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Dendritic Cells Require STAT-1 Phosphorylated at Its Transactivating Domain for the Induction of Peptide-Specific CTL

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Phosphorylation of transcription factor STAT-1 on Y701 regulates subcellular localization whereas phosphorylation of the transactivating domain at S727 enhances transcriptional activity. In this study, we investigate the impact of STAT-1 and the importance of transactivating domain phosphorylation on the induction of peptide-specific CTL in presence of the TLR9-dependent immune adjuvant IC31. STAT-1 deficiency completely abolished CTL induction upon immunization, which was strongly reduced in animals carrying the mutation of the S727 phospho-acceptor site. A comparable reduction of CTL was found in mice lacking the type I IFN (IFN-I) receptor, whereas IFN-γ-deficient mice behaved like wild-type controls. This finding suggests that S727-phosphorylated STAT-1 supports IFN-I-dependent induction of CTL. In adoptive transfer experiments, IFN-I- and S727-phosphorylated STAT-1 were critical for the activation and function of dendritic cells. Mice with a T cell-specific IFN-I receptor ablation did not show impaired CTL responses. Unlike the situation observed for CTL development S727-phosphorylated STAT-1 restrained proliferation of naive CD8+ T cells both in vitro and following transfer into Rag-deficient mice. In summary, our data reveal a dual role of S727-phosphorylated STAT-1 for dendritic cell maturation as a prerequisite for the induction of CTL activity and for T cell autonomous control of activation-induced or homeostatic proliferation. The Journal of Immunology, 2009, 183: 2286–2293.

All members of the STAT family regulate innate and adaptive immune responses (1–3). The prototype member, STAT-1, contributes nonredundantly and essentially to immunity as a central mediator of type I IFN (IFN-I) and IFN-γ. Hence, in mice and humans absence or loss-of-function mutation of STAT-1 strongly reduces innate resistance to intracellular viral and nonviral pathogens (4–6). Activation of STAT-1, i.e., formation of transcription-competent dimers, requires phosphorylation on Y701 by IFNAR-associated JAKs. Whereas the IFN-γ-receptor complex phosphorylates predominantly STAT-1 and causes homodimer formation, the IFN-I receptor (IFNAR) complex also phosphorylates a second STAT, STAT-2, which forms heterodimers with STAT-1 (7). STAT dimers shift their subcellular localization to the nucleus and acquire the ability to bind specific DNA sequences. STAT-1 homodimers bind to target genes with γ-IFN-activated promoter sequences (GAS), and this group of genes dominates responses to IFN-γ. STAT-1/STAT-2 heterodimers associate with a third protein, IFN regulatory factor 9, and the resulting IFN-stimulated gene factor (ISGF)3 complex binds to IFN-stimulated response element sequences (ISRE). Genes with these sequences dominate the transcriptional response to IFN-I.

Modifications other than Y701 phosphorylation modulate the fate of activated STATs. S727 in the transactivating domain of STAT-1 dimers is phosphorylated in IFN-stimulated cells exclusively in the cell nucleus (8–10). In addition, a number of agents that do not cause STAT-1 tyrosine phosphorylation effectuate S727 phosphorylation of predominantly cytoplasmic STAT-1 (11). Among these are TLRs, TNFRs, or lymphocyte Ag receptors. The importance of S727 phosphorylation outside the IFN response has not been clarified.

In the context of STAT-1 dimers, S727 phosphorylation increases the association with the transcriptional coactivator CBP, thus the ability to stimulate histone acetylation and RNA polymerase recruitment (12). Consistent with this ability, IFN-γ-dependent immunity is reduced in cells or mice expressing a S727A mutant of STAT-1 (13). The relevance of STAT-1 S727 phosphorylation in the ISGF3 context and for IFN-I-dependent immunity is suggested by studies with cells expressing a Stat1S727A mutant, but its impact on the complex regulation of an organismic immune response to model Ags or pathogens has not been tested.

STAT-1 exerts much of its immunological potential by directly contributing to the antimicrobial gene signature of cells that are either infected, in danger of being infected, or that have ingested pathogens by phagocytosis (14). However, recent evidence suggests that STAT-1 also regulates T cell activity, hence adaptive...
immunity. In vitro, the transcription factor reduces proliferation and increases apoptosis of splenic T cells (15–17). Experiments in lymphocytic choriomeningitis virus-infected mice suggest a role for STAT-1 in limiting IFN-γ production by Th1 cells or NK cells (18, 19). Reduction of STAT-1 expression in lymphocytic choriomeningitis virus-specific CD8⁺ T cells was shown to render these cells capable of expansion in the presence of otherwise growth-inhibitory, virus-induced IFN-I (17). This is both consistent with observations of the behavior of activated T cells in vitro (20), and with the finding that antiviral CTL in influenza virus-infected mice were found unaffected by the lack of STAT-1 (21). In contrast, in a mouse tumor model the development of CTL activity was shown to require STAT-1 (22). Together these studies suggest an ability of STAT-1 to control multiple aspects of T cell-mediated immunity, but that its impact varies with as yet unknown parameters of the immune response. The same picture emerges when STAT-1-activating cytokines, particularly IFN-I, are analyzed. In vitro, IFN-I can increase survival and the development of effector function, thus replacing IL-12 as a third signal for T cell activation (23, 24). This IFN-I activity is mediated by STAT-1 and may, like IFN-γ production in CD4⁺ T cells, be negatively regulated by STAT-1. In viral or bacterial mouse infection models IFN-I promoted CD8⁺ T cell expansion, but the magnitude of this effect varied strongly with the particular pathogen (25–28). During Chlamydia infection production of IFN-I was negatively correlated with host immunity and suggested to cause suppression of T cell immunity (29).

Like IFN-I, IFN-γ influences T cell-mediated immunity (30–32). The cytokine reportedly contributes to expansion, polarization and contraction of Ag-specific T cells (33, 34). Direct effects on T cells are one way by which STAT-1 and IFN regulate T cell-based immunity. However, alternative targets of their activity are the APCs, most prominently dendritic cells (DC). In fact, several studies show that STAT-1 and IFN-I may influence maturation of DC subsets including their ability to cross-present Ag to naive CD8⁺ T cells and to stimulate Th-dependent Ab production by B cells (35–38).

The literature we briefly reviewed suggests a high degree of variability concerning the influence of STAT-1 on the development of T cell-based immunity which varies with Ag, inflammatory environment, magnitude of IFN-I production and the cellular target of STAT-1-activating cytokines. To reduce complexity and increase interpretability of the Ag-specific immune response we studied the impact of STAT-1 and its serine phosphorylation on the development of CTL responses, elicited by a model peptide Ag and a well-defined immune adjuvant. Under these conditions the development of CTL activity required serine-phosphorylated STAT-1 and IFN-I. A major function of STAT-1 in this setting is to render DC capable of stimulating the expansion of Ag-specific CD8⁺ T cells. In vitro, STAT-1 limits CD8⁺ T cell proliferation. In T cell-deficient animals, the transcription factor reduces homeostatic proliferation.

Materials and Methods

Mice

Wild-type (wt) C57BL/6N, B6.129P2-Stat1tm1 (STAT-1-ko) (4), B6.129P2-Stat1tm1(S172A) (13), B6.129P2-IFNAR1tm1 (IFNAR1-ko) (39), B6.129P2-Iifgtm1 (IFN-γ-ko) (40), C57BL/6-Tg(OVA) OT-1 mice with transgenic TCR for OVA257–264 bound to H2-Kb (OT-1) (41), Stat1S727A knockin/OT-1 mice B6.129P2-Stat1tm1(S172A) (42), IFNAR1TM1CD4 cre mice B6.129P2-IFNAR1tm1 (IFNAR1TM1CD4 cre) were in C57BL/6 background and maintained under specific pathogen-free conditions according to the Federation of European Laboratory Animal Science Associations recommendations at the University of Veterinary Medicine (Vienna, Austria). Experiments were done with gender- and age-matched 6- to 12-wk-old mice. All animal experiments were discussed and approved by the University of Veterinary Medicine Vienna institutional ethics committee and conducted in accordance with protocols approved by the Austrian law (GZ 680/205/67-BerGr/2003 and GZ BMWF-68.205/0204-C/OGT/2007).

Flow cytometric analysis

The following Abs were used: aliphophycocyanin-conjugated anti-CD11c, anti-CD8a, and anti-Annexin V; FITC-conjugated anti-CD80, anti-CD86, and anti-CD4; PE-conjugated anti-MHC class I (MHC I), anti-MHC II, and anti-CD8. All Ab purchased from BD Pharmingen were used at a concentration of 0.2–0.5 μg per one million cells. Samples were analyzed using a FACS Calibur (BD Biosciences).

In vivo CTL assay

Mice were immunized via s.c. injection with SIINFEKL (0.1 mg/mouse) alone, in combination with the adjuvant IC31 or PBS. Seven days later, immunized mice received syngeneic, unfractionated splenic leukocytes as target cells labeled with different concentrations of CFSE (0.025, 0.25, and 2.5 μM/mL; Molecular Probes). The CFSe low population was unpulsed, the CFSe med population was pulsed with an irrelevant peptide (Tpp2181-185 VYDFFVWL; derived from murine tyrosine-related protein 2; Bachem) and the CFSe high population was pulsed with the relevant peptide (SIINFEKL, 10 μg/mL). Target cells and controls were mixed in a 1:1 ratio and 3 × 10⁵ cells were injected into tail veins of mice. At 18 h after injection, spleens and draining lymph nodes were extracted and single cell suspensions were analyzed by flow cytometry. Peptide-specific target cell lysis was quantified by the ratio of the OVA257–264 peptide-pulsed cell population (CFSe high) to the Tpp2181–185-pulsed cell population (CFSe med) as the mean percentage and SD of cell lysis observed for each experimental group.

ELISPOT assay

Whole splenocytes were extracted from immunized or control mice and 1 × 10⁶ cells were seeded per well of a 96-well plate precoated with IFN-γ capture Ab. After overnight incubation in the presence of either the peptide used for immunization, an irrelevant peptide (10 μg/mL), or medium alone (background control), cells were removed and IFN-γ-producing cells were detected by ELISPOT reader (BIOREADER 2000; BioSys) using anti-mouse IFN-γ detection Ab. Spot numbers of background controls were subtracted and results were expressed as the number of IFN-γ-producing cells per 1 × 10⁶ splenocytes including SD of triplicates.

Differentiation of bone marrow-derived DC (BMDC)

The in vitro differentiation of BMDC was adapted from previously published protocols (43, 44). Myeloid DC were obtained by culture of bone marrow cells in DMEM (Life Technologies) containing 10% of heat-inactivated FCS (Life Technologies), penicillin-streptomycin and 10% of X6310 derived GM-CSF as described (45). BMDC were activated by stimulation of 500 U/mL IFN-β and/or pulsed with chicken OVA257–264 (SIINFEKL, 0.2 μg/mL; Bachem).

Adoptive cell transfer

Transfer of BMDC. In vitro differentiated BMDC were pulsed with 0.2 μg/mL OVA257–264 peptide for 1 h or left untreated. The 100 μL of the cell suspension containing 5 × 10⁵ BMDC was s.c. injected into recipient mice. Transfer of T cells. CD8⁺ T cells were isolated from spleens and lymph nodes using MACS (Miltenyi Biotec) according to the manufacturer’s protocol. The purity of the isolated cells was confirmed by flow cytometry. The 5–10 × 10⁶ of purified and CFSE- or non-CFSE-labeled T cells were injected in the tail vein of recipient mice.

Ag presentation by BMDC

BMDC were loaded with 0.2 μg OVA257–264 peptide/ml for 24 h and OVA257–264 peptide-MHC I complexes were detected by flow cytometry using 25-D1 Ab as described (46).

In vitro T cell proliferation

A total of 2 × 10⁵ splenocytes were labeled with CFSE (Molecular Probes) and cultured for 4 days in DMEM (+10% FBS) supplemented with anti-CD28 Abs and IL-2 on anti-CD3-coated plates or medium alone. Alternatively, 2 × 10⁵ lymph node cells from OT-1 mice were labeled with CFSE and cocultivated with 5 × 10⁵ BMDC (pulsed with 0.2 μg/mL OVA257–264 peptide or control peptide) for 4 days. The percentage of proliferating CD8⁺ T cells was analyzed by flow cytometry.
In vivo proliferation of CD8\(^+\) T cells

A total of 1 \times 10^7 CD8\(^+\) T cells were purified from spleens and lymph nodes, labeled with CFSE and transferred into RAG2-ko mice. As controls, naive wt mice were injected with 5 \times 10^5 peptide-pulsed BMDC or left untreated. On day 4 after transfer, mice were sacrificed and splenocytes of recipient mice were stained with anti-CD8 Ab. The cells were analyzed by flow cytometry for proliferating, CFSE-positive cells, gating on CD8\(^+\) T cells.

Apoptosis of T cells

Apoptosis of CFSE-labeled wt and Stat1S727A T cells during proliferation of whole splenocytes was measured by flow cytometry using Abs against CD8 and Annexin V. The percentage of Annexin V-positive cells was determined following injection of CFSE-labeled targets as described in Materials and Methods. Specific killing activity is indicated as the ratio of CTL-mediated lysis of targets pulsed with OVA peptide and targets pulsed with an unrelated peptide.

Statistical analysis

Student’s \(t\) test and SPSS software was used for calculation of statistical significance (***, \(p < 0.001\); **, \(p < 0.01\); or *, \(p < 0.05\)), which is indicated for each experiment.

Results

Impact of STAT-1, Stat1S727 phosphorylation and of IFN stimulation on the induction of Ag-specific CTL activity

A recently established assay was used to assess CTL activity in vivo (47). In brief, animals were immunized s.c. with MHC I-restricted OVA peptide SIINFEKL in presence or absence of IC31. The activity of the IC31 immune adjuvant is based on TLR9 signaling (47). Seven days after immunization, CFSE-labeled targets were injected into the tail veins of recipient mice. Eighteen hours later, target cell killing in the draining lymph node or spleen was analyzed by flow cytometry. Specific target cell lysis was quantified by the killing ratio of OVA peptide-pulsed vs coinjected control peptide-pulsed cells. Target cells loaded with specific peptide were distinguished from the control due to the labeling with different concentrations of CFSE.

Immunization of wt mice with both peptide and IC31 caused a robust CTL response, whereas peptide alone was ineffective to induce an adaptive immune response. In STAT-1-deficient animals the peptide alone caused a slight increase in CTL activity that was not further increased by the presence of IC31. In mice expressing Stat1S727A, the combination of peptide with IC31 enhanced CTL activity compared with controls, but the increase was small compared with wt animals. Both mutant genotypes showed a statistically significant decrease in the adaptive immune response compared with wt controls upon immunization with OVA peptide/IC31 (Fig. 1A). To determine whether serine-phosphorylated STAT-1 acted in the context of an IFN response, mice deficient for either the IFN-I receptor or for IFN-\(\gamma\) were examined. Although absence of the IFNAR1 completely abolished CTL activity, absence of IFN-\(\gamma\) had no significant effect. An IFNAR-STAT-1 pathway thus plays a critical role in driving the development of peptide-specific CTL (Fig. 1, B and C).

CD8\(^+\) T cells reportedly contribute to IFN-\(\gamma\) production during infection (48, 49). To determine whether numbers of IFN-\(\gamma\)-producing CD8\(^+\) T cells were affected by the absence of IFNAR1/STAT-1, or by the lack of Stat1S727 phosphorylation, splenic T cells were restimulated with the same MHC I-restricted OVA peptide or control peptide, and the frequency of IFN-\(\gamma\)-producing cells was determined by ELISPOT assay (Fig. 2). The frequency of IFN-\(\gamma\)-producing cells followed the same genotype-dependent pattern as the development of CTL activity. Absence of STAT-1 or the IFNAR1 abolished, and Stat1S727A mutation reduced the increase in Ag-specific, IFN-\(\gamma\)-producing CD8\(^+\) T cells seen in wt animals upon immunization.

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Effects of STAT-1 and IFN deficiency on the activity of peptide-specific CTL. Mice deficient or mutant for STAT-1 (A), the IFN-I receptor (B), or IFN-\(\gamma\) (C) were immunized with OVA peptide/IC31 as indicated. CTL activity in draining popliteal lymph nodes was determined following injection of CFSE-labeled targets as described in Materials and Methods. Specific killing activity is indicated as the ratio of CTL-mediated lysis of targets pulsed with OVA peptide and targets pulsed with an unrelated peptide.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Splenic IFN-\(\gamma\)-producing cells in mice immunized with OVA peptide/IC31. Splenocytes were isolated from mice 7 days after immunization and restimulated in vitro with control peptide or OVA peptide. Frequency of IFN-\(\gamma\)-producing cells were determined by ELISPOT assay.
FIGURE 3. T cell IFN-I response is not required for the generation of peptide-specific CTL. Mice with a T cell-specific disruption of the IFN-I receptor (IFNAR<sup>−/−</sup> CD4<sup>cre</sup>) or, as controls, wt or mice with complete IFN-I receptor deletion were immunized with OVA peptide/IC31 as indicated. CTL activity in draining popliteal lymph nodes was determined following injection of CFSE-labeled targets as described in Materials and Methods. Specific killing is indicated as the ratio of CTL-mediated lysis of targets pulsed with OVA peptide and targets pulsed with an unrelated peptide.

T cell IFN-I response is not required for the development of OVA-specific CTL

We examined whether the IFNAR-STAT-1 pathway delineated above was necessary in T cells to promote their differentiation into IFN-γ-producing and/or cytolytic CD8<sup>+</sup> T cells. We made use of mice containing floxed ifnar1 loci, bred to express the Cre recombinase under control of the CD4 promoter (50). Such animals efficiently and specifically eliminate IFNAR1 expression in the T cell lineage. Immunization of mice with CD4 Cre-deleted IFNAR1 with OVA/IC31 produced a marginal decrease in CTL activity compared with wt controls. By contrast, ubiquitous IFNAR1 deletion produced the expected complete loss of Ag-specific CTL response (Fig. 3). The experiment clearly shows that IFNAR1 do not act at the level of T cells to produce OVA-specific lytic activity in our experimental system.

STAT-1 is required in DC for the generation of OVA-specific CTL activity

Because mice with a DC-specific IFNAR1 or STAT-1 deletion are not available, we used adoptive transfer to test whether the IFNAR-STAT-1 pathway is important for CD8<sup>+</sup> T cell activation by DC. A different number of myeloid BMDC, either wt, STAT-1-deficient, or Stat1S727A, were pulsed with OVA peptide and injected into wt recipient mice. Cells from wt mice very efficiently stimulated development of OVA-specific CTL. The highest number transferred (10<sup>5</sup>) produced a 7-fold increase in CTL activity compared with naive animals (Fig. 4A). This was higher than the amount of CTL activity achieved by immunization with peptide/IC31. By contrast, the increase in OVA-specific CTL activity caused by administration of pulsed, STAT-1-deficient myeloid DC was ~2-fold. To test whether Stat1S727 phosphorylation in myeloid DC contributed to CD8<sup>+</sup> T cell activation and whether DC activity could be enhanced by treatment with IFN-I, 10<sup>5</sup> myeloid DC from wt, STAT-1<sup>−/−</sup> or Stat1S727A animals were used to immunize wt recipient mice. Before injection the cells were either left untreated, or treated with IFN-β for 24 h. Under these conditions, wt DC stimulated CTL activity to be 6-fold greater than in naive mice and this was increased to 8-fold by IFN-I treatment (Fig. 4B). By contrast, the enhancement of CTL activity caused by STAT-1-deficient myeloid DC was 2- to 3-fold irrespective of IFN-I treatment. Stat1S727A DC produced an intermediate result: between 4- and 5-fold stimulation of CTL activity in absence of IFN-I treatment and an increase of ~6-fold after IFN-I treatment. In line with this result, OVA-pulsed, IFN-I-treated Stat1S727A and even more pronounced STAT-1<sup>−/−</sup> DC showed reduced ability to stimulate the proliferation of OT-1 T cells in vitro (data not shown). Together the results establish that the IFN-I/STAT-1 axis is important for the production of myeloid DC fully competent of driving CTL development in vivo. However, this experiment also shows that STAT-1<sup>−/−</sup> and particularly Stat1S727A myeloid DC display significant residual ability to activate CD8<sup>+</sup> T cells. To directly address the effect of STAT-1 deficiency outside the DC compartment, we immunized STAT-1<sup>−/−</sup> or Stat1S727A mice with wt DC and asked whether these would completely restore CTL development. The results summarized in Fig. 4C and D, clearly show that wt DC do not completely rescue the effect of STAT-1 deficiency or mutation. The combination of wt DC and either STAT-1<sup>−/−</sup> or Stat1S727A mice produced smaller CTL activity than wt DC and wt mice. Therefore, DC may not fully account for the reduced CTL activity observed in STAT-1<sup>−/−</sup> Stat1S727A, or IFNAR1<sup>−/−</sup> mice.
Absence or mutation of STAT-1 reduces cell surface expression of molecules contributing to the immunological synapse

To investigate the causes underlying the reduced ability of STAT-1−/− or Stat1S727A mice to generate a CTL response, we examined myeloid BMDC. Cells of all examined genotypes displayed a similar endocytic activity, measured by the uptake of FITC-labeled dextran. Similarly, expression of the CD86 costimulator or of MHC II was not significantly different (data not shown). Expression of CD80 was similar in resting myeloid DC, but the enhancement of cell surface expression caused by IFN-I in wt DC was reduced in Stat1S727A cells, and almost completely absent in STAT-1−/− DC (Fig. 5A). In agreement with previous reports on other cell types (51, 52), STAT-1 deficiency caused a reduction of MHC I cell surface molecules and inability of IFN-I to increase MHC I expression on DC. In presence of Stat1S727A the reduction of MHC I expression compared with wt was less pronounced and significant up-regulation occurred in response to IFN-I (Fig. 5A). We directly asked whether this resulted in a corresponding reduction of OVA/MHC I complexes by probing the cell surface of myeloid DC with an Ab recognizing the combination of both molecules. Flow cytometry of stained DC demonstrated significant reduction OVA/MHC I complexes on STAT-1−/− DC and a slight reduction on Stat1S727A DC (Fig. 5B, number indicates mean fluorescence of untreated and IFN-I-treated cells). IFN-I treatment for 24 h increased the ability of wt and Stat1S727A cells, but not of STAT-1−/− DC to present OVA peptide. Taken together, decreased MHC I expression on STAT-1−/− DC may contribute to their reduced functional competence in mice. The failure to up-regulate the CD80 costimulator in response to IFN-I may additionally contribute to this phenotype.

Absence or mutation of STAT-1 increases CD8+ T cell proliferation in vitro and in Rag-deficient mice

Populations of total splenic T cells display an enhanced proliferative response to activation signals when deficient for STAT-1. This is consistent with the DC phenotype described, yet counterintuitive considering the complete absence of CTL in STAT-1-deficient mice. To assess whether the reported results similarly apply to purified CD8+ T cells and to examine the consequences of Stat1S727A mutation, OVA-specific, MHC I-restricted splenic CD8+ T cells from OT-1 mice, either wt, STAT-1−/−, or Stat1S727A, were cocultured with myeloid DC in the presence of OVA peptide. This experimental protocol demonstrated an increased proliferative response of both STAT-1−/− and Stat1S727A CD8+ OT-1 T cells (Fig. 6A). In line with this response and with data published on total splenic T cells, normal Stat1S727A CD8+ T cells showed increased proliferation when activated by treatment with anti-CD3/CD28 Abs and simultaneous staining with FITC-Annexin V demonstrated a concomitant reduction in the number of apoptotic cells in both the resting and proliferating population (Fig. 6B).

We examined whether T cell hyperproliferation is an attribute of STAT-1-deficient T cells in vitro or whether a similar phenomenon occurs also in murine hosts. Therefore, 1 × 10⁷ purified CD8+ T cells from OT-1 mice, with the STAT-1 locus either disrupted, mutated at S727, or wt, were injected into wt, Rag2−/−, or OVA/IC31-immunized Rag2−/− recipients. Four days later the proliferative response was determined in absence of further activation. In naive wt animals, this protocol caused a low proliferative response that was slightly increased by S727A mutation and significantly by STAT-1 deficiency of CD8+ T cells (Fig. 7A). In Rag2−/− mice a vigorous homeostatic proliferative response was seen with OT-1/wt T cells and this was increased by S727A mutation and further enhanced by STAT-1 deficiency. Immunization of Rag2−/− mice with OVA/IC31 caused ~80% of OT-1/wt T cells to proliferate and this was again increased by STAT-1 deficiency. Concomitant with the higher proliferative response, much higher levels of OVA-specific CTL activity was observed in immunized Rag2−/− mice 7 days after transfer of OT-1 T cells of STAT-1−/− or Stat1S727A genotype compared with OT-1/wt cells (Fig. 7B). This experiment...
demonstrates a hyperproliferative phenotype of STAT-1 mutant or STAT-1-deficient T cells in vivo and confirms the results in this paper that IFNAR/STAT-1-deficient T cells are perfectly able to differentiate into functional CTLs.

Discussion

The data presented show that IFN-I and their signal transducer STAT-1 essentially contribute to the induction of OVA-specific CTL following immunization with OVA peptide and IC31 immune adjuvant. Surprisingly and contrasting many experimental situations with pathogen-specific CTL, OVA-specific CTL did not require IFN-I/STAT-1 as an activation/survival signal. This was shown by the use of conditional IFNAR1 deletion and by adoptive transfer of wt or Stat1S727A, or STAT-1−/− mice, were labeled with CFSE and activated using OVA peptide-pulsed myeloid DC or, as a control, cocultivated with unpulsed myeloid DC. Proliferation rates after 4 days were measured by flow cytometry. The percentage of proliferating cells of each genotype is indicated. B, Splenic CD8+ T cells, either wt or Stat1S727A, were labeled with CFSE and activated using Abs against CD3 and CD28. Four days later, the cells were stained with allophycocyanin-Annexin V and analyzed by flow cytometry. Percentages of Annexin-positive or -negative cells shown in both the proliferating and nonproliferating cell fractions.

![Figure 6](http://www.jimmunol.org/)

FIGURE 6. STAT-1 deficiency or STAT-1 mutation increases the expansion of CD8+ T cells in vitro. A, OT-1 T cells, either wt, Stat1S727A, or STAT-1−/− were labeled with CFSE and activated using OVA peptide-pulsed myeloid DC or, as a control, cocultivated with unpulsed myeloid DC. Proliferation rates after 4 days were measured by flow cytometry. The percentage of proliferating cells of each genotype is indicated. B, Splenic CD8+ T cells, either wt or Stat1S727A, were labeled with CFSE and activated using Abs against CD3 and CD28. Four days later, the cells were stained with allophycocyanin-Annexin V and analyzed by flow cytometry. Percentages of Annexin-positive or -negative cells shown in both the proliferating and nonproliferating cell fractions.

![Figure 7](http://www.jimmunol.org/)

FIGURE 7. STAT-1 and Stat1S727 phosphorylation regulate homeostatic and Ag-driven proliferation of T cells in Rag2−/− mice. A, A total of 1×10^7 purified CD8+ OT-1 T cells expressing wt, Stat1S727A, or no STAT-1 were labeled with CFSE, and injected i.v. into Rag2-deficient or, as controls, naive wt mice. Where indicated, mice were additionally immunized with OVA peptide and IC31-pulsed DC. Four days later, splenocytes were isolated and stained with Ab to CD8. The percentage of proliferating cells in the spleens of recipient mice, indicated by CFSE dilution, was determined by flow cytometry. Gates were set for CD8+ cells. B, CTL activity in lymph nodes of Rag2-deficient mice was determined 7 days after transfer of 1×10^7 OT-1 T cells expressing wt, Stat1S727A, or no STAT-1 and administration of OVA peptide and IC31-pulsed DC. Specific killing activity is indicated as the ratio of CTL-mediated lysis of targets pulsed with OVA peptide and targets pulsed with an unrelated peptide.

In vitro experiments, because immunization with the OVA/IC31 combination causes predominant entry of the peptide as an external Ag, whereas in vitro at least some of the peptide may be loaded directly onto cell surface MHC I. In line with recent data (51, 52), STAT-1 deficiency caused reduced MHC I expression by myeloid DC even in absence of external IFN-I and this provides further explanation for the reduced CTL stimulatory activity of STAT-1-deficient myeloid DC.

Consistent with results by other investigators our study emphasizes the variable dependence of T cell or DC activation and differentiation on IFN-I (25–29). This variability has been attributed to the cytokine milieu created by a pathogen that may or may not include cytokines able to substitute for IFN-I in the development of functional T cells and DC (27). The cytokine milieu in our experiments most likely results from IC31 action. Because the immunologically active component of this adjuvant is an oligonucleotide, signaling through TLR9 produces the stimulatory effect on CTL responses (47). TLR9 signaling can cause IFN-I production by both plasmacytoid DC and myeloid DC. Therefore, IFN-I/
STAT-1 are likely to, at least in part, transmit the activity of the IC31 immune adjuvant. This view is supported by recent data showing that IC31 enhances DC activation markers (47). It remains an open question which cytokines produced through TLR9 activity make IFN-I dispensable as a third signal for T cell activation. Furthermore the parameters determining whether IFN-I or STAT-1 are required to render DC capable of stimulating naive CTL remain to be explored. Although their importance for maturation, cross-presentation by DC and the shaping of DC subsets has been documented (35, 37, 38), CD8+ CTL were normal in STAT-1−/− mice after infection with influenza virus (21), suggesting that neither the Ag-presenting DC nor the CTL themselves required IFN-I/STAT-1 in this situation. In experimental systems such as the one used in our study it may be of interest to vary the immune adjuvant. This may help to decipher innate immune pathways generating or eliminating the need for IFN-I and STAT-1. For example, TLR2 ligands that do not directly stimulate IFN-I synthesis can be explored for their potential to induce Ag-specific CTL in IFNAR or STAT-1-deficient mice.

STAT-1 phosphorylation at S727 was important for the generation of OVA-specific CTL activity in mice. This shows that STAT-1 serine phosphorylation is important for the IFN-I response driving CTL development and that the S727A mutation in this context represents a hypomorphic STAT-1 allele and a completely unprecedented situation where Stat1S727 phosphorylation is required for the function of the ISGF3 complex during an immune response. However, closer inspection reveals that the S727A mutation may not always, i.e., in all situations and cell types function as a STAT-1 hypomorph. This is exemplified by our results with DC and CTL. Whereas the S727A mutation generally produced weaker versions of the effects of STAT-1 deficiency on the T cells, the impact of the mutation on adoptively transferred myeloid DC was relatively small and the myeloid DC retained much of their IFN-I responsiveness when analyzed in vitro. The involvement of a non-DC, non-T cell compartment in producing STAT-1-dependent adaptive immunity is suggested by the inability of wt myeloid DC to fully reconstitute CTL responses in either STAT-1-deficient or Stat1S727A mice. This compartment may be particularly dependent on Stat1S727 phosphorylation for IFN-I responsiveness. We have no data at present to suggest what this third-party cell compartment may be. Conceivably, it contributes to CTL recirculation and activation. That said, STAT-1 deficiency or S727A mutation do not grossly alter amounts of either CD8+ T cells or CD11c+ DC in draining lymph nodes, as determined by flow cytometry up to 4 days after immunization with OVA/IC31 (data not shown).

Apart from assuming a cell type-dependent influence of Stat1S727 phosphorylation on IFN signaling, a distinct explanation why the requirement for STAT-1 serine phosphorylation in an immune reaction does not always reflect the requirement for IFN may be a role for serine-phosphorylated STAT-1-independent of IFN responses. This notion is supported by recent reports showing phosphorylation of STAT-1 by T cell Ag receptor signaling (53) and the recruitment of S727-phosphorylated STAT-1 to the immunological synapse of Th precursor cells. In these cells S727-phosphorylation is required for Th polarization, but STAT-1 tyrosine phosphorylation is not (54). Determining the extent to which this IFN-independent STAT-1 phosphorylation impinges on T cell-mediated adaptive immunity will require future research with IFN-unresponsive mice expressing Stat1S727A.

Examining the impact of STAT-1 in vitro, we noted in accordance with earlier results (15–17) that purified STAT-1−/− or Stat1S727A CD8+ cells, show reduced apoptosis and increased proliferation upon activation. The effect of STAT-1 on activated T cells is thus contrary to the effect on CTL development in the context of the complete immune system of animals. This result confirms our notion that DC, and possibly non-DC/non-T cells, are primary targets of IFN-I/STAT-1. It poses the question in what context STAT-1 and Stat1S727 phosphorylation are required to limit T cell expansion. The experimental system used by us suggests that both homeostatic T cell proliferation and proliferation of activated cells may be limited by STAT-1 and its phosphorylation on S727 because T cell hyperproliferation caused by STAT-1 mutation was seen in Rag2−/− mice both before and after immunization. Homeostatic proliferation relies on the cytokines IL-7 and IL-15 as well as self-Ag presented by APC. It is required to maintain sufficiently large pools of both naive and memory T cells (55). Our results provide the first evidence that STAT-1 and possibly IFN limit homeostatic T cell proliferation in mice. STAT-1-deficient mice were shown to have an increased propensity for autoimmune responses (56). Although this has been attributed to a role in the generation of regulatory T cells, our data suggest that STAT-1-dependent inhibition of homeostatic proliferation may similarly prevent the development of autoimmunity or leukemia.

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Disclosures
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