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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 actively 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 IgG2a\(^b\) 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 IgG2a\(^b\) are not deleted in the thymus of Ighb animals (which produce IgG2a\(^b\)) and reach peripheral lymphoid organs (9). Nevertheless, there are some evidences that peripheral anti-IgG2a\(^b\) 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-IgG2a\(^b\) T cells. Thus, we investigated the mechanisms and conditions responsible for anti-IgG2a\(^b\) 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 IgG2a\(^b\) 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 IgG2a\(^b\) in association with I-A\(^\delta\) (8). The Tg animals were obtained in different backgrounds: BALB/c, expressing the a and not the b allotype of IgG2a; CB-17 expressing the IgG2a\(^b\); and TCR \(\alpha\)-chain-deficient knockout (KO) BALB/c (aKOTg\(^b\) b\(^+\)) and CB-17 (aKOTg\(^b\) b\(^+\)), to exclude endogenous a rearrangements (9). TCR \(\alpha\)-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 IgG2a\(^b\); 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 (αKOB−/−) 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 (αKOB−/−). αKOB−/− mice were bred with αKOB−/− mice to obtain (αKOB−/− × CB-17 × BALB/c) F1 mice. All animals were kept in specific pathogen-free conditions.

ELISPOT
ELISPOT assay was performed as described (8).

Cell preparations
Naïve TCR Tg anti-IgG2a T cells (2a T cells) were purified from spleen and lymph nodes of αKOTg−/− mice. A total of 106 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 Microspheres (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 unclonable cell suspensions were incubated with anti-B220 (20 μg/ml) and anti-CD11c (20 μg/ml) 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. Unclonable 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 Microspheres (Miltenyi Biotec). Cells were then negatively selected on MS MACS separation columns according to Miltenyi Biotec recommendations.

IgG2a serum levels
To assess the amount of circulating IgG2a 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 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 protein, and incubated overnight at 4°C. Biotinylated anti-mouse IgG2a Ab was then added and incubated for 2 h at 37°C and followed with 20 min incubation with streptavidin-HP conjugate (room temperature). Developing substrate (tetramethylbenzidine; Sigma-Aldrich) was finally added for 20 min (room temperature) and reaction stopped with 2 N H2SO4. Plates were read using a Hewlett-Packard reader. All steps were pipetted with appropriate washing using tap water.

ELISA tests to assess circulating anti-KLH IgG2a were performed in the same way using different preclinical 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−/− mice by magnetic negative selection, washed extensively in PBS, and then injected (1–2 × 107 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 × 106 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 V814; FITC- or PE-coupled anti-mouse CD25; FITC- or biotinylated-coupled anti-mouse CD69; and FITC- or biotinylated-coupled anti-mouse CD44. FACS analysis was performed using a BD FACSscan 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−/− mice or αKOB−/− mice transferred 1 mo earlier with naïve 2a T cells.

Purified 2a T cells (1 × 106 cells/well) and small resting B cells (1 × 106 cells/well) were resuspended in complete IMDM, plated in 24-well plate, and Bpep was added at different concentrations. After 4 h of coculture, Vβ14 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
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 nodes were purified as earlier described and injected (4 × 106 cells/mouse) i.p. in SCID mice together with 2a T cells (1.5 × 106 cells/mouse) derived from αKOB−/− mice transferred 1 mo earlier with naïve 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 were measured by ELISA as previously described.

Ocular infection and scoring of HSK
Corneas of mice were sacrificed 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. Naïve CD4+ 2a T cells, specific for the Bpep of the IgG2a, were purified by negative selection from TCR Tg α-chain-deficient (αKO) BALB/c (y2a+−) animals (αKOTg−/−) (9) and transferred into congenic αKO-CB-17 (IgG2a−) mice (designated αKO-b−), showing an average IgG2a serum level of 4–10 μg/ml. As control, 2a T cells were also transferred into αKOBALB/c (αKO-b−) mice (y2aa+− mice) (Fig. 1A).

As the Bpep is exclusively presented by IgG2aKO−/− mice 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−/− 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−/− 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+Vβ14+, 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 αKOB−/− mice followed by a slight decrease reaching a plateau (Fig. 1B) that persisted for over 9 mo (data not shown). In contrast, in αKOB−/− 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...
followed by analyzing activation marker expression at different times. Early activation markers such as CD25 and CD69 were transiently up-regulated on T cells transferred into αKO-b+ mice (Fig. 1, D and E) and CD44 up-regulation started 7 days after transfer (Fig. 1C). In contrast, T cells injected in αKO-b− control recipients did not show any CD69 and CD25 up-regulation but only a late increase of CD44 expression (Fig. 1). Thus, transferred T cells underwent an Ag-driven proliferation in αKO-b− mice and homeostatic proliferation in lymphopenic αKO-b+ hosts, a behavior previously observed after transfer of T cells in lymphopenic animals (15–17).

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 2a T cells could simply represent the first step of the tolerization process. To test whether 2a 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 2a 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 2a T cell activation.

Other parameters, such as CD25 and CD69 up-regulation, could not be taken into account because the TCR down-regulation hampered a proper identification of the 2a T cells (using anti-Vβ14 Ab). The second parameter we analyzed to verify in vitro 2a T cell responsiveness was the production of effector cytokines, such as IFN-γ. Surprisingly, transferred 2a 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-IgG2a+ T cells show a killing activity vs IgG2a+ B cells (21). Taking advantage of this behavior, the functionality of 2a T cells, 1 mo after transfer, was also investigated in vivo by analyzing their ability to suppress IgG2a+ B cells. To perform this analysis, normal CB-17 (γ2a−) 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 IgG2a measured 10 days later (Fig. 2B).

The assumption was that if 2a T cells recovered from host mice were functional, once transferred into SCID mice they should be able to suppress IgG2a+ B cells derived from KLH immunized CB-17 mice, and no KLH-specific IgG2a should be measured in the serum (although other anti-KLH isotypes should still be present). As shown in Fig. 2C, the KLH-specific IgG2a 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 IgG2a+ B cells are killed after CD4+ anti-IgG2a+ T cell encounter (21), Bpep-presenting cells (IgG2a− B cells) could be quickly cleared after T cell interaction.

In accordance with this hypothesis, serum IgG2a disappeared around 20 days after T cell transfer (Fig. 3A), and no IgG2a− secreting B cells were measurable by ELISPOT (data not shown).

To confirm that Bpep-presenting cells did not persist in 2a T cell transferred animals, naive CFSE-labeled 2a 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 2a
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 proliferate into iKO-b+ hosts (host 2). Control recipients and host 2 mice were first reconstituted with polyclonal T lymphocytes deprived of Vb+ T 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 was chronically presented. We followed two strategies: 1) we took advantage of iKO-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 iKO-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.
cells. By ELISPOT assay we verified that low IgG2a\textsuperscript{b} levels corresponded to low IgG2a\textsuperscript{b+,+} B cell frequency and vice versa high IgG2a\textsuperscript{b} levels corresponded to high IgG2a\textsuperscript{b+,+} B cell frequency (Fig. 4A). Thus, we selected a group of mice showing high (40–100 μg/ml) IgG2a\textsuperscript{b} 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\textsuperscript{+} 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\textsuperscript{b} in host animals at different time points. We observed that the IgG2a\textsuperscript{b} 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\textsuperscript{b+,+} B cell frequency were presumably rendered nonfunctional and were not able to suppress IgG2a\textsuperscript{b+,+} 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\textsuperscript{b+,+} B cells as previously indicated. CB-17 (y2a\textsuperscript{a}) 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\textsuperscript{+} 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\textsuperscript{b} were measured 10 days later. As shown in Fig. 5C, 2a T cells were not able to suppress anti-KLH IgG2a\textsuperscript{b} 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\textsuperscript{+} 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 Vβ14\textsuperscript{+} 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\textsubscript{1} mice (showing very low levels of IgG2a\textsuperscript{b}) received naïve 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\textsuperscript{+} 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\textsuperscript{high} (Fig. 6B). We then tested their functionality in vitro and in vivo. As shown by the experiment in Fig. 2A, 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 naïve 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\textsuperscript{b} level was not suppressed (Fig. 6D) and, second, these cells were unable to
suppress an anti-KLH IgG2a\(^b\) 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 KLH-sensitized T and B cells from CB-17 mice, and recipient animals were challenged with KLH (Fig. 6E). Ten days later anti-KLH IgG2a\(^b\) serum levels were measured. As expected, SCID mice that received 2a T cells from mice subjected to repetitive injections of Bpep-presenting resting B cells were able to mount an anti-KLH IgG2a\(^b\) response, whereas in SCID mice that received T cells from control group, the anti-KLH IgG2a\(^b\) response was suppressed (Fig. 6F), indicating that 2a T cells were tolerized if chronically exposed to the Ag.

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, \(\alpha KO-b^-\) mice showing low or high Bpep-presenting B cell frequency were reconstituted with polyclonal T cells depleted of the V\(\beta 14\) population to be able to track 2a T cells once transferred. Thus, among recipient mice we selected animals that still showed low (<10 \(\mu g/ml\)) and high (40–100 \(\mu g/ml\)) IgG2a\(^b\) serum levels 1 or 2 mo after reconstitution and that had a frequency of 3–4% of total CD4\(^+\) cells in blood and lymph nodes (data not shown). Persisting T cells were all CD44\(^\text{high}\) (Fig. 7A). To test their functionality we investigated their capacity to inhibit in vivo anti-KLH IgG2a\(^b\) responses (Fig. 7B). As shown in Fig. 7C, 2a T cells derived from \(\alpha KO-b^-\) hosts with high Bpep-presenting B cell frequency did not inhibit the anti-KLH IgG2a\(^b\) response, whereas 2a T cells from animals with a low frequency of Bpep-presenting B cells totally inhibited the response.

FIGURE 5. In vitro and in vivo 2a T cell functionality 1 mo after transfer into \(\alpha KO-b^-\) showing persistent and transient Bpep presentation by B cells. A, Ability of 2a T cells to respond in vitro to APCs in presence of different amounts of Bpep 1 mo after transfer into \(\alpha KO-b^-\) mice with transient or persistent Bpep presentation by B cells. Naive cells were added as control. The percentage of 2a T cells showing high levels of V\(\beta 14\) expression was calculated 4 h after coculture with APCs in presence of Bpep. IFN-\(\gamma\) levels in coculture supernatants were measured by ELISA at 24 h. B, Illustration of the experiment performed to test 2a T cell functionality in vivo. KLH-sensitized B and T cells were transferred into SCID mice together with 2a T cells from \(\alpha KO-b^-\) recipients. Immediately after cell transfer SCID mice were challenged with KLH. IgG2a\(^b\) anti-KLH response was measured 10 days later. C, Serum levels of KLH-specific IgG2a\(^b\) in SCID mice that received 2a T cells from \(\alpha KO-b^-\) 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. \(\alpha KO-b^-\) mice with a high frequency of Bpep-presenting B cells were transferred at day 0 with naive 2a T cells (Host 1). Control \(\alpha KO-b^-\) mice were transferred at day 0 with CD4\(^+\) V\(\beta 14\) 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-V\(\beta 14\) Abs and the analyses were performed on gated CD4\(^+\) V\(\beta 14\) cells.
mice were then challenged with KLH. It has been recently proven that CD4 T cells responsible for HSK cross-react with a corneal Ag and the Bpep of IgG2a (10). Ighb animals, such as CB-17 mice, are resistant to HSK and their resistance has been correlated with the tolerant state of anti-IgG2a polyclonal T cells (10). Thus, we used the HSK model 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 tolerated 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
for an epitope in the CH3 region of IgG2a\(^{b}\) 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\(^{b}\) 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\(^{b}\) B cell maturation or induce a further switch to IgE (8).

In our system, we only tested the importance of Ag presentation 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 tolerated 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 homoeostatic 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 naive 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 8. Effect of persistence of Bpep-presenting B cells in susceptibility to HSK. 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


