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A Critical Role for Stat3 Signaling in Immune Tolerance

Fengdong Cheng,[1] Hong-Wei Wang,[1] Alex Cuenca,[1] Mei Huang,[1] Tomar Ghansah,[1] Jason Brayer,[1] William G. Kerr,[1,2] Kiyoshi Takeda,[3] Shizuo Akira,[3] Stephen P. Schoenberger,[4] Hua Yu,[1] Richard Jove,[1,2] and Eduardo M. Sotomayor[1,*]

[1]Department of Interdisciplinary Oncology and
[2]Department of Biochemistry
H. Lee Moffitt Cancer Center & Research Institute
University of South Florida
Tampa, Florida 33612
[3]Department of Host Defense
Osaka University
Osaka 565-0871
Japan
[4]Division of Immune Regulation
La Jolla Institute for Allergy and Immunology
San Diego, California 92121

Summary

Antigen-presenting cells (APCs) can induce T cell activation as well as T cell tolerance. The molecular mechanisms by which APCs regulate this critical decision of the immune system are not well understood. Here we show that Stat3 signaling plays a critical role in the induction of antigen-specific T cell tolerance. Targeted disruption of Stat3 signaling in APCs resulted in priming of antigen-specific $CD4^+$ T cells in response to an otherwise tolerogenic stimulus in vivo. Furthermore, APCs devoid of Stat3 effectively break antigen-specific T cell anergy in vitro. Conversely, increased Stat3 activity in APCs led to impaired antigen-specific T cell responses. Stat3 signaling provides, therefore, a novel molecular target for manipulation of immune activation/tolerance, a central decision with profound implications in autoimmunity, transplantation, and cancer immunotherapy.

Introduction

Bone marrow (BM)-derived APCs play an important role in the initiation of productive antigen-specific T cell responses (Banchereau and Steinman, 1998). However, these cells are also required for the induction of T cell tolerance (Adler et al., 1998; Kurts et al., 1997). Thus, APCs are at the center of a critical decision leading to immune activation versus immune tolerance. A potential explanation for this dual function of APCs is that perhaps a subset of APCs could preferentially induce tolerance (Huang et al., 2000; Munn et al., 2002; Scheinecker et al., 2002) while a different subpopulation may trigger T cell priming. Alternatively, it has been proposed that the state of activation/differentiation of the APC at the time of antigen presentation is the central determinant of T cell priming versus tolerance (Lanzavecchia, 1998), and perhaps each APC's subpopulation may have the potential to induce either T cell outcome. Indeed, Belz et al. (2002) have recently demonstrated that $CD11c^+$, $CD8\alpha^+$ DCs, a subset previously implicated in cross-priming (den Haan et al., 2000), could also induce peripheral tolerance to tissue-associated antigens. In spite of these advances at the cellular level, the intracellular signaling pathways in APCs that lead to T cell activation versus T cell tolerance remains to be elucidated.

In recent years, one of the most important insights into immune regulation was provided by the identification of negative regulatory pathways that, by counteracting positive signaling pathways, greatly influence the magnitude of immune responses (Ravetch and Lanier, 2000). Initiation of productive immune responses is often triggered by APCs capturing antigen in the context of inflammatory or tissue-destructive processes (Pardoll, 2001). In these settings, APCs are exposed to numerous stimuli simultaneously, and it is likely that their final functional outcome would be determined by a delicate interplay among activating and inhibitory intracellular signaling pathways.

Signal transducer and activators of transcription (Stats) are cytoplasmic transcription factors that are key mediators of cytokine and growth factor signaling pathways (Darnell, 1997). Recently, one of the members of the Stat family, Stat3, has emerged as a negative regulator of inflammatory responses. Indeed, Hackenmiller et al. have demonstrated that increased Stat3 activation in c-$fes^{-/-}$ mice is associated with impaired inflammatory responses (Hackenmiller et al., 2000). Conversely, Takeda et al. have found that in mice with a cell type-specific disruption of the Stat3 gene in macrophages, these cells are abnormally activated and produce higher levels of inflammatory cytokines in response to endotoxin (Takeda et al., 1999). Further highlighting the regulatory role of Stat3 signaling in immune responses, Welte et al. have demonstrated that tissue-specific disruption of this signaling pathway during hematopoiesis is associated with an overactivated innate immunity leading to severe inflammatory bowel disease and lethality in mutant mice (Welte et al., 2003).

In this study, we evaluated, therefore, whether Stat3 signaling may play a role in the ability of APCs to determine T cell activation versus T cell tolerance. Indeed, disruption of this signaling pathway leads to the generation of APCs that effectively prime naive antigen-specific T cells and restore the responsiveness of anergic $CD4^+$ T cells. Conversely, increased Stat3 activity in APCs resulted in impaired T cell responses. Importantly, our findings that induction of T cell tolerance occurs in mice with an intact Stat3 signaling in APCs, but not in mice with targeted disruption of this signaling pathway, further emphasizes an important regulatory role for Stat3 in the in vivo induction of antigen-specific T cell tolerance.

Results

Enhanced Antigen-Presenting Cell Function of Tyrphostin AG490-Treated Macrophages

To assess whether Stat3 signaling in APCs may influence antigen-specific T cell responses, we first treated

*Correspondence: sotomed@moffitt.usf.edu

peritoneal elicited macrophages (PEM) with increasing concentrations of the JAK-selective inhibitor, tyrphostin AG490, a compound known to block Stat3 activation in malignant cells (Catlett-Falcone et al., 1999). Following this treatment, naive $CD4^+$ T cells specific for a MHC class II-restricted epitope of influenza hemagglutinin (HA) were added to these APCs and stimulated or not with cognate HA-peptide. As shown in Figure 1A, clonotypic T cells encountering cognate peptide on AG490-treated PEM display an enhanced HA-specific proliferation (Figure 1Aa) and IL-2 production (Figure 1Ab) as compared to T cells encountering cognate peptide on either untreated PEM (No LPS) or PEM treated with LPS alone. More importantly, AG490-treated PEM trigger effector function of clonotypic $CD4^+$ T cells, as determined by the capacity of these T cells to produce IFN-γ in response to HA-peptide stimulation (Figure 1Ac). In sharp contrast, antigen-specific T cells encountering HA-peptide on untreated or LPS-treated PEM were unable to produce IFN-γ. This ability of AG490-treated PEM to trigger IFN-γ production was not just the result of the drug speeding up cytokine expression in naive T cells since kinetic studies showed that even after 72 hr of incubation, T cells encountering HA-peptide on untreated PEM were still unable to produce IFN-γ (Figure 1B).

Utilizing a T cell receptor transgenic model, we have previously demonstrated that $CD4^+$ T cells specific for an MHC class II epitope of influenza hemagglutinin (HA) are rendered unresponsive during the progression of A20 B cell lymphoma expressing HA as a model tumor antigen (A20HA) (Staveley-O'Carroll et al., 1998). In this system, clonotype-positive T cells were found to be tolerant by their failure to be primed in vivo as well as by their diminished proliferation and IL-2 and IFN-γ production in response to in vitro restimulation with cognate antigen. However, as shown in Figure 1C, incubation of these same tolerant T cells (reisolated from A20HA lymphoma-bearing mice) with AG490-treated PEM resulted in restoration of T cell responsiveness to cognate HA antigen. Indeed, PEM treated with either 16.6 or 50 μM of AG490 + LPS were able to trigger HA-specific proliferation (Figure 1Ca), IL-2 (Figure 1Cb), and IFN-γ production by tolerant $CD4^+$ T cells (Figure 1Cc). In sharp contrast, tolerant T cells encountering HA antigen on either untreated (No LPS) or LPS-treated PEM remained fully unresponsive.

Strikingly, tolerized T cells display an enhanced antigen-specific proliferation and produce significantly more IL-2 and IFN-γ on a per cell basis than naive antigen-specific T cells in response to antigen presented by AG490-treated PEM. Indeed, HA-specific proliferation by tolerized T cells was almost 8-fold higher relative to the response of naive T cells to cognate antigen presented by PEM treated with LPS + 50 μM of AG490 (3283 cpm versus 425 cpm per 100 clonotype+ T cells, respectively, Figures 1Ca and 1Aa). A 10-fold increase in IFN-γ production by tolerized T cells relative to naive T cells was also observed (37.8 pg/ml versus 3.7 pg/ml per 100 clonotype+ T cells respectively, Figures 1Cc and 1Ac). IL-2 production by tolerized T cells was also increased, but only by 2-fold as compared to naive clonotypic T cells (21.7 pg/ml versus 10 pg/ml, respectively, Figures 1Ab and 1Cb). This greater magnitude of response of tumor-tolerant T cells than that of naive T cells suggests that AG490-treated PEM are capable of overcoming T cell unresponsiveness rather than just enhancing the activation of those naive T cells that may have escaped tolerance induction in vivo.

The ability of tyrphostin AG490 to enhance the antigen-presenting capabilities of PEM was associated with a complete inhibition of Stat3 DNA binding activity in these cells. Shown in Figure 1D is the Stat3 DNA binding activity of untreated or treated PEM. Untreated PEM display constitutive activation of Stat3. Following stimulation with LPS, Stat3 activity was enhanced in PEM, reminiscent of previous studies in human monocytic cells (Benkhart et al., 2000). In sharp contrast, PEM treated with LPS in the presence of 50 μM of AG490 display almost complete inhibition of Stat3 DNA binding (Figure 1D, $AG490^+/LPS^+$), an effect that correlates with the ability of these APCs to restore the responsiveness of tolerant $CD4^+$ T cells (Figure 1C). Treatment of PEM with lower doses of AG490 also resulted in inhibition of Stat3 DNA binding activity, although this effect was not as dramatic as with the 50 μM dose (see Supplemental Data at http://www.immunity.com/cgi/content/full/19/3/425/DC1).

Inhibition of Stat3 Signaling in Dendritic Cells Restores the Responsiveness of Tolerant $CD4^+$ T Cells

Given our results with PEM, we evaluated next whether inhibition of Stat3 signaling in BM-derived DCs may also influence the ability of these cells to present cognate antigen to naive (Figure 2A) or tolerant T cells (Figure 2B). Analysis of $CD4^+$ T cell responses to HA-peptide presented by untreated or LPS-treated DCs showed that these APCs can induce full activation of naive T cells, as determined by their capacity to proliferate and produce IL-2 as well as IFN-γ (Figures 2Aa–2Ac, No LPS or LPS). In contrast with our observations in PEM treated with LPS + AG490 (Figure 1A), similar treatment of BM-derived DCs did not result in further enhancement of either HA-specific proliferation (Figure 2Aa) or IL-2 production (Figure 2Ab) by naive T cells. A modest increase in IFN-γ production was observed, however, when naive T cells encountered HA-antigen on DCs treated with LPS + 50 μM of AG490 as compared to those T cells encountering antigen on DCs treated with LPS alone (Figure 2Ac).

Analysis of the response of tolerant $CD4^+$ T cells to cognate antigen presented by BM-derived DCs, however, unveiled important findings (Figure 2B). First, untreated or LPS-treated BM-derived DCs were unable to restore the responsiveness of tolerant T cells isolated from tumor-bearing mice. Although tolerant T cells were capable of proliferating in response to HA-peptide (Figure 2Ba, No LPS or LPS), this response was of a lesser magnitude relative to the proliferative response of naive T cells encountering antigen on untreated or LPS-treated DCs (Figure 2Aa, No LPS or LPS). Furthermore, tolerant T cells were unable to produce IL-2 (Figure 2Bb) or IFN-γ (Figure 2Bc) in response to cognate antigen presented by untreated or LPS-treated DCs. Only when DCs were treated with LPS plus AG490 were they capable of restoring the responsiveness of tolerant T cells. Indeed, in these tolerant T cells, HA-specific proliferation was no longer inhibited (Figure 2Ba), and they re-

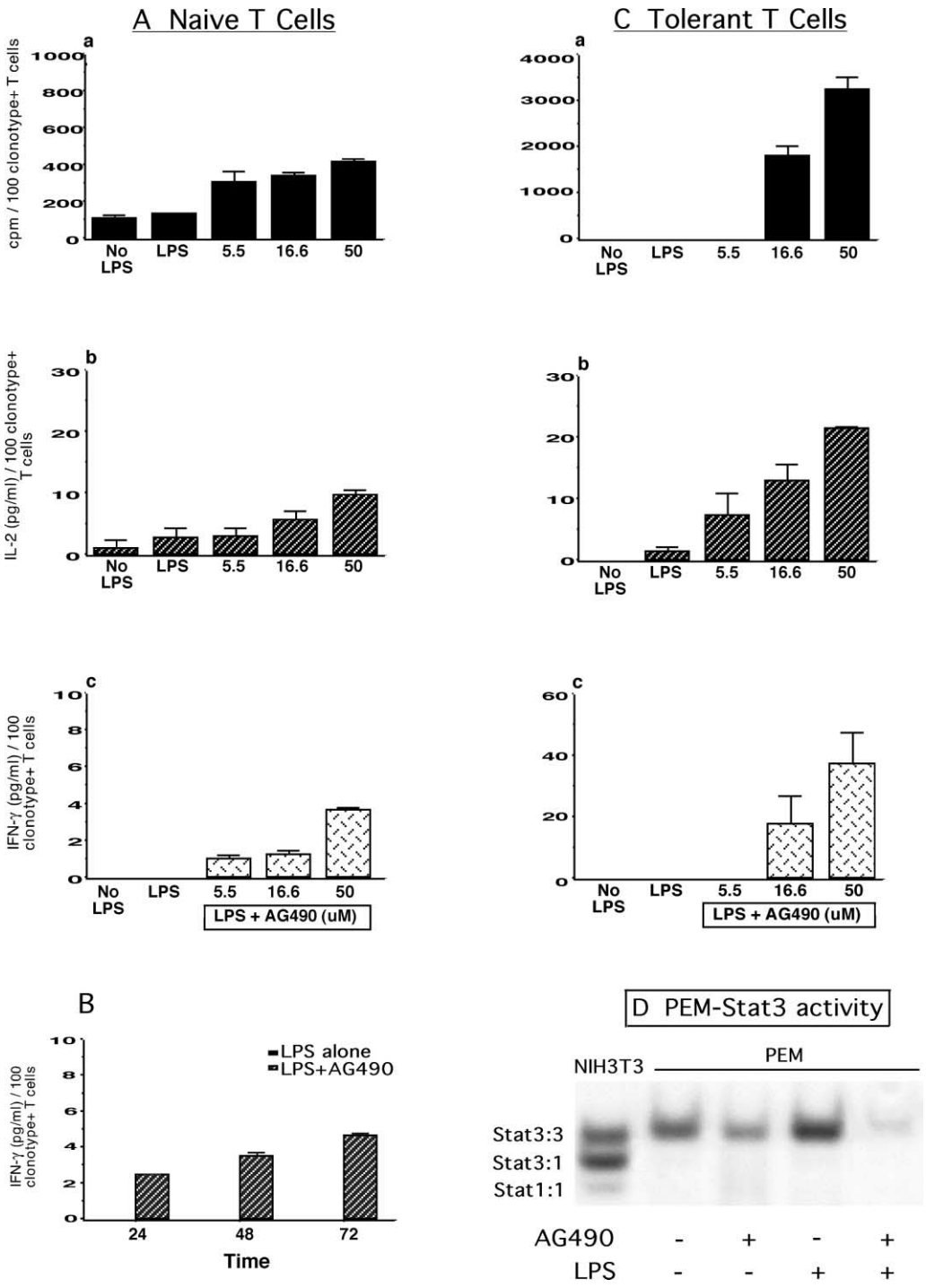


Figure 1. Antigen-Specific CD4$^+$ T Cell Responses to Cognate Antigen Presented by AG490-Treated PEM

(A) PEM from BALB/c mice (1 $\times$ 10^5/well) were cultured for 48 hr in media alone (No LPS), LPS (5 μg/ml), or LPS+ AG490 (0–50 μM). Then, supernatants were collected, and 5 $\times$ 10^4 purified naive CD4$^+$ T cells from the spleen of anti-HA TCR mice were added to PEM in the presence or not of 12.5 μg of HA peptide. (a) [^{3}H]thymidine incorporation was determined after 3 days in culture. Values represent the mean $\pm$ SE of the cpm per 100 clonotype+ T cells per well. In a parallel plate, IL-2 (b) and IFN-γ production (c) by antigen-specific T cells was determined by ELISA. Data represent mean $\pm$ SE of triplicate cultures and are expressed as the amount of cytokine produced per 100 clonotype+ T cells. Shown is a representative experiment of four independent experiments with similar results.

(B) Kinetics of IFN-γ production by AG490-treated PEM. Naive CD4$^+$ T cells were cultured with PEM treated with LPS or LPS + AG490 in the presence or not of HA-peptide. Supernatants were collected and IFN-γ production was determined by ELISA.

(C) PEM (1 $\times$ 10^5/well) were cultured with 5 $\times$ 10^4 CD4$^+$ T cells isolated from the spleen of A20HA-bearing mice in the presence or not of HA-peptide. (a) HA-specific proliferation, (b) IL-2 production, and (c) IFN-γ production by 100 clonotype+ T cells. Shown is a representative experiment of four independent experiments with similar results.

(D) Nuclear extracts from PEM were isolated and Stat3 activity was determined by EMSA. Nuclear extracts from EGF receptor-expressing NIH3T3 cells stimulated with EGF were used as positive control.

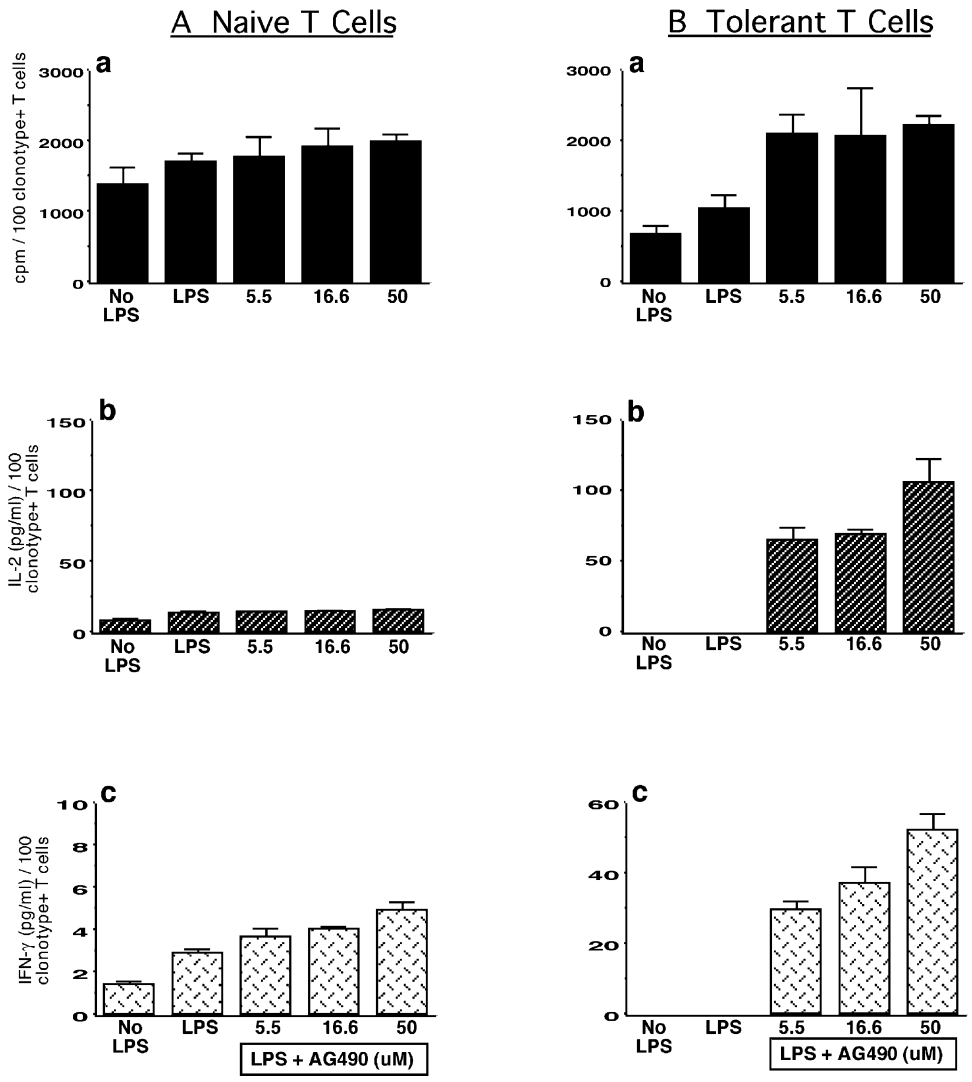


Figure 2. CD4$^+$ T Cell Responses to Cognate Antigen Presented by AG490-Treated DCs

(A) BM-derived DCs (1 $\times$ 10^4/well) were cultured for 48 hr in media alone (No LPS), LPS (5 μg/ml), or LPS+ AG490 as indicated. Then, 5 $\times$ 10^4 purified naive anti-HA CD4$^+$ T cells were added in the presence or not of 12.5 μg of HA peptide. (a) HA-specific proliferation, (b) IL-2 production, and (c) IFN-γ production by 100 clonotype+ T cells.

(B) BM-derived DCs (1 $\times$ 10^4/well) were cultured as above with 5 $\times$ 10^4 purified CD4$^+$ T cells from the spleen of A20HA-bearing mice in the presence or not of 12.5 μg of HA peptide. (a) HA-specific proliferation, (b) IL-2 production, and (c) IFN-γ production. Data are representative of two independent experiments with similar results.

gained the ability to produce IL-2 (Figure 2Bb) and IFN-γ (Figure 2Bc) in response to cognate peptide. The ability of tolerized antigen-specific T cells to produce significantly more IL-2 and IFN-γ on a per cell basis than naive antigen-specific T cells (Figures 2B versus 2A) suggests again that treatment of APCs with AG490 renders them capable of overcoming T cell unresponsiveness.

Increased Stat3 Activity in APCs Resulted in Impaired Antigen-Specific T Cell Responses

Previous studies have shown that Stat3 is tyrosine phosphorylated in response to stimulation with IL-10 (Finbloom and Winestock, 1995). Indeed, as shown in Figure 3A, PEM treated with IL-10 display significantly higher Stat3 activity as compared to untreated PEM. This increased Stat3 activity was associated with impairment in their T cell stimulatory capabilities, since clonotypic T cells encountering cognate peptide on these APCs produce less IL-2 than those T cells encountering antigen on untreated PEM (Figure 3B). Additional support for a regulatory role of Stat3 signaling in APC function was provided by the assessment of the T cell stimulatory capabilities of DCs transiently transfected with Stat3c, a mutant form of Stat3 that is constitutively activated without tyrosine phosphorylation (Bromberg et al., 1999). As shown in Figure 3C, nontransfected or empty vector-transfected DC cell line 2.4 (DC 2.4) efficiently

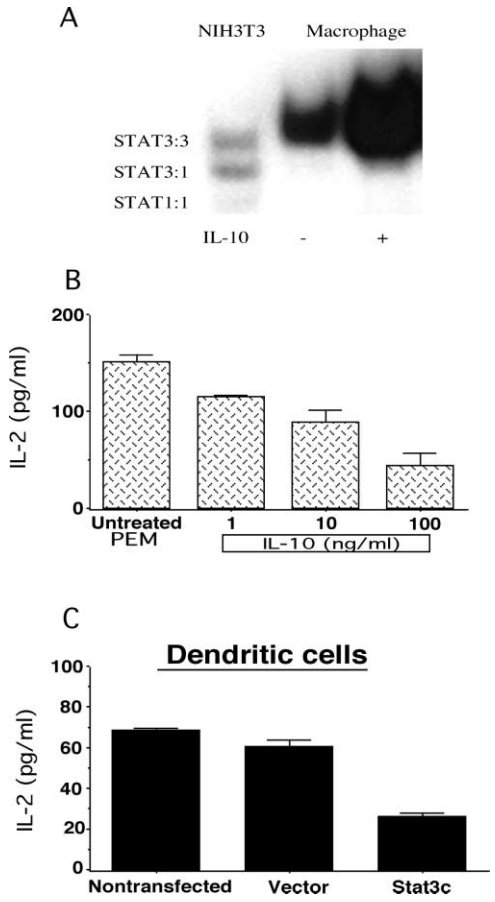


Figure 3. $CD4^+$ T Cell Responses to Antigen Presented by APCs with Increased Stat3 Activity

(A) Stat3 binding activity was determined by EMSA of nuclear extracts from untreated or PEM treated with IL-10 (10 ng/ml) for 24 hr.

(B) PEM (1×10^5/well) were cultured for 24 hr in media alone or IL-10 as indicated. Then, supernatants were discarded, and 5×10^4 naive anti-HA $CD4^+$ T cells were added in the presence or not of 12.5 μg of HA peptide. After 48 hr, IL-2 production was determined by ELISA. Data is representative of three independent experiments with similar results.

(C) DC 2.4 cells were transfected with either pcDNA3 empty vector or Stat3c expression vector. Then, 1×10^4 DCs were incubated with 5×10^4 purified anti-OVA transgenic $CD4^+$ T cells in the presence (or not) of 3 μg/ml of OVA peptide. Forty-eight hours later, IL-2 production was determined by ELISA. Values represent mean $\pm$ SE of triplicate cultures and are representative of two independent experiments.

triggers IL-2 production by $CD4^+$ T cells. In contrast, antigen presentation by DC 2.4 cells transfected with Stat3c led to diminished IL-2 production by antigen-specific T cells rather than effective T cell priming.

PEM with Genetic Disruption of Stat3 Efficiently Prime Naive Antigen-Specific T Cells and Restore the Responsiveness of Tolerant T Cells

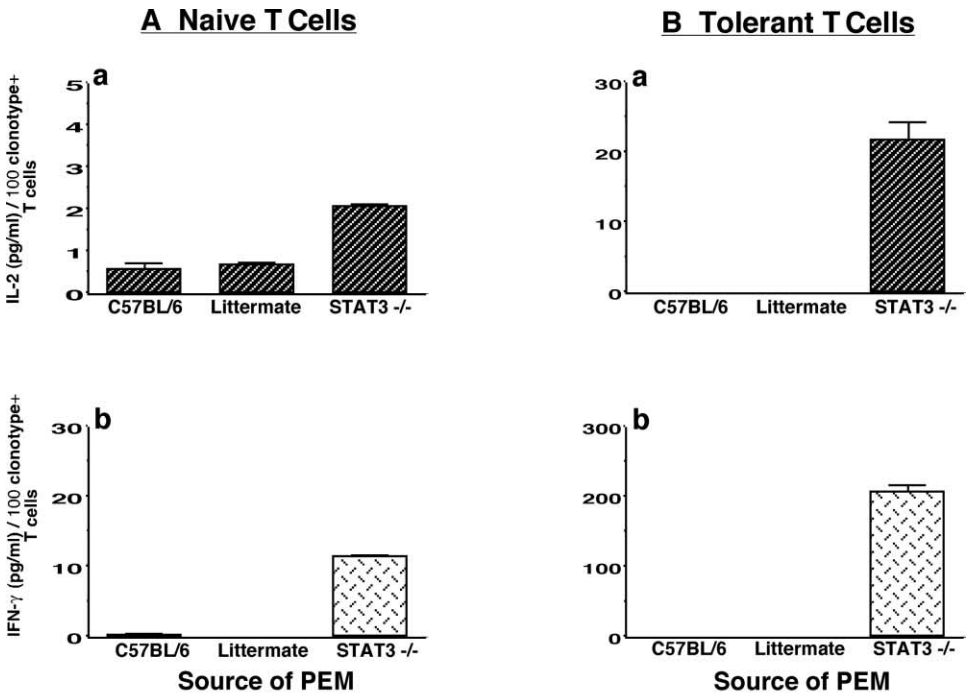
Given the above results, we asked next whether macrophages genetically devoid of Stat3 (Takeda et al., 1999) can effectively prime naive antigen-specific T cells and/or restore the responsiveness of tolerized T cells. Because of the background of LysMcre/$Stat3^{flox/-}$ mutant mice (H-2^b), we assessed the ability of their PEM to present ovalbumin (OVA) peptide to transgenic $CD4^+$ T cells expressing an $\alpha\beta$ TCR specific for OVA (Barnden et al., 1998). As shown in Figure 4A, anti-OVA $CD4^+$ T cells encountering cognate antigen on LPS-stimulated PEM from LysMcre/$Stat3^{flox/-}$ ($Stat3^{-/-}$ PEM) produced higher levels of IL-2 (Figure 4Aa) and IFN-γ (Figure 4Ab) than those antigen-specific T cells encountering OVA-peptide on PEM from C57BL/6 mice ($Stat3^{+/+}$) or littermate LysMCre/$Stat3^{flox/+}$. It should be pointed out that the ability of $Stat3^{-/-}$ PEM to efficiently prime T cell responses was not the result of potential minor genetic differences between OT-II mice and LysMcre/$Stat3^{flox/-}$ mice, since no cytokine production was detected in the absence of OVA peptide stimulation (data not shown).

PEM devoid of Stat3 were also able to restore the responsiveness of tolerant $CD4^+$ T cells from tumor-bearing mice. As shown in Figure 4B, anti-OVA $CD4^+$ T cells reisolated from animals harboring an OVA-expressing melanoma tumor (B16-OVA) were found to be tolerant by their lack of IL-2 (Figure 4Ba) and IFN-γ production (Figure 4Bb) in response to OVA-peptide presented by syngeneic PEM. Similarly, tolerant T cells remained unresponsive to stimulation with cognate antigen presented by LPS-stimulated PEM from littermate controls. In sharp contrast, presentation of OVA-peptide by LPS-stimulated PEM from $Stat3^{-/-}$ mice resulted in restoration of responsiveness of tolerant T cells as determined by the ability of these cells to produce significant levels of IL-2 and IFN-γ. Similar to our findings with AG490-treated APCs, tolerized T cells encountering cognate antigen on $Stat3^{-/-}$ PEM produce significantly more IL-2 and IFN-γ on a per cell basis than naive antigen-specific T cells (Figure 4B versus 4A). Therefore, $Stat3^{-/-}$ PEM seem to be capable of reversing T cell unresponsiveness rather than just enhancing the activation of those T cells that may have escaped tolerance induction in vivo.

EMSA of nuclear extracts isolated from aliquots of $Stat3^{-/-}$ PEM and control PEM used in the above experiments are shown in Figure 4C. As expected, PEM from mutant mice lack activated Stat3 as compared to PEM isolated from control mice. Interestingly, this lack of Stat3 activity in PEM from LysMcre/$Stat3^{flox/-}$ mice was associated with an increased activity of the proinflammatory Stat1 signaling pathway (Figure 4C, Stat 1:1).

Injection of a Tolerogenic Dose of Peptide Led to Antigen-Specific T Cell Activation Rather Than T Cell Tolerance in Mice with Targeted Disruption of Stat3 Signaling

In a well-characterized model of high dose peptide-induced antigen-specific T cell tolerance (Kearney et al., 1994), we determined next whether Stat3 signaling may play a role in the in vivo induction of T cell tolerance. Naive HA-specific $CD4^+$ T cells were transferred into LysMcre/$Stat3^{flox/-}$ mutant mice or littermate LysMCre/$Stat3^{flox/+}$ (backcrossed to H-2^d background) as well as into BALB/c control mice. Two days later, mice were injected i.v. with a tolerogenic dose of HA-peptide, and 2 weeks later animals were sacrificed and antigen-specific T cell responses were analyzed. Although no significant differences in the percent of clonotype+ $CD4^+$



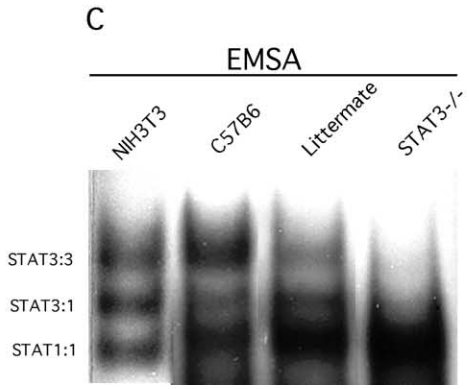


Figure 4. Antigen-Specific CD4$^+$ T Cell Responses to Cognate Antigen Presented by Stat3$^{-/-}$ PEM

(A) PEM (1 $\times$ 10^5/well) from C57BL/6, LysMCre/Stat3$^{flox/+}$ (littermate), or LysMcre/Stat3$^{flox/-}$ mutant mice (Stat3$^{-/-}$) were treated with 5 μg/ml of LPS and incubated with 5 $\times$ 10^4 purified anti-OVA CD4$^+$ T cells in the presence of 3 μg/ml of OVA peptide. Twenty-four hours later, supernatants were collected and assayed for IL-2 (a) or IFN-γ production (b) by ELISA.

(B) PEM from the mice described in (A) were cultured with 5 $\times$ 10^4 CD4$^+$ T cells isolated from B16-OVA-bearing mice. Twenty-four hours later, supernatants were collected and assayed for IL-2 (a) and IFN-γ (b) by ELISA. Data represent mean $\pm$ SE of triplicate cultures and are expressed as the amount of cytokine produced per 100 OT-II transgenic T cells. Shown is a representative experiment of three independent experiments with similar results.

(C) EMSA of nuclear extracts from PEM used in (B).

T cells was observed in the spleen of either untreated (black bars) or in vivo HA peptide-treated (stippled bar) BALB/c mice (Stat3$^{+/+}$), littermate controls, or LysMcre/Stat3$^{flox/-}$ mutant mice (Stat 3$^{-/-}$) (Figure 5A), functional analysis of T cells from these mice showed striking differences. Antigen-specific CD4$^+$ T cells isolated from untreated BALB/c mice or littermate controls produce IL-2 in response to in vitro stimulation with HA-peptide (Figure 5B, black bars). However, this response was absent in T cells isolated from BALB/c mice or littermate control mice that received a single i.v. injection of high dose peptide (Figure 5B, stippled bars), indicative that these antigen-specific T cells were rendered tolerant in vivo. Although T cells reisolated from untreated littermate controls produce low amounts of IFN-γ in response to HA-peptide (Figure 5C, black bar, littermate),

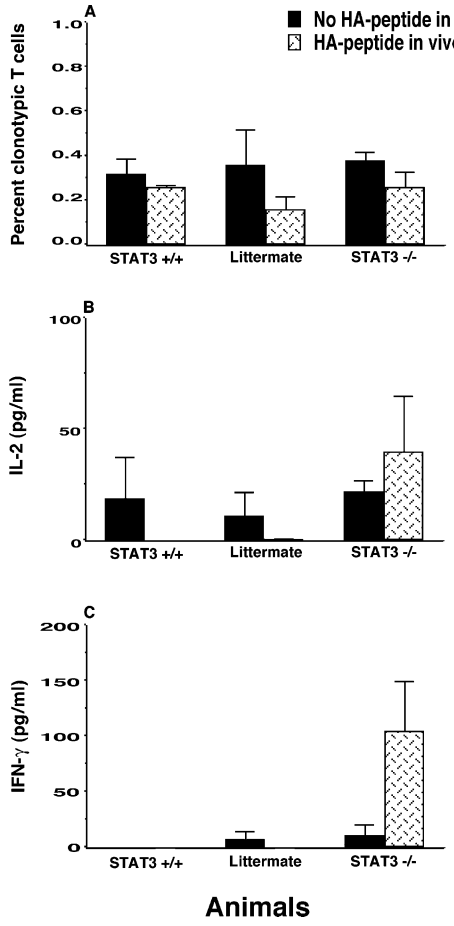


Figure 5. Effect of Disruption of Stat3 Signaling on the In Vivo Response to High Dose HA-Peptide

LysMcre/Stat3$^{flox/-}$ mutant mice, LysMCre/Stat3$^{flox/+}$ littermate, or BALB/c mice received 2.5 $\times$ 10^6 anti-HA TCR$^+$ transgenic CD4$^+$T cells i.v. Two days later half the mice received HA-peptide (275 μg/i.v.) (stippled bars) or vehicle alone (black bars) given i.v. Animals were sacrificed 2 weeks later, and T cells were purified from their spleens.

(A) Two-color flow cytometry staining for CD4 versus anti-HA TCR clonotype. Data represent mean $\pm$ SE of the percentage of T cells coexpressing CD4 and the clonotypic TCR for four mice/group. Purified T cells were also stimulated with HA-peptide plus fresh splenocytes for 48 hr. Supernatants were collected and assayed for IL-2 (B) and IFN-γ production (C) by ELISA. Data represent mean $\pm$ SE of triplicate cultures from three mice in each group. Data are representative of two independent experiments with similar results.

this response was also blunted in T cells isolated from littermate controls treated with a tolerogenic dose of peptide (Figure 5C, stippled bar). Therefore, antigen-specific T cells from either BALB/c mice or littermate controls were rendered fully tolerant following injection of high dose HA-peptide in vivo. In sharp contrast, induction of antigen-specific CD4$^+$ T cell tolerance was not observed in mice with a targeted disruption of Stat3. As seen in Figure 5B (black bars), antigen-specific CD4$^+$ T cells isolated from untreated Stat3$^{-/-}$ mice produce similar amounts of IL-2 as T cells from either BALB/c or littermate controls in response to restimulation with HA-peptide. But, unlike T cells isolated from HA-treated control mice, CD4$^+$ T cells from in vivo HA-treated Stat3$^{-/-}$ mice were still capable of producing significant amounts of IL-2 in response to cognate peptide (Figure 5B, stippled bars). More importantly, these same CD4$^+$ T cells produce significant levels of IFN-γ upon restimulation with cognate peptide in vitro (Figure 5C, stippled bar), indicative that they were primed in vivo in response to an otherwise tolerogenic dose of peptide.

Disruption of Stat3 Signaling Pathway Leads to the Generation of Inflammatory APCs

To elucidate the mechanism(s) by which macrophages devoid of Stat3 induce priming rather than tolerance of antigen-specific T cells, we evaluated next the phenotypic characteristics and the cytokine/chemokine profile of these APCs. Freshly isolated PEM from Stat3$^{-/-}$ mice display an increased expression of MHC class II molecules as well as B7.1 and B7.2 costimulatory molecules relative to PEM from control mice (Figure 6A). In spite of these phenotypic changes, no constitutive expression of inflammatory mediators was observed in PEM from mutant mice (Figure 6B, $-$LPS). Following stimulation with LPS, no further changes in the expression of MHC class II molecules or costimulatory molecules were observed in either PEM from mutant mice or control mice (data not shown). However, LPS-stimulated PEM from Stat3$^{-/-}$ mice display significantly higher mRNA levels of the chemokines RANTES, MIP-1α, MIP-1β, MIP-2, IP-10, and the cytokine IL-6 as compared to LPS-stimulated PEM from control mice (Figure 6B). In addition, IL-12 mRNA (Figure 6B) as well as IL-12 protein (Figure 6C) was detected in LPS-stimulated Stat3$^{-/-}$ PEM at the time that no IL-12 mRNA or protein could yet be detected in control PEM. Importantly, no IL-10 was detected in the supernatants of LPS-stimulated Stat3$^{-/-}$ PEM, cytokine that was clearly present in the supernatants of Stat3$^{+/+}$ PEM controls (Figure 6D).

Supernatants from Stat3$^{-/-}$ PEM Restore the Responsiveness of Tolerant Antigen-Specific CD4$^+$ T Cells

Next, to determine the potential contribution of soluble inflammatory mediator(s) in the ability of PEM from Stat3$^{-/-}$ mice to influence T cell responses, we transferred supernatants from LPS-stimulated Stat3$^{-/-}$ PEM into tolerant T cells cultured with syngeneic PEM and cognate peptide. Tolerized T cells were obtained from two well-characterized models of tolerance induction: antigen-specific CD4$^+$ transgenic T cells rendered anergic during tumor progression (Staveley-O'Carroll et al., 1998) and CD4$^+$ T cell clones (AE.7 clone) rendered tolerant in vitro by stimulation with anti-CD3 antibodies in the absence of costimulation (Jenkins et al., 1990). As shown in Figure 7A, transfer of supernatant from LPS-stimulated Stat3$^{-/-}$ PEM, but not supernatants from controls, restores the responsiveness of tolerant T cells isolated from tumor-bearing mice as determined by the ability of these T cells to produce significant levels of IFN-γ. Of note, the magnitude of IFN-γ production by these tolerant T cells was similar to that of naive T cells primed in vivo with recombinant vaccinia-encoding HA (data not shown). Tolerized AE.7 T cells also regain their ability to produce IL-2 in response to cognate antigen only when supernatants from Stat3$^{-/-}$ PEM were added

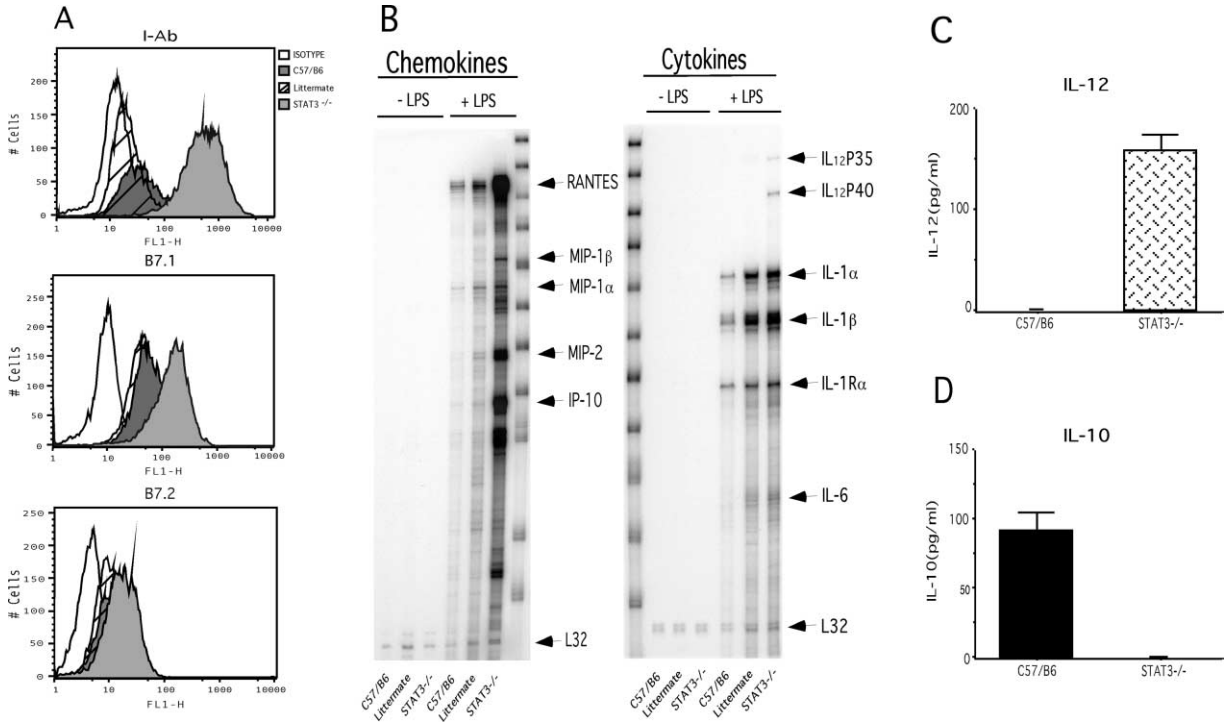


Figure 6. Phenotypic and Functional Characteristics of Stat3$^{-/-}$ PEM

(A) Freshly isolated PEM from either C57BL/6, littermate, or LysMcre/Stat3$^{flox/-}$ mutant mice were stained for the expression of I-A^b, B7.1, and B7.2. Open histogram in each figure represents staining with isotype antibody. Data is representative of two independent experiments. (B) PEM from the mice in (A) were treated or not with LPS (5 μg/ml) for 24 hr. Then total RNA was isolated and RPA was performed. In a parallel culture, supernatants were collected and the production of IL-12 (C) and IL-10 (D) was determined by ELISA. Data represent mean ± SE of triplicate cultures and are representative of two independent experiments.

to the cultures (Figure 7B, Stat3$^{-/-}$). However, the magnitude of this recovery was approximately 50% relative to the levels of IL-2 produced by nontolerized AE.7 T cells (Figure 7B, Control).

In order to identify the putative factor(s) responsible for restoring the responsiveness of tolerant T cells, we performed experiments with neutralizing antibodies specific for the inflammatory mediators produced by LPS-stimulated Stat3$^{-/-}$ PEM (Figure 6B). Specific blockade of either IL-12 (Figure 7C) or RANTES (Figure 7D) partially diminished the positive effect of Stat3$^{-/-}$ supernatants upon tolerant T cells. More importantly, the concurrent blockade of these two inflammatory mediators abrogates almost completely the ability of Stat3$^{-/-}$ supernatants to break T cell anergy (Figure 7E). Therefore, IL-12 and RANTES produced by Stat3$^{-/-}$ PEM play a dominant role in restoring the responsiveness of tolerant CD4$^+$ T cells.

Discussion

It is now becoming clear that antigen encounter in the periphery by cells of the immune system does not always lead to immune activation and can result instead in immunologic unresponsiveness (Kearney et al., 1994; Pardoll, 2001). APCs lie at the center of this critical decision of the immune system, since these cells have been shown to capture antigens in the periphery, migrate to the lymphoid organs, and present processed peptides to T cells in a way that may lead to either priming (Huang et al., 1994) or tolerance induction (Adler et al., 1998; Kurts et al., 1997; Sotomayor et al., 2001). In this study we have unambiguously identified Stat3 signaling in APCs as a critical pathway influencing the functional outcome of antigen-specific T cells. Furthermore, we have found that in mice lacking functional Stat3 in macrophages, the in vivo response to a tolerogenic dose of peptide is T cell priming rather than T cell tolerance. This effect of Stat3 deficiency unveiled, therefore, a previously unknown role for this signaling pathway in the induction of antigen-specific T cell tolerance.

The findings that PEM with a targeted disruption of Stat3 have a constitutively activated phenotype and are more prone to producing inflammatory mediators in response to LPS point to Stat3 signaling as a negative regulatory pathway in these cells. Interestingly, PEM devoid of Stat3 display increased Stat1:1 homodimers (Figure 4C), a functionally active complex that positively regulates genes encoding inflammatory factors (Ramana et al., 2000). Previous studies have demonstrated a frequent coactivation of Stat1 and Stat3 by the same ligand (Bromberg and Darnell, 2000), leading to formation of Stat1:Stat3 heterodimers that may have a higher association constant than the Stat1:Stat1 homodimeric complex (Kotenko and Pestka, 2000). It is plausible, therefore, that Stat3 activation in APCs, by binding activated Stat1, may control the formation of functionally active Stat1:Stat1 homodimers, thus limiting the magnitude and/or intensity of an inflammatory response. Perhaps, only in those scenarios when the level of Stat1

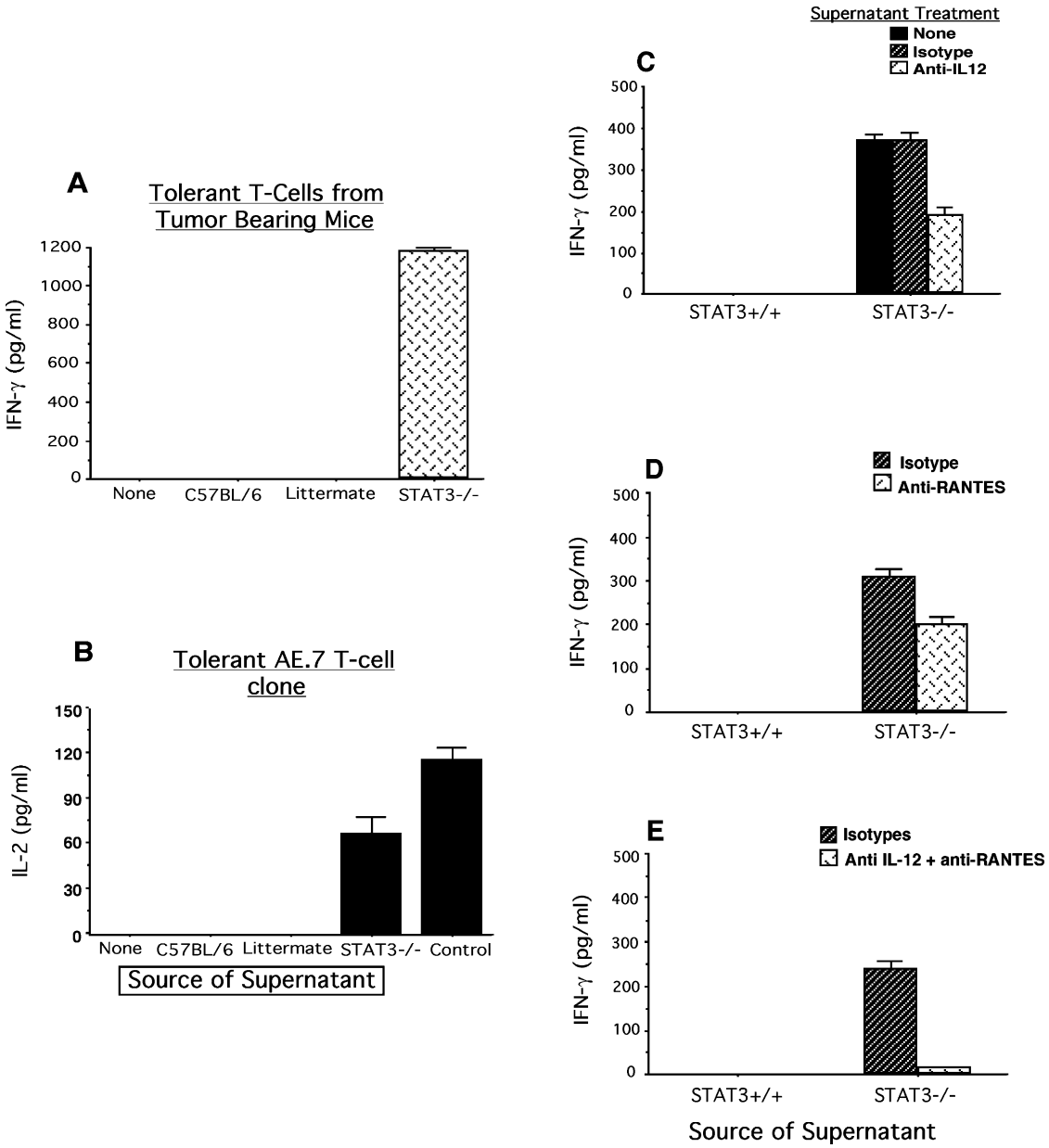


Figure 7. IL-12 and RANTES Produced by Stat3$^{-/-}$ PEM Breaks CD4$^+$ T Cell Tolerance

PEM (1 $\times$ 10^5 cells/well) from Stat3$^{-/-}$ mice or controls were stimulated with LPS (5 μg/ml) for 24 hr. Then supernatants were collected, centrifuged, and stored at -70°C.

(A) Supernatants were thawed, and 50 μl was added to tolerant anti-OVA CD4$^+$ T cells cultured with syngeneic PEM and 3 μg/ml of OVA peptide. Forty-eight hours later, supernatants were collected and assayed for IFN-γ production by ELISA.

(B) Tolerized AE.7 CD4$^+$ T cells (5 $\times$ 10^4 cells/well) were cultured with 1 $\times$ 10^5 PEM from B10.BR mice in the presence (or not) of 50 μl of the supernatants described above and specific PCC peptide (1 μM). After 48 hr, supernatants were collected and assayed for IL-2 production by ELISA. Production of IL-2 by nonanergized AE.7 CD4$^+$ T cells is displayed as Control.

(C–E) In additional experiments, supernatants from LPS-stimulated PEM were thawed and treated with either (C) anti-IL-12 neutralizing antibodies (R&D systems), (D) anti-RANTES neutralizing antibodies (R&D systems), or a combination of anti-IL-12 plus anti-RANTES (E) as well as their respective isotype controls. Untreated or treated supernatants (50 μl) were then added to tolerant CD4$^+$ T cells cultured with fresh PEM in the presence or not of specific peptide. Forty-eight hours later, supernatants were collected and assayed for IFN-γ production by ELISA. Data represent mean $\pm$ SE of triplicate cultures and are representative of two independent experiments with similar results.

exceeds the level of activated Stat3 or when Stat3 signaling is disrupted, Stat1 homodimers may trigger the downstream signals that APCs need to efficiently activate T cells.

An additional important feature of Stat3$^{-/-}$ PEM is their lack of production of IL-10 (Figure 6D). Previous studies have demonstrated that binding of activated Stat3 to the IL-10 promoter is required for efficient expression of the IL-10 gene (Benkhart et al., 2000). Importantly, IL-10 has been shown to downregulate the expression of cytokines and costimulatory molecules in activated APCs (Ding et al., 1993). Furthermore, this

cytokine has been implicated in the induction of T cell anergy (Groux et al., 1996). Therefore, it is plausible that the absence of the inhibitory effects of IL-10 may explain the constitutive inflammatory phenotype of Stat3$^{-/-}$ PEM and their ability to induce T cell priming rather than T cell tolerance. However, different lines of evidence suggest that the phenotypic and functional changes in Stat3$^{-/-}$ PEM may not just be the result of lack of IL-10 production by these APCs. First, similar to our findings in PEM devoid of Stat3, alveolar macrophages from IL-10$^{-/-}$ mutant mice display increased expression of B7.1 and B7.2 costimulatory molecules (Soltys et al., 2002). However, while no changes in the expression of MHC class II molecules are observed in macrophages devoid of IL-10, a significant increase in the expression of MHC class II molecules is a characteristic of macrophages devoid of Stat3 (Figure 6A). Second, recent studies have found that pathways other than IL-10 signaling, such as NF-κB activation by Toll-like receptors, M-CSF signaling, and NADPH oxidase function, which are also negatively regulated by Stat3, may play a more important role in the enhanced innate immunity observed in mice with disruption of this signaling pathway (Welte et al., 2003).

It is interesting that Stat3$^{-/-}$ PEM share more phenotypic and functional characteristics with macrophages from mice lacking the inhibitory Tyro-3 family of receptor tyrosine kinase, Tyro-3, Axl, and Mer (TAM) (Lu and Lemke, 2001). Freshly isolated macrophages from TAM mice display increased expression of MHC class II molecules, produced elevated amounts of IL-12, and induce strong lymphocyte activation, reminiscent of our findings in Stat 3$^{-/-}$ PEM. It is noteworthy, however, that while genetic disruption of all three inhibitory Tyr-3 receptors (triple mutant mice) is required to generate inflammatory macrophages, a similar outcome can be achieved by just disrupting Stat3 signaling pathway in these cells. The common findings in macrophages from Stat3$^{-/-}$ mice and IL-10$^{-/-}$ mice, as well as in TAM triple mutant mice, raise the interesting possibility that Stat3 may represent a common signaling pathway linking different inhibitory receptors with their downstream intracellular targets. It is plausible, therefore, that the activated phenotype of Stat3$^{-/-}$ PEM could be related not only to their lack of IL-10 production but also to the enhanced activity of different proinflammatory pathways tightly regulated by an intact Stat3 signaling in these APCs. Although our in vitro studies have identified IL-12 and RANTES as important inflammatory signals by which Stat3$^{-/-}$ PEM can overcome T cell tolerance, the potential role of overactivated proinflammatory signaling pathways (i.e., Stat1 signaling, among others) in the generation of these inflammatory mediators, however, remains to be elucidated.

A previously unknown role for Stat3 signaling in influencing antigen-specific T cell responses in vivo was uncovered by our findings that in mice with a genetic disruption of Stat3 in macrophages, the response to a tolerogenic dose of peptide is T cell priming rather that T cell tolerance (Figure 5). It was surprising, that just by disrupting the Stat3 signaling pathway predominantly in one APC subpopulation, such a dramatic effect in T cell responses could be observed. It is possible that the constitutively activated phenotype of Stat3$^{-/-}$ macrophages (Figure 6A), together with their enhanced ability to produce inflammatory mediators (Figure 6B), may render these cells quite efficient professional APCs capable of presenting the tolerogenic peptide in the appropriate inflammatory context leading to T cell priming rather than T cell tolerance in vivo. Alternatively, macrophages devoid of Stat3 may provide the inflammatory microenvironment in which other APCs, such as DCs, could be fully activated to efficiently present HA-peptide in vivo in a way that resulted in CD4$^+$ T cell priming rather than unresponsiveness. In support of this possibility, we have recently found an increased population of CD11c$^+$, CD8α$^-$ DCs expressing high levels of MHC class II in the spleen as well as in the lymph nodes of LysMcre/Stat3$^{flox/-}$ mice (data not shown). Although the phenotype of these DCs could be just the result of the adjuvant effect provided by Stat3$^{-/-}$ macrophages, it is also possible that this specific DC subpopulation may have floxed out the Stat3 gene, indicative that they have passed through a developmental stage in which the LysM promoter is active. Alternatively, perhaps Stat3 signaling represents an important checkpoint limiting the differentiation/maturation of myeloid cells, and therefore, in its absence some monocyte/macrophages may become CD11c$^+$, CD8α$^-$ DCs. Of note, such an expansion of DCs expressing high levels of MHC class II molecules was restricted to myeloid DCs, since no changes were observed in CD11c$^+$, CD8α$^+$ DCs isolated from the lymphoid organs of LysMcre/Stat3$^{flox/-}$ mice (data not shown).

In the immune response to self-antigens expressed in peripheral tissues, host's APCs capture apoptotic cellular fragments and then migrate to the T cell zone of secondary lymphoid organs for presentation of peptide antigens to antigen-specific T cells. In the absence of inflammatory signals, this process has been proposed to play a critical role in the induction and maintenance of peripheral tolerance to self-antigens (Steinman et al., 2003). Noninflammatory APCs, by having low levels of MHC, costimulatory molecules, and other adhesion molecules that participate in T cell priming induce instead T cell unresponsiveness, a default outcome that typifies how the immune system normally responds to antigens expressed in the periphery. In mice devoid of Stat3 signaling, however, this default response toward tolerance induction seems to be significantly altered. The proinflammatory phenotype displayed by APCs in these mice converted a T cell encounter with antigen/APC from a tolerizing event into a priming event. Furthermore, the demonstrated ability of Stat 3$^{-/-}$ APCs to promote T cell priming and cell-mediated immunity rather than T cell tolerance provides an explanation to earlier observations of increased incidence of autoimmune inflammatory bowel disease in mice with targeted disruption of Stat3 in macrophages (Takeda et al., 1999) as well as in mice with disruption of this signaling pathway in all hematopoietic cells (Welte et al., 2003).

Given the central role of bone marrow-derived APCs in the induction of tolerance to tumor antigens (Sotomayor et al., 2001), defining the signaling mechanisms by which these cells induce unresponsiveness rather than activation of T cells is critical for the continued development of effective immune-enhancing therapeutic strategies. Our demonstration that disruption of Stat3

signaling in either macrophages (Figures 1C and 4B) or bone marrow-derived DCs (Figure 2B) renders these APCs capable of restoring the responsiveness of tolerant T cells from tumor-bearing mice has, therefore, important implications for cancer immunotherapy.

Taken together, our results establish a novel role for Stat3 signaling in APC function and regulation of T cell responses. Strategies to manipulate Stat3 signaling in either direction (blockade or stimulation) may be able, therefore, to influence immune activation versus immune tolerance, a critical decision with profound implications in autoimmunity, transplantation, and cancer immunotherapy.

Experimental Procedures

Mice

Six- to eight-week-old BALB/c, C57BL/6, and B10.BR mice were obtained from the NIH (Frederick, MD). Stat3$^{flox/+}$, LysMcre/Stat3$^{+/-}$, and LysMcre/Stat3$^{+/+}$ mice (H-2^b) were provided by Dr. S. Akira (Osaka University, Japan). LysMcre/Stat3$^{+/-}$ mice were mated with Stat3$^{flox/+}$ mice to generate LysMcre/Stat3$^{flox/-}$ mice (experimental group). LysMcre/Stat3$^{flox/+}$ from these crosses were used as littermate controls. TCR transgenic mice expressing an $\alpha\beta$ TCR specific for peptide 323–339 from ovalbumin (OVA) presented by MHC class II, I-A^b (Barnden et al., 1998) were provided by Dr. W. Heath (The Walter and Eliza Hall Institute, Australia). All the experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committees of the University of South Florida.

Cell Lines

A20 lymphoma cells expressing HA as a model tumor antigen were selected and grown in vitro as previously reported (Staveley-O'Carroll et al., 1998). B16 melanoma cells transfected with ovalbumin (B16-OVA) were provided by Dr. P. Dellabona (Instituto San Rafaelle, Milan, Italy). For in vivo tumor challenge experiments, tumor cells were washed in HBSS and injected via the tail vein (A20HA) or s.c. (B16-OVA) into BALB/c or C57BL/6 mice, respectively, in a total volume of 0.2 ml, 1×10^6 tumor cells per mouse.

The dendritic cell line, DC 2.4 (Shen et al., 1997), kindly provided by Dr. K. Rock (University of Massachusetts), and BM-derived DCs were transiently transfected with either pcDNA3 empty vector or Stat3c expression vector (Bromberg et al., 1999) using Lipofectamine methods (InVitrogen, Carlsbad, CA). Transfection efficiency was between 10%–15% for DC 2.4 cells and 10% for bone marrow-derived DCs.

Isolation of Peritoneal Elicited Macrophages

Mice were injected intraperitoneally (i.p.) with 1 ml of thyoglicollate (DIFCO Laboratories, Detroit, MI). Four days later, PEM were obtained by peritoneal lavage. MHC class II expression (I-A^b) on PEM was determined by staining with a FITC-conjugated AF6-120.1 antibody (BD Pharmingen, San Diego, CA). The expression of B7.1 and B7.2 molecules was determined by staining with either a FITC-conjugated anti-CD80 or a FITC-conjugated anti-CD86 (BD Pharmingen) MoAbs. Ten thousand gated events were collected on a FACScan (Becton Dickinson, San Jose, CA) and analyzed using Flow-Jo software (Treestar Inc).

Generation of Bone Marrow-Derived DCs

In brief, DCs were generated from murine bone marrows using RPMI 1640 medium supplemented with 10% FCS, 20 ng/ml murine recombinant GM-CSF, and 10 ng/ml IL-4 (both from RDI, Flanders, NJ). The cultures were maintained at 37°C in 5% CO_2 humidified atmosphere in 24-well plates. On day 3 of culture, floating cells were gently removed and fresh medium with cytokines was replaced. On day 5, cells were collected, and DCs were enriched by centrifugation over a 13.5% metrizamide gradient (Accurate Chemicals, Westbury, NY).

Antigen Presentation Studies

PEM (1×10^5/well) or BM-derived DCs (1×10^4/well) were cultured with 5×10^4 CD4$^+$ T cells in the presence or not of cognate peptide (either synthetic HA-peptide$_{110\text{-}120}$ SFERFEIFPKE or OVA peptide$_{323\text{-}339}$, ISQAVHAAHAEINEAGR). After 72 hr, cells were pulsed with [^{3}H]thymidine (1 μCi/well, NEN Life Science, MA). Eighteen hours later, cells were harvested with a Packard Micromate cell harvester. Thymidine incorporation into DNA was measured as counts per minute (cpm) in the peptide-pulsed group minus cpm from cells cultured in medium alone divided by the number of clonotype+ T cells in the wells as determined by FACS. In a parallel plate, supernatants were collected at different time points (24–72 hr) and stored at −70°C until assayed for IL-2 and IFN-γ production by ELISA (R&D Systems, Minneapolis, MN). Values for T cells cultured in media alone are usually less than 10% of the values for antigen-stimulated T cells. Data are expressed as the amount of cytokine produced by 100 clonotype+ T cells/well.

Nuclear Extracts and Electrophoretic Mobility Shift Assay

APCs were treated with LPS (5 mcg/ml) and/or increasing concentrations of the JAK-selective inhibitor, tyrphostin AG490 (Meydan et al., 1996) or IL-10 alone before isolation of nuclei. Nuclear extracts were prepared and electrophoretic mobility shift assay (EMSA) determination was performed as previously described (Catlett-Falcone et al., 1999).

RNA Isolation and RNase Protection Assays

Total RNA was isolated from PEM by TRIzol reagent (Life Technologies, Inc., Grand Island, NY). RNase protection assays (RPAs) were carried out using the PharMingen's cytokine and chemokine multiprobe templates according to the manufacturer's protocol.

Tolerance Models

The following experimental models were utilized.

Tumor-Induced Antigen-Specific T Cell Tolerance

In brief, 2.5×10^6 CD4$^+$ anti-HA transgenic T cells were injected i.v. into tumor-free mice or A20HA-bearing mice. Twenty-one days after T cell transfer, animals were sacrificed and T cells were reisolated from their spleens as previously described (Staveley-O'Carroll et al., 1998). To determine the percent of isolated anti-HA clonotypic CD4$^+$ T cells, purified T cells were stained with FITC-conjugated goat anti-mouse CD4 (Caltag, Burlingame, CA) and biotinylated rat anti-clonotypic TCR antibody mAb 6.5 followed by PE-conjugated streptavidine (Caltag). For this analysis, 50,000 gated events were collected on a FACScan and analyzed using Flow-Jo software. Antigen-specific proliferation and cytokine production by clonotypic CD4$^+$ T cells in response to HA-peptide presented by APCs was determined as described above. For induction of antigen-specific T cell tolerance in H-2^b tumor-bearing mice, a similar experimental approach was utilized, the only difference being that 1×10^6 anti-OVA CD4$^+$ transgenic T cells (OT-II) were transferred i.v. into tumor-free or animals bearing an OVA-expressing tumor (B16OVA). Fourteen days after T cell transfer, animals were sacrificed and OT-II cells were reisolated from their spleens. The percent of anti-OVA transgenic CD4$^+$ T cells (OT-II) was determined by staining with Cychrome-conjugated anti-mouse CD4 (BD, Pharmingen), anti-Vα2-PE and Vβ5-FITC conjugated monoclonal antibodies (BD, Pharmingen).

High Dose Peptide Model

In brief, 2.5×10^6 anti-HA/I-E^d TCR$^+$ transgenic T cells were transferred into BALB/c mice, littermates, or LysMcre/Stat3$^{flox/-}$ mutant mice (backcrossed for ten generations to H-2^d background). Two days later, half the mice were injected i.v. with a tolerogenic dose of HA-peptide$_{110\text{-}120}$ (275 mcg), and 2 weeks later all the animals were sacrificed. T cells from the spleen of these animals were isolated, and their phenotypic and functional characteristics were analyzed as described above.

In Vitro Induction of CD4$^+$ T Cell Tolerance

AE.7 CD4$^+$ T cells were stimulated for 5 days with anti-CD3 antibody in the absence of costimulation. As positive control, AE.7 CD4$^+$ T cells were stimulated with anti-CD3 plus anti-CD28 antibodies as previously described (Jenkins et al., 1990).

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