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Article

Constitutive Crosspresentation of Tissue Antigens by Dendritic Cells Controls CD8$^+$ T Cell Tolerance In Vivo

Nancy Luckashenak,[1,6] Samira Schroeder,[1,6] Katrin Endt,[1] Darja Schmidt,[1] Karsten Mahnke,[2] Martin F. Bachmann,[3] Peggy Marconi,[4] Cornelia A. Deeg,[5] and Thomas Brocker[1,*]
[1]Institute for Immunology, Ludwig-Maximilian-University, 80336 Munich, Germany
[2]Department of Dermatology, University Heidelberg, 69115 Heidelberg, Germany
[3]Cytos Biotechnology AG, 8952 Schlieren-Zuerich, Switzerland
[4]University of Ferrara, Department of Experimental and Diagnostic Medicine, 44100 Ferrara, Italy
[5]Institute for Physiology and Physiological Chemistry, Ludwig-Maximilian-University, 80538 Munich, Germany
[6]These authors contributed equally to this work.
*Correspondence: tbrocker@med.uni-muenchen.de
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SUMMARY

Immature dendritic cells (DCs) sample tissue-specific antigens (TSAs) and process them for "cross-presentation" via major histocompatibility complex (MHC) class I and II molecules. Findings with adoptively transferred T cell receptor (TCR)-transgenic CD8$^+$ T cells in transgenic mice expressing model TSA indicate that this process contributes to tolerance induction of CD8$^+$ T cells, a phenomenon termed "crosstolerance." However, up to now it has been unknown whether "crosstolerance" can also control autoimmune T cells specific for physiological nontransgenic TSA. Here, we showed that a DC-specific deficiency in uptake of apoptotic material inhibits crosspresentation in vivo. This defect allowed the accumulation of fully functional autoreactive CD8$^+$ T cells that could be activated for autoimmune attack in peripheral lymphoid organs. Thus, our data demonstrate the importance of crosstolerance induction by DCs as a vital instrument for controlling self-reactive T cells from the peripheral repertoire and preventing autoimmune disease.

INTRODUCTION

Control of autoreactive T cells by tolerance induction is essential to avoid autoimmune disease. The majority of self-specific T cells are deleted by negative selection in the thymus. Here, peripheral tissue-specific antigens (TSAs) are expressed in the thymic medulla by specialized epithelial cells that confront developing thymocytes with self-antigen (Ag) in the context of major histocompatibility complex (MHC) class I and II molecules and delete those with high avidity self-reactive T cell receptors (TCRs) (Kyewski and Klein, 2006). Although this process is very efficient, some self-reactive T cells are spared and can reach peripheral organs (Gallegos and Bevan, 2006; Liu et al., 1995; Zehn and Bevan, 2006). Here, different peripheral tolerance mechanisms are in place to protect organs from autoimmune damage (Redmond and Sherman, 2005). Because naive T cells remain sequestered in lymphoid organs and blood (Mackay, 1993), they do not normally recognize TSA directly on parenchymal tissue cells and are therefore considered to be "ignorant." Instead, Ag-capturing DCs continuously migrate from peripheral tissues such as stomach, intestine, pancreas, lung, and skin (Adler et al., 1998; Belz et al., 2002; Huang et al., 2000; Kurts et al., 1996; Scheinecker et al., 2002; Turley et al., 2003; Vermaelen et al., 2001) to lymph nodes, transporting TSA for presentation to circulating naive T cells. It is thought that under noninflammatory conditions, constitutive presentation of self-Ags by DCs stimulates CD8$^+$ T cells to proliferate abortively, thereby resulting in their deletion or inactivation (Redmond and Sherman, 2005). Because DCs do not synthesize TSA proteins themselves, the presentation of exogenous Ag in the context of MHC class I molecules to CD8$^+$ T cells must occur via the crosspresentation pathway (Bevan, 1976; Heath and Carbone, 2001). This mechanism is thought to be crucial for generating immune responses to Ags that are exclusively produced by nonprofessional APC, e.g., tumor, cellular, or tissue tropic viral proteins (Huang et al., 1994; Kurts et al., 1996; Li et al., 2001; Sigal et al., 1999).

However, evidence for the involvement of crosspresentation in tolerance induction is based entirely on transgenic mice expressing model TSA and adoptively transferred TCR-transgenic CD8$^+$ T cells with rather high avidity for self-TSA (Belz et al., 2002; Hernandez et al., 2001; Kurts et al., 1996; Morgan et al., 1999). So far, it is unknown whether "crosstolerance" also operates under more physiological conditions because several lines of evidence argue against an important role of this mechanism. For example, in contrast to the adoptive T cell transfer systems used to study "crosstolerance," endogenous self-reactive T cells might be of rather low avidity for self (Zehn and Bevan, 2006). Further, only tissue Ag that is expressed at sufficiently high amounts will be crosspresented for tolerance induction (Kurts et al., 1998), and the amounts at which natural TSA are expressed is predominantly unknown. In addition, the stability of TSA also determines crosspresentation (Wolkers et al., 2004), and instable TSAs are likely to be crosspresented inefficiently or not at all.

More recently, naive T cells were shown to migrate very efficiently through peripheral tissues (Cose et al., 2006) and consequently may encounter TSAs directly on parenchymal tissue rather than on DCs in lymph nodes. Likewise, lymph node stromal cells can express and directly present TSAs and tolerize self-reactive $CD8^+$ T cells (Lee et al., 2007). Taken together, the physiological contribution of "crosspresentation" by DCs to tolerance and prevention of autoimmunity is unclear at the moment.

Here, we showed that DCs with a defect in uptake of soluble protein and apoptotic cellular Ag were unable to crosspresent TSA and were inefficient at cros-tolerizing $CD8^+$ T cells in vivo. This led to the accumulation of fully functional self-reactive $CD8^+$ T cells in peripheral lymphoid organs ready to be activated for autoimmune attack. Thus, our data demonstrate a considerable contribution of crosspresentation to tolerance induction as a vital instrument to eliminate self-reactive T cells from the peripheral repertoire and prevent autoimmune disease.

RESULTS

Characterization of Crosspresentation Capacities of Rac Mice

The constitutive uptake of exogenous protein as well as T cell priming by DCs is dependent on the GTPase Rac1 (Benvenuti et al., 2004; West et al., 2000). To investigate the relevance of crosspresentation in vivo, we analyzed transgenic mice expressing a dominant-negative (N17) mutant of the GTPase Rac1 under control of the DC-selective CD11c-promoter [CD11c-Rac1(N17) mice or short Rac mice] (Kerksiek et al., 2005; Neuenhahn et al., 2006). Because several receptors for apoptotic material are functionally dependent on Rac1 (Albert et al., 2000; Lauber et al., 2004; Park et al., 2007), DCs from Rac mice are unable to initiate $CD8^+$ T cell responses against apoptotic cellular Ag (Kerksiek et al., 2005) or proteins expressed by recombinant *Listeria monocytogenes* (Kerksiek et al., 2005; Neuenhahn et al., 2006). Uptake of apoptotic material facilitates entry of self-Ag into the crosspresentation pathway (Albert et al., 1998a; Albert et al., 1998b; Kurts et al., 1998; Steinman et al., 2000), and tissue-derived apoptotic material is considered a major source for self-TSA crosspresented by DCs (Moser, 2003). Because Rac mice have a defect in crosspresenting apoptotic cellular protein Ag (Kerksiek et al., 2005), we next asked whether these animals could crosspresent soluble protein Ag. To this end, we immunized transgenic and control mice with the soluble protein-Ag ovalbumin (OVA) and monitored activation and expansion of adoptively transferred OVA-specific TCR-transgenic $CD8^+$ T cells (OT-I) (Figures 1A–1C). In nontransgenic littermates, OT-I T cells expanded substantially to crosspresented Ag, whereas the expansion of OT-I T cells in Rac mice was severely inhibited (Figures 1A–1C). In addition, the capacity of OT-I T cells to produce IFN-γ in vitro (Figure 1D), as well as their cytolytic activity in vivo (Figure 1E), were strongly reduced as compared to those from nontransgenic littermates. Similar results were obtained with OVA-coated polystyrene beads and OVA:IgG-immune complexes (data not shown).

Crosspresentation of OVA-protein by DCs is mediated via the mannose-receptor (Burgdorf et al., 2007; Burgdorf et al., 2006), and targeting of OVA to the DEC-205 receptor also leads to efficient crosspresentation by $CD8^+$ DCs (Bonifaz et al., 2002). To analyze whether crosspresentation of Ag directly targeted to the DEC-205 receptor was also defective in Rac-DCs, we immunized mice with αDEC205:OVA complexes. However, crosspresentation of αDEC-205:OVA was also strongly reduced in Rac mice, and the proliferation of OT-I cells was diminished as compared to nontransgenic mice (Figure 1F). This lead to a >60% reduction in the accumulation of OT-I T cells in lymph nodes and spleens of immunized Rac mice as compared to nontransgenic mice (Figure 1G).

We have shown previously that although the total numbers of $CD11c^+$ DCs are unaltered in Rac mice, the relative frequency of $CD8^+$ DCs is reduced by 20%–30% (Kerksiek et al., 2005). Therefore, the reduced crosspresentation observed above could be due to (1) decreased uptake and internalization of exogenous protein, (2) reduced numbers of crosspresenting $CD8^+$ DCs in lymphoid organs, or (3) a combination of both. To differentiate between these possibilities, we immunized mice with OVA and isolated $CD8^+$ DCs from spleens. The isolated $CD8^+$ DCs were then incubated with CFSE-labeled OT-I T cells in vitro at identical DC numbers (Figure 1H). Under these conditions, Rac-$CD8^+$ DCs crosspresented OVA with severely reduced efficiency as compared to Tg^- $CD8^+$ DCs (Figure 1H). Together, these findings indicate that the inhibition of crosspresentation in Rac mice is a consequence of diminished protein uptake (Kerksiek et al., 2005) as well as reduced cell numbers of $CD8^+$ DCs.

Next, we analyzed endogenous polyclonal CTL responses in Rac mice. $CD8^+$ T cell responses against virus-like particles (vlps) have previously been shown to depend on crosspresentation (Ruedl et al., 2002; Storni and Bachmann, 2004). Accordingly, when Rac mice were immunized with vlps carrying the gp33-41 epitope of LCMV, we detected significantly reduced numbers of $H2D^b$-gp33-specific $CD8^+$ T cells (Figures 1I and 1J, $p = 0.01$, Student's t test), which were not significantly elevated above those found in nonimmunized control mice (Figures 1I and 1J, $p = 0.17$, Student's t test). Accordingly, vlp vaccination could not elicit significant cytotoxic activity in Rac mice as compared to control mice (Figure 1K, $p = 0.14$, Student's t test), whereas in nontransgenic mice, gp33-coated target cells were lysed specifically (Figure 1K, $p = 0.01$, Student's t test as compared to controls). Taken together, the above immunizations demonstrate that Rac mice are deficient in mounting optimal CTL responses via crosspresentation.

The Rac Transgene Does Not Affect Direct MHC Class I or MHC Class II Presentation

To further characterize immune responses in Rac mice, we immunized mice with peptide Ag, which can be directly presented on MHC class I molecules (Figures 2A–2D). The expansion of OT-I T cells (Figures 2A and 2B) and the induction of effector functions (Figures 2C and 2D) in Rac mice were indistinguishable from OT-I T cells in nontransgenic littermates. We also observed in Rac mice OT-I expansion that was comparable to Tg^- mice after immunization with replication incompetent HSV-OVA, further demonstrating normal direct MHC class I presentation (Figures S1A and S1B available online). In addition, Rac mice showed no defect in the ability to stimulate OVA-specific TCR-transgenic $CD4^+$ T cells (OT-II) in response to whole protein and peptide as compared to Tg^- mice (Figures S1C and S1D). Because the

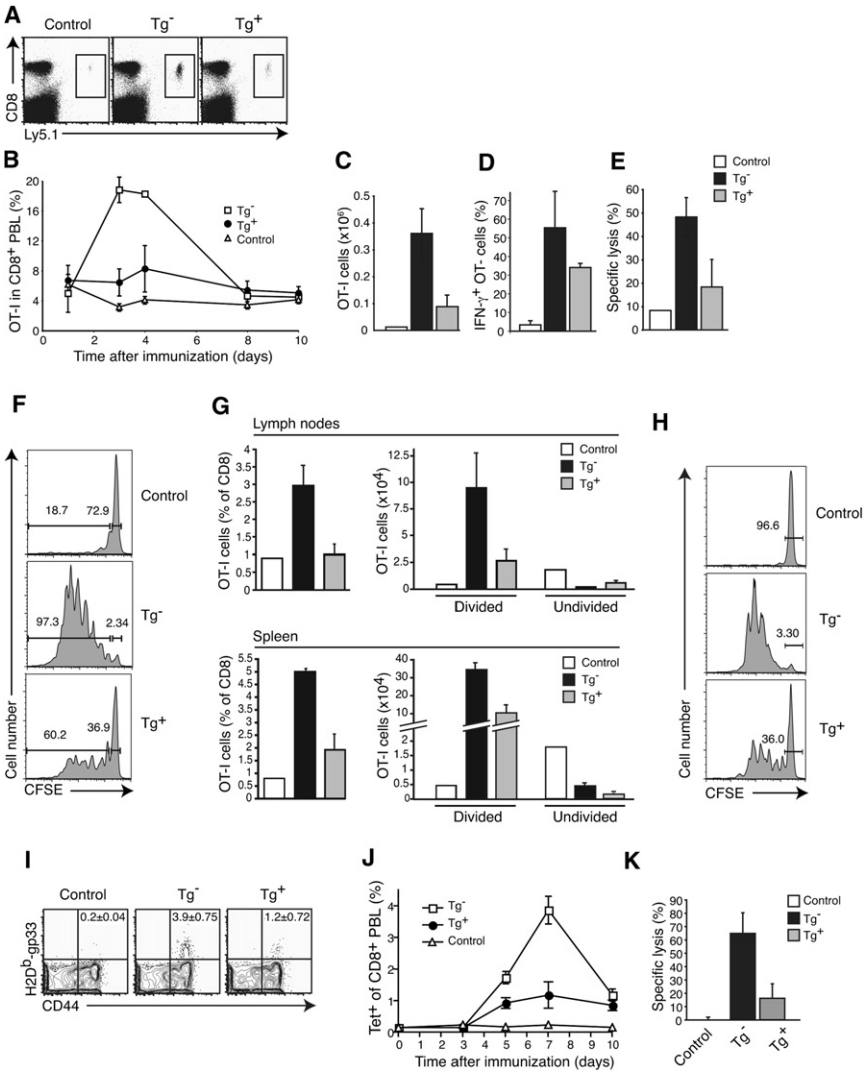

Figure 1. Rac Mice Exhibit a Reduced Ability to Crosspresent

CD11c-(N17) Rac1 transgenic mice (Tg^+) or nontransgenic littermates (Tg^-) were adoptively transferred with 1×10^6 purified Ly5.1$^+$ OT-I T cells (A–G) and immunized i.v. 1 day later with 100 μg soluble OVA and LPS (A–E) or 10 μg of anti-DEC205-OVA conjugate and LPS (F and G). Control mice received LPS only. As shown in (A) and (B), the frequency of OT-I T cells from PBL was determined by flow cytometry by gating on CD8$^+$Ly5.1$^+$OT-I T cells at different time points after immunization. A cohort of mice was sacrificed on day 4 to determine (C) total numbers and (D) intracellular IFN-γ production of OT-I T cells. (E) shows that at day 10, an in vivo killing assay was performed. (F) shows that 3 days after immunization with anti-DEC205-OVA, lymph nodes and spleens were harvested and analyzed for OT-I T cell proliferation by flow cytometry. Prior to CFSE dilution analysis, adoptively transferred cells were identified as the Ly5.1$^+$, CD8$^+$ population (not shown). As shown in (G), frequencies and total numbers of divided and undivided OT-I cells were calculated from the total cell number of each spleen and pooled inguinal lymph nodes from each mouse. In (H), to determine the priming capacities of Tg^+ DCs in vitro, we immunized mice with 5 mg whole OVA protein in PBS or PBS alone. The following day, CD8$^+$ DCs were purified from spleen by magnetic separation. Equal numbers of CD8$^+$ DCs were then cocultured with purified CFSE-labeled Ly5.1$^+$OT-I T cells at DC:T cell ratios of 1:2.5 for 4 days. Proliferation was visualized by flow cytometry as CFSE dilution of the Ly5.1$^+$ cells, and numbers indicate the percent of undivided OT-I T cells from each culture. As shown in (I)–(K), mice were immunized s.c. with 150 μg CpG-containing virus-like particles derived from bacteriophage Qß, and such particles were chemically linked to the LCMV peptide epitope gp33-41. As shown in (I), 7 days after immunization, the frequency of D^b-restricted, gp33-specific CD8$^+$ T cells was determined from PBL with H2D^b-gp33-tetramers. The kinetics of such a response is shown in (J). As shown in (K), 12 days after immunization, gp33-specific cytolytic activities in the different recipients were measured with an in vivo killing assay. Each symbol or bar represents the average of at least three mice per group. Error bars indicate the SEM within a group. The experiments shown here have been repeated more than three times (A–E) or twice (F–K) with similar results.

reduction of OVA protein uptake in Rac mice is restricted to CD8$^+$ DCs (Kerksiek et al., 2005), the CD8$^-$ DC subtype that is particularly efficient at presentation of soluble Ag to CD4$^+$ T cells (Pooley et al., 2001) is possibly responsible for normal OT-II responses in Rac mice. Taken together, our findings indicate that Rac mice show a specific defect in crosspresentation (Figure 1) but are able to mount normal CD4$^+$ and CD8$^+$ T cell responses to directly presented or virus-derived MHC class I and class II epitopes (Figure 2 and Figure S1). These properties render Rac mice as a suitable tool to study the relevance of crosspresentation for tolerance induction in vivo.

The Role of Crosspresenting DCs in an Autoimmune Diabetes Model

Next, we bred Rac mice to the RIP-mOVA strain (RIP for short) expressing high amounts of membrane-bound OVA as a surrogate TSA under control of the rat insulin promoter selectively in the pancreas, kidney, and thymus (Gallegos and Bevan, 2004; Kurts et al., 1996). Central thymic CD8$^+$ T cell tolerance is very efficient in RIP mice because OVA-reactive CD8$^+$ T cells are undetectable upon immunization (Bennett et al. [1997] and Figure 3). Although thymic deletion of OVA-specific CD8$^+$ T cells is mediated by epithelial cells rather than thymic DCs (Gallegos and Bevan, 2004), we had to formally exclude an influence of Rac DCs on negative selection in Rac mice before studying peripheral tolerance. To this end, we bred OT-I mice with Rac mice to create the OT-IxRac line and generated bone-marrow chimeras (Figure 3A). In OT-IxRac > RIP bone-marrow chimeras, central deletion of OT-I T cells was similarly efficient as compared to OT-I > RIP chimeras indicating normal negative selection (Figure 3A). Immunization of double-transgenic Rac-RIP mice with OVA-encoding HSV confirmed this result because expansion of endogenous OVA-specific CTL (Figures 3B and 3C) was undetectable. In contrast, HSV-gB-specific CTL were

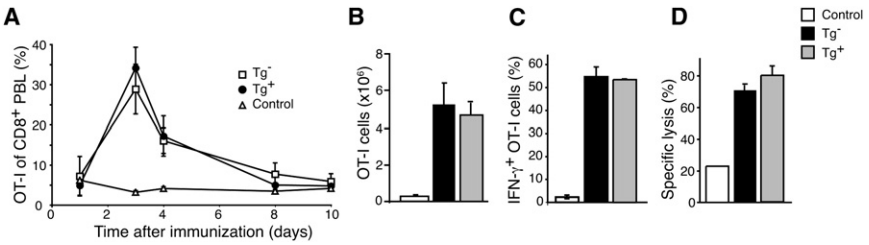

Figure 2. The Rac Transgene Does Not Affect Direct MHC Class I Presentation

CD11c-(N17) Rac1 transgenic mice (Tg^+) or nontransgenic littermates (Tg^-) were adoptively transferred with 1×10^6 purified Ly5.1$^+$ OT-I T cells and immunized i.v. 1 day later with 10 μg SIINFEKL-peptide and LPS. Control mice received LPS only. The frequency of OT-I T cells from PBL was determined by flow cytometry by gating on CD8$^+$Ly5.1$^+$OT-I T cells as shown in Figure 1A, and their frequency was determined at different time points after immunization (A). A cohort of mice was sacrificed on day 4 in order to determine (B) the total numbers and (C) intracellular IFN-γ production of OT-I T cells in spleens. As shown in (D), at day 10, an in vivo killing assay was performed. Each symbol or bar represents the average of at least three mice per group. All error bars for this figure indicate the SEM within a group (n = 2–3 mice per group). The experiments shown here have been repeated at least three times with similar results.

readily detectable at similar amounts as in wild-type controls (Figures 3B and 3C). Because none of the mice developed diabetes (data not shown), these results indicate that OVA-specific central tolerance induction was not affected in Rac-RIP mice and does not depend on crosspresentation by DCs, as published previously (Gallegos and Bevan, 2004). Therefore, a contribution of endogenous OVA-specific CTL can be formally excluded in Rac-RIP mice. In a similar approach, we monitored the capacity of thymic Rac-DCs to negatively select CD4$^+$ T cells. Likewise, negative selection of OT-II thymocytes was not affected in OT-IIxRac > RIP bone-marrow chimeras (Figure S2); the numbers and frequencies of OT-II cells were similarly reduced as compared to thymi and peripheral organs of OT-II > RIP chimeras. This result is not surprising given the ability of Rac DCs to prime normal CD4$^+$ T cell responses (Figure S1C and S1D) and previous findings on the role of thymic DCs in negative selection of self-reactive CD4$^+$ thymocytes (Brocker et al., 1997; Gallegos and Bevan, 2004).

Because induction of CD8$^+$ T cell deletional peripheral tolerance is accompanied by abortive proliferation (Kurts et al., 1997; Kurts et al., 1998), we labeled OT-I T cells with the proliferation marker CFSE and monitored their proliferation upon injection into wild-type, RIP, or Rac-RIP mice (Figure 4). OT-I T cells proliferated vigorously in pancreatic and renal lymph nodes of RIP mice (Figure 4A). The highest rate of proliferation was confined to lymph nodes draining TSA-expressing tissues but not skin-draining inguinal nodes (Kurts et al., 1996). In contrast, OT-I T cells in Rac-RIP recipients proliferated at a much slower rate (Figure 4A), and less dividing OT-I T cells could be found in Rac-RIP mice as compared to RIP mice (Figure 4B). Taken together, these data showed that the inhibition of crosspresentation in DCs of Rac mice impairs constitutive TSA presentation to CD8$^+$ T cells in vivo.

Defective Peripheral Crosstolerance in Rac-Rip Mice

Next, we tested whether the failure of DCs in Rac-RIP mice to crosspresent TSA and to induce efficient proliferation would obstruct peripheral crosstolerance. We injected naive OT-I T cells into different recipients and analyzed the mice 3–4 weeks later (see protocol, Figure 5A). OT-I T cells were present at ~10-fold higher frequencies in wild-type compared to RIP recipients because it has been previously reported that their deletion is near complete after 3 weeks in RIP mice (Kurts et al., 1996) (Figures 5B and 5C). In contrast, the frequency of OT-I T cells in Rac-RIP mice was comparable to that found in wild-type recipients (Figures 5B and 5C). To assess the cells' state of tolerance in the different mice, we challenged them with HSV-OVA. As expected, the remaining OT-I T cells in RIP recipients had been rendered unresponsive and showed no marked Ag-specific expansion after immunization (Figures 5B and 5C). In contrast, OT-I T cells expanded vigorously in wild-type and in Rac-RIP- mice (Figures 5B and 5C), indicating that crosstolerance to OVA was not established in Rac-RIP mice. Wild-type mice were not expected to develop disease because of the lack of OVA in their pancreas (Figure 5D), and RIP-mice had effectively crosstolerized OT-I T cells and were protected from diabetes (Figure 5D). In contrast, all mice of the double-transgenic Rac-RIP group showed elevated urine glucose concentrations at 4 days and maximal levels of diabetes at 5 days after HSV-OVA immunization, confirming the absence of protective crosstolerance (Figure 5D). These data show that Rac-RIP mice are defective in crosstolerizing adoptively transferred OT-I T cells, specific for OVA expressed in the pancreas. Because of the transgenic limitations of this system, the questions concerning a contribution of crosspresentation to tolerance against normal nontransgenic TSA in endogenous polyclonal postthymic CD8$^+$ T cells remain unanswered.

Self-Reactive T Cells Accumulate in Rac Mice

If crosstolerance by DCs is physiologically relevant, potentially self-reactive CD8$^+$ T cells should be detectable in peripheral lymphoid organs of Rac mice. To detect these cells, we transferred CFSE-labeled polyclonal T cells from Rac mice or nontransgenic littermates into wild-type, Thy1.1-congenic recipients, in which they could encounter APC continuously crosspresenting TSA. Fifteen days after transfer, CD8$^+$Thy1.2$^+$ T cells were quantified in spleens (Figure 6A) and lymph nodes (data not shown) of Thy1.1 recipients. We detected a more than 6-fold increase in the frequency of CD8$^+$Thy1.2$^+$ T cells from Rac mice as compared to those originating from nontransgenic littermates (Figure 6A). More importantly, the total cell numbers of CD8$^+$ Thy1.2$^+$ T cells from Rac mice found in spleens (Figure 6B) and lymph nodes (data not shown) were 6- to 8-fold higher as compared to those of CD8$^+$Thy1.2$^+$ from nontransgenic mice. These differences were due to a strong increase in the fraction of CD8$^+$Thy1.2$^+$CFSE^{lo} T cells and were caused by proliferative expansion (Figures 6A and 6B) probably in response to TSA. However, despite the proliferative expansion of TSA-specific

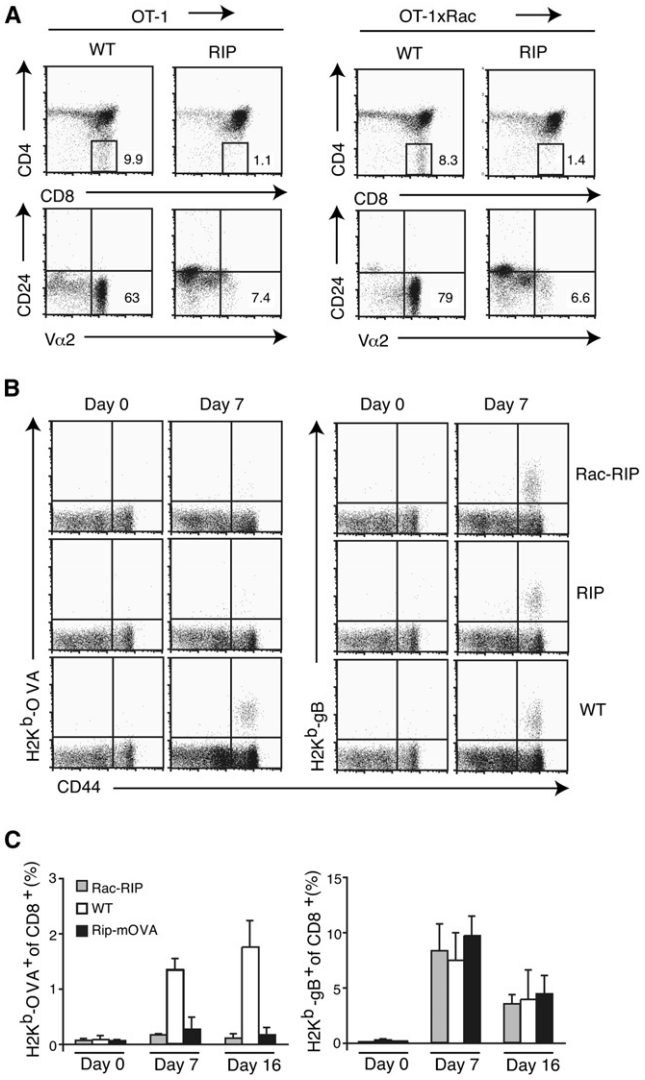

Figure 3. The Rac Transgene Does Not Affect Deletion of OT-I T Cells in RIP Thymi

(A) CD8 SP thymocytes and OT-I cells from OT-I and OT-IxRac donors are equally reduced in chimeric RIP thymi. Grafting OT-I- or OT-IxRac bone marrow into lethally irradiated wild-type or RIP recipients generated [OT-I→WT], [OT-IxRac→WT], [OT-I→RIP], and [OT-IxRac→RIP] chimeric thymi. Thymocytes from the indicated mice were analyzed for expression of CD4 and CD8 by flow cytometry. The numbers indicate the percentage of cells in each gate (A) $CD8^+$ thymocytes (cells in gate) were further analyzed for TCR-Vα2, the TCR-α chain of the OT-I TCR, and expression of heat stable Ag (CD24) as a maturation marker (lower panels). Numbers indicate the percentages of most mature $CD24^{low}V\alpha2^+$ thymocytes as average of n = 2–3 thymi per type of chimera (lower panels).

(B) Wild-type, RIP-mOVA, and RacxRIP mice were immunized with 4×10^6 particles of HSV-OVA i.v. The polyclonal endogenous $CD8^+$ T cell responses of the respective mice were monitored from PBL by flow cytometry. Examples for these analyses from each type of mouse are shown. Percentages of ([B], left panel) OVA-specific cells and ([B], right panel) HSV-gB-specific $CD8^+$ T cells were obtained by gating on $CD8^+$ cells (data not shown) and analysis of $CD44^+$MHC-tetramer$^+$ frequencies. The results are shown in (C) as mean ± SEM (n = 3 mice per group). Shown is one representative experiment of three.

$CD8^+$ T cells, recipient mice did not show signs of disease or weight loss (data not shown). We therefore assumed that under steady-state conditions, i.e., in the absence of inflammatory stimuli, TSA-specific $CD8^+$ T cells would transiently expand, similar to OT-I cells in the RIP-OVA model (Figure 4), but could not develop autoaggressive capacities.

Therefore, we next tested whether autoimmunity could be triggered in the presence of nonspecific inflammatory stimuli. Anti-CD40 treatment has been shown to induce tissue-specific autoimmune reactions to endogenous crosspresented model Ag, such as OVA (Vezys and Lefrancois, 2002) or LCMV glycoprotein (Roth et al., 2002). In addition, anti-CD40 treatment could replace the otherwise required $CD4^+$ T cell help for productive crosspriming of $CD8^+$ T cells (Bennett et al., 1998). Thus, the above experiment (Figures 6A and 6B) was repeated in the presence of agonist CD40 mAb (Figure 6C). However, in contrast to TCR-transgenic T cells (Roth et al., 2002; Vezys and Lefrancois, 2002) or $CD8^+$ T cells with specificity for foreign Ag (Bennett et al., 1998), the addition of anti-CD40 could not elicit adequate crosspriming of Rac-$CD8^+$ T cells for autoimmunity (Figure 6C). Therefore, autoreactive peripheral CD8 T cells from Rac mice behave similar to low-avidity OVA-specific CD8 T cells, which could not be activated by inflammatory stimuli plus self-Ag (Zehn and Bevan, 2006). These low-avidity OVA-specific CD8 T cells need high amounts of OVA expressed by recombinant bacteria to be activated for autoimmunity. Because the cognate Ag(s) of T cells from Rac mice are currently unknown, specific priming conditions need to be defined in order to reveal their specificities.

Rac T Cells Develop Autoimmunity under Lymphopenic Conditions

The conditions for effective crosspriming of polyclonal self-specific T cells accumulating in the absence of peripheral tolerance are unknown. Deductions from the crosspriming conditions defined for $CD8^+$ T cells specific for foreign Ag suggest dependence on help by $CD4^+$ T cells (Bennett et al., 1997). Furthermore, the precursor frequencies of such self-specific T cells are not known. Although a mouse may contain 100–200 naive $CD8^+$ T cells specific for a given viral epitope (Blattman et al., 2002), highly efficient thymic negative selection against self (Mathis and Benoist, 2004) should drastically reduce the frequencies of peripheral self-reactive T cells. Taking this into account, we next analyzed the autoimmune potential of Rac T cells in lymphopenic hosts. *Rag1*-deficient mice have been used extensively in adoptive-transfer studies of T cell-induced colitis. To improve the conditions for activation of Rac-$CD8^+$ T cells, we eliminated $CD25^+$ regulatory T (Treg) cells before transfer. In this model, the absence of suppression by Treg cells in combination with lymphopenia allows even self-tolerant wild-type T cells to induce autoimmunity (Powrie, 1995). If T cells from Rac mice have a greater autoimmune potential, we would expect either faster onset of disease or accelerated mortality. As expected, $Rag1^{-/-}$ mice receiving T cells exhibited diarrhea, weight loss, and other general signs of illness (Figure 7A, left panel, and data not shown). However, illness started significantly earlier in the groups of mice receiving T cells from Rac donors ($p = 0.0256$, log rank test; Figure 7A, right panel). In addition, the rate of early mortality before day 30 in these experiments was three times more elevated

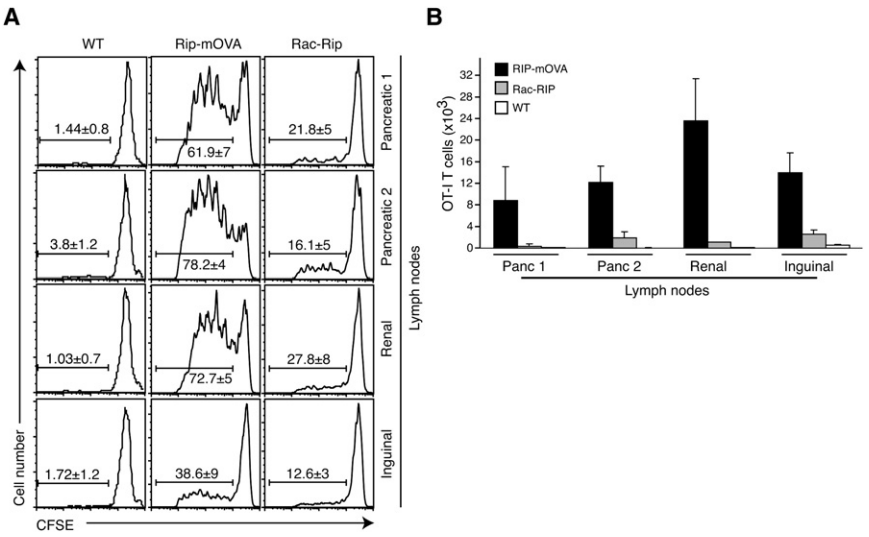

Figure 4. Reduced Proliferation of OVA-Reactive CD8$^+$ T Cells in Draining Lymph Nodes of Rac-RIP Mice

Wild-type, RIP-mOVA transgenic mice and CD11c-(N17) Rac1xRIP-mOVA double-transgenic mice (Rac-RIP mice) received 9 × 10^6 CFSE-labeled purified Ly5.1$^+$OT-I T cells i.v. Lymph nodes were analyzed 68 hr later. The frequency of dividing cells among OT-I T cells in the respective lymph nodes was determined by gating on CD8$^+$Ly5.1$^+$ cells (not shown). (A) shows undivided CFSE$^+$ OT-I T cells and daughter cells with diluted CFSE contents. The frequency of cells that have undergone one or more divisions is indicated (average of n = 3 mice; (data are represented as mean ± SEM)). (B) shows the total amounts of divided OT-I T cells found in the respective lymph nodes. The results from one out of four experiments with similar outcome are shown.

in Tg$^+$ → *Rag1*$^{-/-}$ as compared to Tg$^-$ → *Rag1*$^{-/-}$ mice (35% versus 10%, Figure 7A, left panel, and data not shown). Moreover, when we increased the numbers of Rac donor mice further and transferred pooled T cells from ten mice into only three recipients, two out of three mice receiving T cells from Rac donors, but not those from Tg$^-$ donors, developed simultaneous eye and skin disease 3 weeks after transfer. Histological analyses of eye and skin sections showed strong inflammatory infiltration (Figures 7B and 7C, upper panels). Further analysis revealed infiltration of CD8$^+$ T cells, but absence of CD4$^+$ T cells in both skin and eyes of the same animals (Figures 7B and 7C, lower panels). In addition, many of the CD8$^+$ T cells in skin were positive for Ki67, indicating proliferative expansion of CD8$^+$ T cells in situ (Figure 7C, low panel).

Together, these data show that absence of crosstolerization to apoptotic self-Ag allows autoreactive peripheral T cells to accumulate. These cells can contribute to autoimmune disease when activated under the appropriate conditions.

DISCUSSION

The actual contribution of crosstolerance to the control of self-reactive T cells in physiological situations is currently unclear. We have investigated this question by using a model of adoptive transfer of autoreactive T cells as well as analyzing endogenous polyclonal T cells in crosspresentation-deficient mice. Our study establishes a functional role for extrathymic crosspresentation of TSA to circulating peripheral CD8$^+$ T cells and identifies DCs as key players in peripheral tolerance induction.

We employed mice with a DC-specific defect in uptake of exogenous and apoptotic proteins due to inhibition of the GTPase Rac1 (Kerksiek et al., 2005; Neuenhahn et al., 2006). DCs from *Rac1*$^{-/-}$*Rac2*$^{-/-}$ mice have a general defect in T cell priming because they are unable to interact optimally with CD4$^+$ T cells (Benvenuti et al., 2004). In contrast, DCs from Rac mice utilized in this study displayed normal capacities to prime CD8$^+$ and CD4$^+$ T cell responses to HSV and peptide Ag. However, cell-associated (Kerksiek et al., 2005), soluble, or DEC-205-targeted protein was not efficiently crosspresented by Rac DCs. In addition to reduced uptake of antigenic material, steps further downstream of Ag internalization may also be Rac dependent. For example, we detected no defect in DEC-205 internalization (data not shown), although crosspresentation of DEC205-OVA was severely reduced. Therefore, N17Rac may inhibit actin-dependent events during receptor internalization and trafficking such as constriction of vesicle necks and scission from the plasma membrane that we were unable to detect in our assay (reviewed in Kaksonen et al. [2006]). In addition, actin-independent steps of crosspresentation may also be defective in Rac mice. It has been shown that the NAPDH-oxidase NOX2 generates reactive oxygen species (ROS) to maintain alkalinization of the phagosomal lumen in DCs. This preserves epitopes for crosspresentation (Savina et al., 2006), and activated Rac1 is essential for NOX2-complex function (Hordijk, 2006; Segal, 2005). Accordingly, DCs from *Vav1*$^{-/-}$ mice, deficient for a regulator of Rac1, do not produce ROS and fail to crosspresent particulate Ag, but show functional Ag uptake (Graham et al., 2007). Taken together, although direct MHC class I and class II presentation were normal, Rac mice displayed a selective crosspresentation deficiency.

Uptake and presentation of self-Ag by DCs also play important roles in central tolerance induction of self-reactive CD4$^+$ but not CD8$^+$ thymocytes (Gallegos and Bevan, 2004). Unexpectedly, we found that negative selection of self-reactive CD4$^+$ OT-II thymocytes was normal in Rac mice. Thymic DCs probably pick up TSA from thymic medullary epithelial cells (mTECs) (Kyewski and Klein, 2006). However, neither the mechanism of TSA transfer nor the stability or form of thymic self-Ag are currently known. If TSA was transferred as cell-associated protein from apoptotic cells, we might expect an uptake defect for Rac DCs. However, although mTEC might die after 2–3 weeks (Gaebler et al., 2007), it is unknown whether TSA transfer to thymic DCs depends on mTEC apoptosis. In addition, DCs are able to pick up membrane from live cells for Ag presentation (Harshyne et al., 2003). If TSA was secreted or otherwise released by mTECs, distinct uptake mechanisms could simultaneously bring TSA into separate intracellular compartments responsible for presentation to CD4$^+$ or CD8$^+$ T cells, similar to peripheral DCs (Burgdorf et al., 2007). Here, the pathway for crosspresentation via MHC class I may

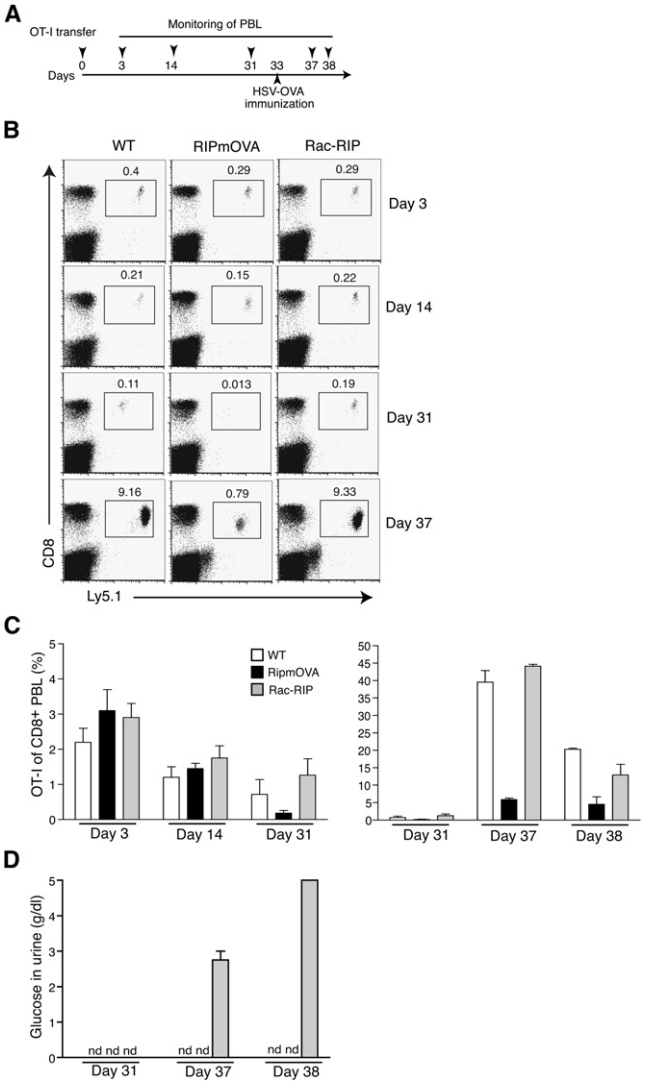

Figure 5. Defective Peripheral Tolerance Induction in Rac-RIP Mice

(A) Wild-type, RIP-mOVA, and CD11c-(N17) Rac1xRIP-mOVA (Rac-RIP) mice received 8 × 10^6 purified Ly5.1$^+$ OT-I T cells i.v. and were then treated and monitored.

(B) PBL from all mice were analyzed by flow cytometry 3, 14, and 31 days later for the presence of CD8$^+$Ly5.1$^+$OT-I T cells. Numbers indicate the percentage of OT-I T cells of PBL (cells in gate).

(C) The respective frequencies of OT-I T cells of CD8$^+$ PBL are shown (left panel, average [of n = 2] ± SD). All mice were immunized with 4 × 10^6 HSV-OVA i.v. 2 days later (day 33) as described in Figure 1. Changes in frequencies of OT-I T cells were monitored 4 (day 37; [B] and [C], right panel) and 5 (day 38; [C], right panel) days after immunization.

(D) In parallel, we monitored all mice for the onset of diabetes by measuring glycosuria (nd = not detectable). Bar graphs represent the average of n = 3 mice (data are represented as average ± SD). One of three experiments with similar results is shown.

be more dependent on Rac1 function than the MHC class II pathway. Taken together, our findings revealed that Rac mice displayed a normal ability to negatively select self-reactive CD4$^+$ and CD8$^+$ thymocytes.

Most importantly, the Rac model allowed the investigation of endogenous, peripheral self-reactive CD8$^+$ T cells, accumulating due to defective crosspresentation. The finding that anti-CD40 was insufficient to induce autoimmunity confirms observations with transferred L^d-alloreactive 2C TCR-transgenic T cells, which also could not be primed by anti-CD40 treatment (Buhlmann et al., 2007). In contrast, for OT-I and P14 T cells, anti-CD40 was sufficient to induce autoimmunity (Roth et al., 2002; Vezys and Lefrancois, 2002). These discrepancies could either be due to the different nature and amounts of Ag expressed in the respective models or the different affinities of TCR (OT-I, P14 versus 2C). Likewise, low-avidity polyclonal T cells from Vß5xRIP-mOVA mice also could not be triggered to cause diabetes in RIP-mOVA mice, when global viral or bacterial inflammatory stimuli were employed (Zehn and Bevan, 2006). Only when the critical density of Ag-MHC class I complexes is increased by infection with OVA-expressing bacteria do low-avidity T cells become autoaggressive (Zehn and Bevan, 2006). Because the specific cognate TSA recognized by Rac T cells are currently unknown, further work is ongoing to identify these TSA in Rac mice.

When Treg cells were depleted, Rac T cells could expand homeostatically in lymphopenic *Rag1*$^{-/-}$ mice and autoimmunity could be developed. Under these conditions, T cells gain memory phenotypes but do not normally transit through an activated effector cell state (Cho et al., 2000; Goldrath et al., 2004). However, if amounts of IL-2 and IL-15 rise (Cho et al., 2007), or if they recognize cognate Ag, T cell effector functions may develop. Rac T cells became effector cells in *Rag1*$^{-/-}$ mice, probably because they encountered self-Ag during expansion. For efficient crosspriming of CD8$^+$ T cells, CD4$^+$ T cells must recognize Ag on the same DC that crosspresents cellular Ag to the CD8$^+$ T cells (Bennett et al., 1997). The development of true memory function by CD8$^+$ T cells expanding in lymphopenic hosts also requires CD4$^+$ T cell help (Hamilton et al., 2006). Therefore, autoreactive Rac-CD8$^+$ T cells are certainly CD4 dependent. Because Rac mice perform normal negative selection, peripheral tolerance to apoptotic TSA might be similarly defective for CD4$^+$ and CD8$^+$ T cells.

Neither the identity of TSA nor the tissues that are normally protected by crosstolerance are known. The accumulation of self-reactive peripheral T cells in Rac mice argues for an important role of crosstolerance in vivo. It could account for tolerance to self-proteins that are not efficiently expressed or presented in the thymus (Kyewski and Klein, 2006). Several studies have investigated the possibility that DCs express and directly present TSA. However, the conclusions from these studies are contradictory. Expression of genes encoding for TSA such as proinsulin, glutamic acid decarboxylase 65, myelic basic protein, thyroid peroxidase, and pancreatic Ag IA-2 was found in CD11c$^+$ cells from peripheral blood and spleen (Garcia et al., 2005). In contrast, another study did not find expression of insulin, GAD65, and IA-2 in DCs (Derbinski et al., 2001). Therefore, it remains to be clarified whether and which DCs express and directly present certain TSA or whether they are entirely dependent on uptake of exogenous TSA. The accumulation of self-specific CD8$^+$ T cells in Rac mice indicates an important role for crosspresentation of exogenous TSA by DCs. Certain TSA epitopes such as signal sequences (Wolkers et al., 2004) or TSA with insufficient stability might be "ignored" by peripheral crosstolerance. Hence, CD8$^+$ T cell tolerance to these epitopes must rely on direct MHC class

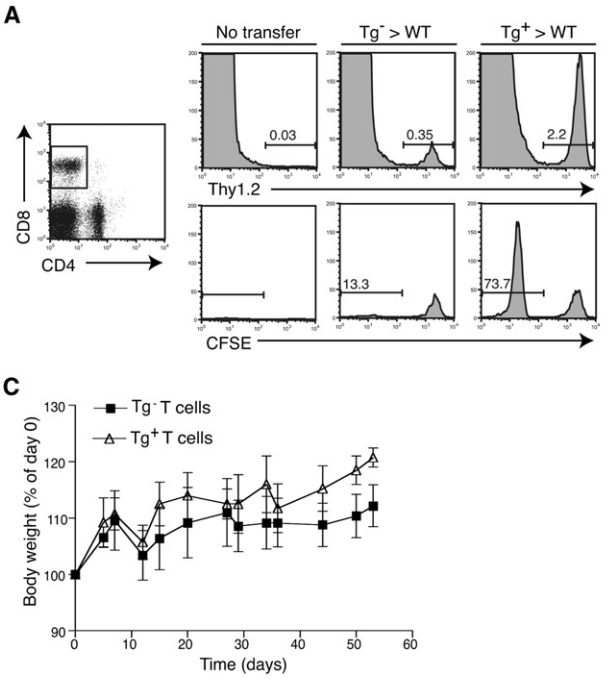

Figure 6. Accumulation of Peripheral, Self-Reactive CD8⁺ T Cells in Rac Mice

Peripheral T cells were isolated from Thy1.2⁺ Rac-mice (Tg⁺) or Thy1.2⁺ nontransgenic littermates (Tg⁻), CFSE-labeled, and transferred i.v. into Thy1.1⁺ congenic recipients (WT). As shown in (A), 15 days later, spleens and lymph nodes (not shown) were analyzed for the presence of CD8⁺Thy1.2⁺ donor T cells. CD8⁺Thy1.2⁺ T cells were further analyzed for proliferation by gating on cells that had diluted their CFSE label (lower panel). (B) shows total numbers of CD8⁺Thy1.2⁺ donor T cells were enumerated from spleens (n = 3–5 mice per group, data are represented as average ± SD). One out of three experiments with similar outcome is shown. As shown in (C), mice received 100 μg CD40 mAb (FGK) i.p. on day −2 and day −1. On day 0, 8 × 10^6 T cells from Rac-Tg⁺ or -Tg⁻ mice were transferred as described in (A). Mice were monitored 2× week for signs of disease such as fur ruffling, alopecia, diarrhea, hunched posture (not shown), and weight loss (average [of n = 5] ± SD).

I presentation, as executed for central tolerance induction by thymic epithelial cells (Kyewski and Klein, 2006) and for peripheral tolerance induction by lymph node stromal cells (Lee et al., 2007) and parenchymal tissue cells, encountered on the normal migratory pathways of naive CD8⁺ T cells (Cose et al., 2006).

Another factor possibly limiting crosstolerization by DCs is the accessibility of tissues. Recent studies have shown that tolerance against proinsulin-2 does not depend on the thymus because a thymic proinsulin-2 gene deficiency was insufficient to induce diabetes (Faideau et al., 2006). In this study, an increase of insulin-specific T cells was observed, and peripheral tolerance mechanisms were sufficient to prevent autoimmunity (Faideau et al., 2006). However, in another study, lack of expression of the interphotoreceptor retinoid-binding protein gene in the thymus caused spontaneous eye-specific autoimmune disease (DeVoss et al., 2006). Because the eye is an immunologically privileged site, there may be limited access for DCs to acquire eye-specific TSA for crosstolerance induction. These studies exemplify that if thymic imperfection allows the generation of autoreactive T cells, peripheral crosstolerance may correct this failure only if TSAs are accessible. However, when tissues share TSAs, then crosstolerance induced by the more accessible tissue might also protect the immune-privileged one. In contrast, TSA that are neither expressed in the thymus nor part of the "cross-presentation repertoire" of DCs might be of particular interest for the induction of tumor-specific T cell-immunity, because they have been spared from tolerance (Speiser et al., 1997).

Interfering with the TSA-uptake function of DCs renders peripheral crosstolerance inefficient, allowing survival of TSA-reactive CD8⁺ T cells that menace peripheral tissues and increase the risk for autoimmune attack. Therefore, pathways controlling uptake of TSA by DCs might turn out to be an Achilles heel of the immune system for functional peripheral crosstolerance induction.

EXPERIMENTAL PROCEDURES

Mice

CD11c-(N17)Rac1 mice have been described previously (Kerksiek et al., 2005) and were backcrossed to C57BL/6 for at least 14 generations. C57BL/6, C57BL/6.Thy1.1⁺, OT-I (CD8⁺ TCR-Tg specific for the ovalbumin [OVA]-derived SIINFEKL peptide) and OT-I in Ly5.1, and *Rag1*$^{-/-}$ mice were originally obtained from the Jackson Laboratory. RIP-mOVA mice expressing transgenic OVA under control of the rat insulin promoter in the pancreas were a kind gift from W. Heath, Australia. All mice were bred and maintained at the animal facilities of the Institute for Immunology (LMU, Munich) in accordance with established guidelines of the Regional Ethics Committee of Bavaria.

Adoptive Transfer and CFSE Labeling of OT-I Cells

OT-I T cells were prepared from lymph nodes and spleens of transgenic mice. In brief, spleen and lymph nodes were harvested, and single-cell suspensions were prepared. Spleen red blood cells were removed with ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA [pH 7.4]) for 4 min at room temperature, and OT-I cells were isolated by negative selection (>96% purity, CD8⁺ T cell isolation kit, MACS LS Separation Columns, Miltenyi Biotec). Enriched cells were labeled with CFSE (5 μM per 1 to 50 × 10^6 cells/ml, Molecular Probes) for 10 min at 37°C. After determining the percentage of OT-I TCR-transgenic T cells by flow cytometry, we injected the indicated numbers of T cells into the lateral tail veins of age- and sex-matched recipient mice or cultured them in vitro under the conditions described.

Immunizations

Mice were immunized i.v. via the tail vein with the indicated dose of Ag in 200 μl PBS (100 μg or 5 mg OVA-protein and 10 μg LPS [Sigma]; 10 μg SIINFEKL-peptide and 10 μg LPS). A total of 10 μg of αDEC205-OVA antibody conjugate (Bonifaz et al., 2002) and 10 μg LPS was injected i.v. via the tail vein. Replication incompetent Herpes simplex virus expressing OVA (TOH-OVA) has been described previously (Lauterbach et al., 2004) and was injected at 4 × 10^6 particles per mouse i.v. Virus-like particles (VLPs) have been described previously (Storni et al., 2004) and were used at a concentration of 150 mg per subcutaneous injection.

Monitoring of Mice for Signs of Illness

Glucosuria in RIP-mOVA mice was determined daily with test stripes (Diabur 5000, Roche Diagnostic). Signs of illness following T cell transfer were defined

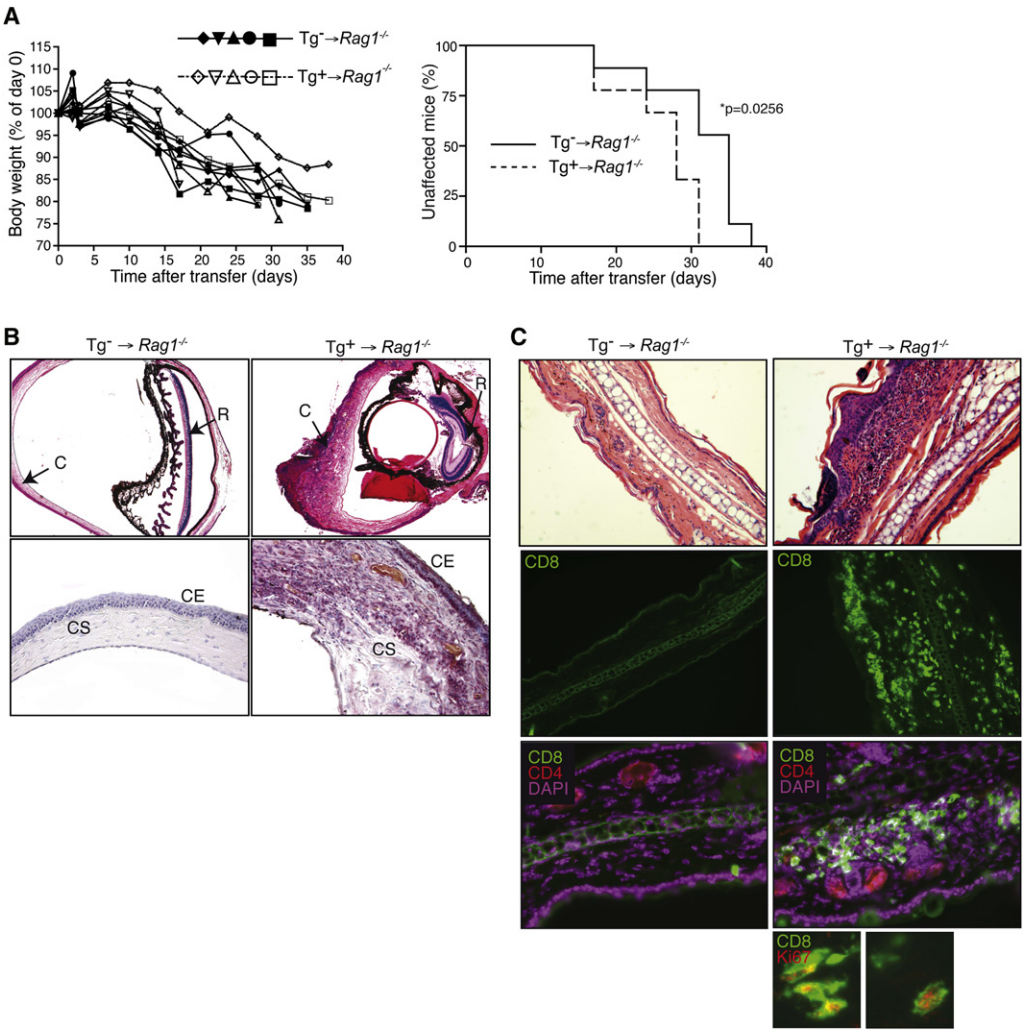

Figure 7. T Cells from Rac Donors Induce Early Onset of Illness in *Rag1*-Deficient Mice

(A) *Rag1*$^{-/-}$ mice received 8 × 10^6 CD25-depleted T cells from either Rac-Tg$^+$ (left panel, open symbols, dotted line) or -Tg$^-$ (left panel, filled symbols) donors. Mice were monitored for illness 2× a week (fur ruffling, alopecia, diarrhea, hunched posture, weight loss, etc.). Kinetics of weight loss is shown from one experiment (n = 5 mice per group) out of three with a similar outcome (left panel). Pooled data from two experiments (total of n = 10 mice per group) show a significant difference (p = 0.0256) in the percentage of mice exhibiting early onset of illness in mice that received T cells from Rac donors (right panel, Tg$^+$→*Rag1*$^{-/-}$).

(B) In the upper panels, 8 × 10^6 T cells pooled from 10 Tg$^-$ or Tg$^+$ donors were transferred into 3 *Rag1*$^{-/-}$ recipients. Three weeks after transfer, two out of three mice developed simultaneous eye and skin disease. The upper-left panel shows H & E reference staining of Tg$^-$→*Rag1*$^{-/-}$ control mice, showing normal corneal (C) and retinal (R) architecture; the upper-right panel shows a representative section of Tg$^+$→*Rag1*$^{-/-}$ diseased eye with disruption of the corneal epithelium and thickening of corneal stroma and marked infiltration of leucocytes, with H & E staining. The lower-left panel shows anti-CD8-staining and hematoxylin counterstain of Tg$^-$→*Rag1*$^{-/-}$ mice, in the cornea; no CD8$^+$ cells were detectable. The lower-right panel shows infiltration of the corneal epithelium (CE) and stroma (CS) by CD8$^+$ cells (violet) in Tg$^+$→*Rag1*$^{-/-}$ mice eyes. Counterstaining with anti-CD3 revealed that all CD3$^+$ cells were also CD8$^+$ (not shown).

(C) In the upper panels, H & E staining of ears from Tg$^-$→*Rag1*$^{-/-}$ (left) or Tg$^+$→*Rag1*$^{-/-}$ (right). Only in the Tg$^+$→*Rag1*$^{-/-}$ ears were inflammatory infiltration with epidermal participation and crusting detectable. In the second row (CD8), these infiltrations contained CD8$^+$ cells. In the third row (CD8/CD4/DAPI), CD4$^+$ cells could not be detected. As shown in the bottom row, several CD8$^+$ T cells were Ki67 positive, indicating proliferation in situ.

as the following: diarrhea (determined by the presence of fecal matter on the perianal region), hunched posture, fur ruffling, alopecia, weight loss, respiratory distress, and skin or eye lesions.

Histology

Formalin fixed eyes were embedded in paraffin (Microm). Ag retrieval was performed at 99°C for 15 min in 0.1 M EDTA-NaOH buffer (pH 8.8). We used rat anti-mouse CD8 hybridoma supernatant (clone CD8-2) for CD8 staining and rat anti-mouse CD3 hybridoma (clone CD3-12, both antibodies kindly provided by E. Kremmer, Helmholtz Center, Munich, Germany) for CD3 staining overnight at 4°C in a humidified chamber. After washing, sections were incubated with biotinylated secondary Ab; goat anti rat IgG (Linaris) for 1 hr. After washing, peroxidase-conjugated streptavidin was applied (Vectastain ABC-Elite:HRP kit; Linaris). Binding was visualized with the Vector HRP-substrate kit VIP, which results in violet color (Linaris). Subsequently, the slides were counterstained with Mayer's hematoxylin (Microm) and mounted. Images were obtained with a Leica microscope DMR (Leica), and digital images were obtained with the Leica DFC320 R2 camera.

For cryosections, skin was embedded in O.C.T. medium (no. 4583; Miles) and snap frozen; 5 μm sections were cut with a cryostat (Jung Frigocut 2800 E, Leica). Sections were air-dried overnight, acetone fixed (−20°C for 10 min), and air-dried for a minimum of 12 hr. Sections were rehydrated for

15 min in PBS containing 0.25% BSA and blocked for 15 min in PBS containing 0.25% BSA and 10% mouse serum. Abs, rat anti-mouse CD8-Alexa 488 (53-6.7), rat anti-mouse CD 4-biotin (L3T4), and mouse anti-human Ki67-PE (B56) (all purchased from BD PharMingen) diluted in blocking buffer were added directly onto the sections and incubated for 30 min. After washing, sections were either incubated with Streptavidin-Alexa 555 (Molecular Probes) and/or with DAPI (1 μg/ml, Molecular Probes) and directly mounted in Fluoromount (Southern Biotechnology Assoc.). Sections were analyzed on an Olympus BX41TF-5 microscope (Olympus CELL-BND-F software) equipped with F-View II Digital Mikro camera (Olympus).

In Vivo CTL Assay

This assay was performed as described previously (Coles et al., 2002). Syngeneic C57BL/6 spleen cells were depleted of erythrocytes by osmotic lysis. Cells were washed and split into two populations. One population was pulsed with 10^{-6} M $OVA_{257-264}$-peptide for 1 hr at 37°C, washed, and labeled with a high concentration of CFSE (1.6 mM) ($CFSE^{high}$ cells). The second control population was labeled with a low concentration of CFSE (0.24 mM) ($CFSE^{low}$ cells). For i.v. injection, an equal number of cells from each population ($CFSE^{high}$ and $CFSE^{low}$) were mixed, such that each mouse received a total of 2×10^7 cells. At the indicated time points, mice were sacrificed and spleen and lymph nodes were removed. Cell suspensions were analyzed by flow cytometry; ~5×10^5 CFSE-positive cells were collected for analysis. Peptide-pulsed and unpulsed target cells were recognized according to their different CFSE intensities. To calculate specific lysis, we used the following formulas: ratio = (percentage $CFSE^{low}$/percentage $CFSE^{high}$); percentage-specific lysis = (1 − (ratio unprimed/ratio primed) × 100).

Bone-Marrow Chimera Model for OT-I Negative Selection

Bone-marrow cells were collected from femurs and tibiae of donor mice 6–8 weeks of age. Red blood cells were removed with ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA [pH 7.4]) for 4 min at room temperature, and 5×10^6 cells were injected i.v. into lethally irradiated (split dose day −2 and day 0: 450 rad) recipient mice (age 10–15 weeks). Upon irradiation, and for 5 weeks after, chimeric mice were given drinking water supplemented with neomycin trisulfate (Sigma). Chimeras were analyzed 8–9 weeks after reconstitution.

Rac T Cell Transfer

C57BL/6 in Thy1.1 mice received 1×10^7 CFSE-labeled (5 μM per 1 to 50×10^6 cells/ml), pan T cells (purified to >96% T cells, with the Pan T cell isolation kit, Miltenyi Biotec) from either Tg^- ($Thy1.2^+$) or Tg^+ ($Thy1.2^+$) mice. Mice were sacrificed 15 days later, and spleen and lymph nodes were analyzed for proliferation and activation of $Thy1.2^+$ donor T cells determined by CFSE dilution and high expression levels of CD44 and low levels of CD62L.

Rag1$^{-/-}$ donors received 8×10^6 CD25-depleted, purified pan T cells from the indicated donors via the tail vein. Donor T cells were harvested from spleen and lymph nodes, and pan T cells were negatively selected by magnetic separation as described above. The T cells were then depleted of $CD25^+$ cells with the CD25 microbead kit (<0.5% $CD25^+$ cells final, Miltenyi Biotec). After cell transfer, mice were monitored two times a week for signs of illness as described above.

In Vitro Stimulation

Mice were immunized i.v. with 5 mg OVA in PBS or PBS alone. The next day, animals were sacrificed and spleens were removed and digested in a solution of LiberaseCI and DNase I (Roche) in serum-free medium for 20 min at 37°C. Spleens were passed through a 70 μM nylon mesh strainer and $CD8^+$ DCs were magnetically isolated with the MACS $CD8^+$ dendritic cell isolation kit (Miltenyi Biotec). The cell preparations routinely consisted of 60%–80% $CD11c^+$, $CD8^+$ cells with <5% of the cells being $CD11c^+$, $CD8^-$. Purity was lower after protein immunization of all groups. Equal numbers of $CD8^+$ DCs were cocultured in vitro with CFSE-labeled OT-I in Ly5.1 T cells (prepared as described above) at DC:T cell ratios of 1:2.5 and 1:5 in the presence of GM-CSF for 4 days.

Antibodies and Multimers

The following Ab or reagents from BD PharMingen were used for flow cytometry: CD4-PerCP (RM4-5), CD8α-FITC and -PerCP (53-6.7), CD44-FITC and APC (IM7), CD62L-APC (MEL-14), Vα2-PE (B20.1), Vβ5-FITC (MR9-4), IFN-γ-PE (XMG1.2), isotype-control rat IgG1-PE (R3-34), Streptavidin-APC, Ly5.1-FITC and -biotin (A20), CD8-APC (CT-CD8α), CD44-PE (IM7.8.1), CD90.1-PE (53-2.1), and CD16/32 (2.4G2). PE-conjugated MHC class I multimer reagents (H2-Kb/SIINFEKL; H2-Kb/gB; H2Kb/B8R 20-27) were purchased from Proimmune. αDEC205-OVA antibody conjugate was produced as previously described (Bonifaz et al., 2002).

Staining of Cells for Flow Cytometry

Staining of surface molecules was performed with 1×10^6 to 6×10^6 cells in cold staining buffer for 30 min at 4°C (15 min at room temperature in the dark when MHC multimers were used). Dead-cell exclusion was attained by incubation with 1 μg/ml ethidium monoazide bromide (EMA, Molecular Probes) prior to surface staining or the addition of 0.8 mg/ml propidium iodide (PI, Sigma). Intracellular staining for cytokines was performed with the Cytofix/Cytoperm kit (PharMingen). Flow cytometry was performed with a FACSCaliburor FACSaria (Becton Dickinson), and data were analyzed with FlowJo software (Tree Star).

Stimulation of Cytokine Production by Epitope-Specific T Cells

Splenocytes (2×10^8/well in 2 ml RPMI medium) were incubated with 1 μg/ml SIINFEKL peptide (Neosystems) and 1 μl/ml Golgiplug (PharMingen) in a 24-well plate for 5 hr at 37°C. Stimulated cells (5×10^7) were washed and then incubated for 20 min with 250 μg/ml Fc block (2.4G2) (PharMingen) and 1μg/ml EMA on ice. After washing, surface molecules were stained for 20 min on ice. Cells were washed and resuspended in Cytofix and incubated for 20 min on ice. Cells were then washed in Perm wash buffer, and intracellular staining (0.4 μg/ml anti-IFN-γ mAb) was performed in Permwash buffer for 20 min on ice.

Statistical Analysis

Data were analyzed with Student's t test for all analysis except the Kaplan Meier plot for which the log rank test was employed. A p value of $p < 0.05$ was considered to be significant.

SUPPLEMENTAL DATA

Two figures are available at http://www.immunity.com/cgi/content/full/28/4/521/DC1/.

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