versa high IgG2a<sup>b</sup> levels corresponded to high IgG2a<sup>b+</sup> B cell frequency (Fig. 4A). Thus, we selected a group of mice showing high (40–100 μg/ml) IgG2a<sup>b</sup> serum levels and we investigated whether in this condition, in which the frequency of Bpep-presenting B cells is much higher and their persistence should be prolonged, tolerance could be induced in 2a T cells. We transferred naive Tg 2a T cells into high frequency Bpep-presenting αKO-b<sup>+</sup> mice and we followed their condition over time. As shown in Fig. 4B, 2a T cells underwent activation and proliferation similar to 2a T cells transferred into mice with a low frequency of Bpep-presenting B cells. We then tested the functionality of transferred 2a T cells after B cell encounter by measuring the serum levels of self-IgG2a<sup>b</sup> in host animals at different time points. We observed that the IgG2a<sup>b</sup> level was down-regulated during the first 7 days followed by an increase during the T cell expansion phase and finally, around 20 days after transfer when the frequency of transferred T cells reached the plateau level, it returned close to the original serum concentration (Fig. 4C). This observation suggested that 2a T cells introduced in mice with a high IgG2a<sup>b+</sup> B cell frequency were presumably rendered nonfunctional and were not able to suppress IgG2a<sup>b+</sup> B cells in contrast to 2a T cells transferred in mice with a low frequency of Bpep-presenting B cells. According to this prediction, 2a T cells recovered 1 mo after transfer in mice with a high frequency of Bpep-presenting B cells were not functional as indicated by in vitro restimulation (Fig. 5A). To test in vivo the functionality of 2a T cells 1 mo after transfer, we analyzed their ability to suppress IgG2a<sup>b+</sup> B cells as previously indicated. CB-17 (y2<sup>a</sup>) mice were immunized with KLH. Two weeks after immunization KLH-sensitized T and B cells were transferred into SCID recipient mice together with 2a T cells recovered from αKO-b<sup>+</sup> hosts showing a high frequency of Bpep-presenting B cells (Fig. 5B). SCID recipient mice were then treated with KLH, and serum levels of KLH-specific IgG2a<sup>b</sup> were measured 10 days later. As shown in Fig. 5C, 2a T cells were not able to suppress anti-KLH IgG2a<sup>b</sup> response. All together, these results indicate that 2a T cells are tolerized when transferred into mice showing a high frequency of Bpep-presenting B cells.

To be sure that the Ag presentation persisted in mice showing high frequency of Bpep-presenting B cells we repeated the experiment shown in Fig. 3, B and C. Thus, naive CFSE-labeled 2a T cells were transferred in αKO-b<sup>+</sup> mice showing high frequency of Bpep-presenting B cells 2 wk after the first 2a T cell transfer, and their capacity to proliferate was analyzed 1 wk later (Fig. 5D). If Bpep-presenting APCs persisted following the first 2a T cell transfer, the transferred CFSE-labeled 2a T cells should proliferate. 2a T cells were able to proliferate in lymph node (Fig. 5E) and spleen (data not shown) of mice that had received a first 2a T cell transfer (host 1), whereas they did not proliferate in control mice not expressing the cognate Ag (control). Control recipients were first reconstituted with polyclonal T lymphocytes deprived of VB14<sup>T</sup> T cells to impede homeostatic proliferation. This result indicates that cells able to present the Bpep persisted in animals showing a high frequency of Bpep-presenting B cells.

**Fate of 2a T cells in mice that received repetitive injections of Bpep-presenting B cells**

αKO-CB-17 × αKO-BALB/c F<sub>1</sub> mice (showing very low levels of IgG2a<sup>b</sup>) received naive 2a T cells and were subjected to injections of small resting B cells from BALB/c mice either pulsed or not with the Bpep every other day for a total of four consecutive treatments (Fig. 6A). 2a T cells were then followed over time and showed a proliferation activity and an activation marker profile very similar to the ones observed for 2a T cells transferred into αKO-b<sup>+</sup> and described in Fig. 1 (data not shown). One month after transfer, 2a T cells were present in all the animals that received the injections and they were all CD44<sup>high</sup> (Fig. 6B). We then tested their functionality in vitro and in vivo. As shown by the experiment in Fig. 6A, 2a T cells were recovered from lymph nodes, challenged with Bpep-loaded B cells, and their responsiveness measured by means of TCR down-regulation and IFN-γ production in comparison to naive 2a T cells. In contrast to the activity observed for T cells recovered from hosts that received repetitive injections of control B cells, 2a T cells derived from hosts that received repetitive injections of Bpep-presenting B cells did not show any activity (Fig. 6C). Thus, chronic Bpep presentation rendered 2a T cells nonresponsive.

The nonfunctionality of 2a T cells recovered from mice that received repetitive injections of Bpep-presenting B cells was also confirmed in vivo 1 mo after transfer. First, the IgG2a<sup>b</sup> level was not suppressed (Fig. 6D) and, second, these cells were unable to...
suppress an anti-KLH IgG2ab response (Fig. 6F) in the same experimental setting shown in Figs. 2C and 5C. In particular, 2a T cells from the two groups of animals were transferred into SCID mice together with 2a T cells from αKO-b+ recipients. Immediately after cell transfer SCID mice were challenged with KLH. IgG2a+ anti-KLH response was measured 10 days later.

C. Serum levels of KLH-specific IgG2a in SCID mice that received 2a T cells from αKO-b+ with persistent or transient Bpep presentation. Control mice received, other than KLH-primed T and B cells, CD4+ T cells from CB-17 animals.

D. Diagram of the experiment performed to investigate the persistence of Bpep-presenting APCs. αKO-b+ mice with a high frequency of Bpep-presenting B cells were transferred at day 0 with naive 2a T cells (Host 1). Control αKO-b+ mice were transferred at day 0 with CD4 V-H925214 T cells from BALB/c mice. Two weeks later, CFSE-labeled naive 2a T cells were transferred into these two recipients and their ability to proliferate was measured 1 wk later. E. Flow cytometry analyses of lymph node cells derived from mice that received CFSE-labeled naive 2a T cells. Cells were stained with anti-CD4 and anti-Vβ14 Abs and the analyses were performed on gated CD4+ Vβ14+ cells.

The extent of Ag persistence is responsible for inducing T cell activation or tolerance in a nonlymphopenic environment

It is known that the behavior of T cells can be influenced by lymphopenic conditions (23). Thus, we determined whether the extent of Ag persistence could be responsible for inducing T cell activation or tolerance also in a nonlymphopenic environment. For this purpose, αKO-b+ mice showing low or high Bpep-presenting B cell frequency were reconstituted with polyclonal T cells depleted of the Vβ14+ population to be able to track 2a T cells once transferred. Among recipient mice we selected animals that still showed low (<10 μg/ml) and high (40–100 μg/ml) IgG2a+ serum levels (low or high Bpep-presenting B cell frequency) 1 or 2 mo after reconstitution and that had a frequency of ~10% CD4+ and 5% CD8+ cells in the blood. Into those mice, 2a T cells were transferred and followed over time. Early after transfer, 2a T cells expanded in both groups of animals, then around 1 mo later they stabilized at a frequency of 3–4% of total CD4+ cells in blood and lymph nodes (data not shown). Persisting T cells were all CD44high (Fig. 7A). To test their functionality we investigated their capacity to inhibit in vivo anti-KLH IgG2a+ responses (Fig. 7B). As shown in Fig. 7C, 2a T cells derived from αKO-b+ hosts with high Bpep-presenting B cell frequency did not inhibit the anti-KLH IgG2a+ response, whereas 2a T cells from animals with a low frequency of Bpep-presenting B cells totally inhibited the response.
mice were then challenged with KLH. It has been recently proven that CD4 T cell-mediated autoimmune disease of the eye can be induced in susceptible mice by ocular infection with HSV-1 (3, 24). It has been shown that T cells responsible for HSK cross-recognize a corneal Ag and the Bpep of IgG2ab (10). Ighb animals, such as CB-17 mice, are resistant to HSK and their resistance has been correlated with the tolerant state of anti-IgG2ab polyclonal T cells (10). Thus, we used the HSK model as readout to test whether activation or tolerance of polyclonal T cells. HSK is a CD4 T cell-mediated autoimmune disease of the eye that can be induced in susceptible mice by ocular infection with HSV-1 (3). As shown in Fig. 8A, mice that received a single injection of CB-17 B cells were more susceptible to HSK than control animals that received only C.AL-20 B cells. In contrast, mice that received sufficient repetitive injections of CB-17 B cells were totally resistant. Thus, transient exposure to Bpep-presenting B cells made keratogenic T cells hyperresponsive, whereas chronic exposure completely tolerized keratogenic T cells, confirming that, also at the polyclonal level, the extent of Ag persistence is responsible for inducing T cell activation or tolerance.

The HSK model
Persistence of Ag presentation is responsible for inducing T cell activation or tolerance of polyclonal T cells. HSK is a CD4 T cell-mediated autoimmune disease of the eye that can be induced in susceptible mice by ocular infection with HSV-1 (3, 24). It has been recently proven that CD4 T cells responsible for HSK cross-recognize a corneal Ag and the Bpep of IgG2ab (10). Ighb animals, such as CB-17 mice, are resistant to HSK and their resistance has been correlated with the tolerant state of anti-IgG2ab polyclonal T cells (10). Thus, we used the HSK model as readout to test whether the extent of persistence of Bpep-presenting B cells was involved in the activation or tolerization of keratogenic anti-IgG2ab T cells. This experiment predicted that if Bpep-presenting B cells persist, they should tolerize keratogenic T cells and mice should become resistant to HSK. In contrast, transient exposure of keratogenic T cells to the Ag should induce priming and differentiation in memory cells.

Rag2−/− mice were reconstituted with T cells from C.AL-20 mice (γ2a), which are CB-17 congenic and highly susceptible to HSK. In this experiment we used CAL.20 T cells for two reasons: 1) Vβ14 2a T cells are not keratogenic and 2) by using keratitis as a readout system, we could use a polyclonal non-Tg population to confirm the data on activation and tolerance we observed with Tg T cells. Reconstituted Rag2−/− mice also received single or repetitive injections of B cells from CB-17 mice or from C.AL-20 mice as control, so that all the recipients were injected with the same total number of B cells (Fig. 8A). One month after reconstitution, mice were infected with HSV-1 (strain KOS) and the incidence and severity of HSK was scored 7, 10, 14, and 19 days after infection (3). As shown in Fig. 8B, mice that received a single injection of CB-17 B cells were more susceptible to HSK than control animals that received only C.AL-20 B cells. In contrast, mice that received sufficient repetitive injections of CB-17 B cells were totally resistant. Thus, transient exposure to Bpep-presenting B cells made keratogenic T cells hyperresponsive, whereas chronic exposure completely tolerized keratogenic T cells, confirming that, also at the polyclonal level, the extent of Ag persistence is responsible for inducing T cell activation or tolerance.

Discussion
The goal of this study was to investigate whether the Ag persistence per se could be an inductive mechanism of the T cell tolerization process. To this aim, we transferred CD4 T cells specific
FIGURE 7. Ag persistence is responsible for inducing T cell activation or tolerance in a nonlymphopenic environment. A, CD44 expression by 2a T cells transferred into T lymphocyte reconstituted αKO-b⁺ mice with persistent Bpep presentation (left panels) or into T lymphocyte reconstituted αKO-b⁺ mice with short-term Bpep presentation (right panels). CD44 expression on naïve 2a T cells (thin line histogram) and CD44 expression on 2a T cells after transfer (thick line histogram). B and C. In vivo 2a T cell functionality after transfer in nonlymphopenic hosts. B, SCID mice received KLH-sensitized T and B cells from CB-17 mice together with 2a T cells from αKO-b⁺ mice with chronic Ag presentation (persistent) or αKO-b⁺ mice with transient Ag presentation (transient). Recipient SCID mice were then challenged with KLH and the levels of anti-KLH IgG2a measured 10 days later. C, Anti-KLH IgG2a⁺ serum measured by ELISA in the experiment described in B. Control: SCID mice that received, other than KLH-sensitized T and B cells from CB-17 animals, CD4⁺ T cells from CB-17 mice. Memory: SCID mice that received, other than KLH-sensitized T and B cells from CB-17 animals, memory 2a T cells. The experiment was repeated twice with similar results. Three mice per group were used in each experiment. Error bars represent the SD from the mean of three mice studied.

for an epitope in the CH3 region of IgG2a⁺ into mice showing chronic or transient Ag presentation by B cells. As already observed in previous studies (13), T and B cells efficiently find each other in lymph nodes and spleen in absence of inflammation.

We show in this study that the consequences of T cell-B cell interaction depend on the relative frequency of Ag-presenting B cells and Ag-specific T cells. In our model system, when Ag-presenting B cells are highly frequent and persist for a long time, Ag-specific T cells are tolerated, whereas transient B cell Ag presentation leads to T cell activation and eventually to a T memory phenotype. This result occurs also at the polyclonal T cell level and it is relevant in the protection against autoimmunity. Susceptibility to HSK is, indeed, totally inhibited if B cells presenting the Bpep persist in highly susceptible mice.

The disappearance of IgG2a⁺ B cells is presumably due to the killing activity shown by 2a T cells. Nevertheless, we cannot exclude that 2a T cells interfere with IgG2a⁺ B cell maturation or induce a further switch to IgE (8).

In our system, we only tested the importance of Ag presentation persistence in inducing T cell tolerance. It is likely that also the Ag dose could play an important role. It is, indeed, well known that i.v. injection of high doses of soluble Ags can induce tolerance of Ag-specific CD4⁺ and CD8⁺ T cells (25). The importance of the balance between Ag dose and specific T cells has also been stressed in experiments performed in TCR Tg animals in which, given the high frequency of Ag-specific T cells, peripheral tolerance by injection of soluble Ags can be reached only if the number of T cells is decreased by anti-CD4 Ab treatment (26).

If the persistence of the Ag itself represents one of the main conditions for peripheral T cell tolerance, the adaptive immune system would be capable of differentiating the response to Ags that rapidly change in concentration (non-self) vs Ags that slowly accumulate or that persist after a rapid increase (self). This reasoning is particularly relevant for all of the self-Ags expressed only by B cells such as peptide-MHC complexes derived from variable components of Ig (27). Moreover, this observation, although made in an in-fact artificial model system, could explain how T cells responsible for inducing HSK are tolerized in peripheral lymphoid organs in nonsusceptible mice. We have previously shown that T cells expressing a high density of the TCR specific for the Bpep are not subjected to central tolerization and reach lymph nodes as functional cells in Ighb mice (9). Keratogenic T cells cross-recognize the Bpep and a not-yet identified peptide in the cornea (10). On the basis of our observation we can hypothesize that because corneal Ags are not accessible, tolerization of keratogenic T cells in nonsusceptible Ighb mice could be due to the persistence of Bpep Ag presented either by activated or resting B cells.

Tolerized 2a T cells persist in vivo, even in nonlymphopenic conditions where they possibly compete with functional T cells. This finding is in agreement with other studies in which the persistence in vivo of anergic T cells has been observed for at least 3 wk (28). It is probable that, in analogy to what was found for homeostatic proliferation (29, 30), the critical limiting requirement for persistence is the availability of specific self-ligands and not the absence of general competition with functional T cells. Thus, if T cells are present that compete for the same ligands, anergic T cells are eliminated, otherwise, they persist. The persistence of anergic T cells could not be a useless phenomenon. Anergic T cells that are cells with an increased activation threshold (4) may still have the potential to react to multiple exogenous peptides presented in particularly favorable contexts. The deletion of T cells on the basis of self-reactivity could eliminate the possibility to respond to different exogenous antigenic stimuli.

Activation and differentiation of naïve 2a T cells to memory lymphocytes in αKO-b⁺ animals in absence of inflammatory stimuli is in agreement with the Localization, Dose and Time model (22). In our experimental conditions, the Ag is, indeed, presented in the appropriate organ for the appropriate time to induce T cell activation and memory formation. Our observations cannot be explained within the Danger model, unless we assume that, together with different types of costimulators induced by danger signals such as soluble and membrane molecules, (31) high TCR affinity could work as a full activation stimulus. Because costimulation could represent simply a synergism with signal one (32, 33), in our system the strength of signal one could be high enough to allow T cell activation and differentiation.

Our observations confirm the hypothesis that self and non-self are changing properties of the individual and that the distinction between self and non-self resides in the differential rate of change (34). Self-Ags have a slow rate of change whereas non-self Ags have a rapid rate of change and are interpreted as perturbations by the adaptive immune system.

Disclosures

The authors have no financial conflict of interest.
FIGURE 1. Effect of persistence of Bpep-presenting B cells in susceptibility to HS-K. A, Scheme of experimental design. SCID mice were transferred with $10^7$ CD4$^+$ T cells from C.AL-20 mice and subjected to single or repetitive injections of B cells from CB-17 animals. Recipients also received repetitive injections of B cells from C.AL-20 mice so that all of them were reconstituted with the same total number of B cells. B, Incidence and severity of the disease were scored on days 7, 10, 14, and 19 after HSV-1 infection. Data are the mean of five mice per group. The experiment was repeated twice with similar results.

References


