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STAT1 Signaling in CD8 T Cells Is Required for Their Clonal Expansion and Memory Formation Following Viral Infection In Vivo

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Recent advances have shown that direct type I IFN signaling on T cells is required for their efficient expansion in response to viral infections in vivo. It is not clear which intracellular signaling molecule is responsible for this effect. Although STAT1 has been shown to mediate many of the type I IFN-dependent biological effects, its role in T cells remains uncertain in vivo. In this study, we demonstrated that STAT1 signaling in CD8 T cells was required for their efficient expansion by promoting the survival of activated CD8 T cells upon vaccinia viral infection in vivo, suggesting that the direct effect of type I IFNs on CD8 T cells is mediated by STAT1. Furthermore, effector CD8 T cells that lack STAT1 signaling did not survive the contraction phase to differentiate into long-lived memory cells. These results identify a critical role for type I IFN-STAT1 signaling in multiple stages of CD8 T cell response in vivo and suggest that strategies to activate type I IFN-STAT1 signaling pathway may enhance vaccine potency. The Journal of Immunology, 2008, 180: 2158–2164.

Type I IFNs are a family of cytokines that constitute 13 and 17 IFN-α subtypes in mice and humans, respectively, and one IFN-β in both species (1). All type I IFNs signal through a heterodimeric receptor composed of two subunits, IFN-α receptor 1 and receptor 2 (2). Receptor binding results in tyrosine phosphorylation and activation of the transcription factors, STAT1 and STAT2, leading to the transcription of >100 IFN-stimulated genes. In addition, other STAT family members including STAT4, STAT3, and STAT5 can also be activated to mediate an array of complex, sometimes paradoxical effects by type I IFNs (2–4).

Although originally discovered for their potent direct antiviral function, type I IFNs also mediate a wide spectrum of biological effects including growth inhibition and immune regulation (5). It has been shown that efficient activation of NK cells is dependent on type I IFNs (6). Type I IFNs also play an important role in promoting adaptive T cell responses (7). In particular, recent studies have demonstrated that the direct action of type I IFNs on T cells is critical for their clonal expansion in response to viral infections in vivo (8–10). It is not clear which STAT member mediates the direct effect of type I IFNs on T cell expansion in vivo. Among many STAT family members that have been implicated in the type I IFN-triggered signaling, STAT1 is the best characterized transcription factor that mediates many of the type I IFN-dependent biological effects in response to viral infections in vivo (5). Indeed, STAT1-deficient (STAT1−/−) mice are highly susceptible to viral infections because of the absence of direct antiviral defense mediated by type I IFNs (11, 12). STAT1 signaling is also required for type I IFN-dependent activation of NK cells (13, 14). However, STAT1 has been shown to mediate the antiproliferative effect of type I IFNs on NK cells in vitro (4, 15). Although a recent report has proposed that selective down-regulation of STAT1 in CD8 T cells might be related to efficient expansion of virus-specific T cells in the presence of type I IFNs in vivo (15), it remains to be defined what the precise role of STAT1 in mediating the direct effect of type I IFN on clonal expansion of Ag-specific CD8 T cells during viral infections in vivo.

In this study, we showed that although STAT1 signaling indeed mediated antiproliferative effect of type I IFN on T cells in vitro, type I IFN-STAT1 signaling on CD8 T cells was required for efficient expansion of Ag-specific CD8 T cells in response to infection with vaccinia virus (VV)3 in vivo. This response was achieved by promoting the survival of activated CD8 T cells. Furthermore, long-lived memory CD8 T cells could not develop in the absence of STAT1 signaling. These results suggest that the direct effect of type I IFNs on multiple stages of CD8 T cell response is mediated through STAT1.

Materials and Methods

Mice

B10.D2 mice were purchased from The Jackson Laboratory. Wild-type (WT) 129/Sv mice were obtained from Charles River Breeding Laboratories. IFN-αβ receptor-deficient (IFN-αβR−/−) mice on the 129/Sv background were purchased from B&K Universal. STAT1−/− mice on the 129/Sv background were purchased from Taconic. The clone 4 influenza hemagglutinin (HA)-TCR transgenic mice, on the Thy1.1, B10.D2 background, that express a TCR recognizing a Kd-restricted HA epitope (518IYSTVASSL526) were previously described (16–18). We intercrossed clone 4 HA-TCR mice with STAT1−/− and IFN-αβR−/− mice to generate the respective STAT1−/− and IFN-αβR−/− clone 4 HA-TCR F1 mice as well as WT clone 4 HA-TCR F1 littermates used in experiments. B10.D2–129/Sv F1 mice served as recipients in all of the experiments using these mice.

Abbreviations used in this paper: VV, vaccinia virus; HA, hemagglutinin; WT, wild type.

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clone 4 HA-TCR T cells. All mice used for experiments were between 8 and 12 wk of age. All experimental procedures involving the use of mice were done in accordance with protocols approved by the Animal Care and Use Committee of Duke University Medical Center.

Stimulation of T cells in vitro

The 96-well flat-bottom plates were coated with anti-CD3 (5 μg/ml) and anti-CD28 (10 μg/ml) at 4°C overnight. Plates were then washed twice with PBS to remove unbound Abs. Polyclonal CD8 T cells were purified from WT, STAT1−/−, or IFN-αβR−/− 129/Sv mice by positive selection using anti-CD8 microbeads (Miltenyi Biotec). Following selection, 2 × 10⁵ cells were CFSE labeled and cultured in anti-CD3- and anti-CD28-coated plates in the presence or absence of 1 × 10⁴ U/ml recombinant IFN-α (PBL Biomedical Laboratories) in 200 μl of medium supplemented with murine IL-2 (100 U/ml).

Adaptive transfer and viral infection

For adaptive transfer of clone 4 CD8 T cells, single cell suspensions were prepared from pooled spleen and peripheral lymph nodes of mice and subjected to positive selection of CD8+ T cells via anti-CD8 microbeads (Miltenyi Biotec) with a purity of >98%. Purified T cells were then transferred into recipient mice through tail vein. In some experiments, clone 4 T cells were labeled with CFSE as described (16–18) before injection.

The Western Reserve strain of VV, the recombinant VV encoding HA (rVV-HA), and the recombinant adenovirus encoding HA (Ad-HA) were grown and purified, and the viral titers were determined by plaque assay as previously described (16, 17, 19). In all experiments, mice were i.p. infected with VV, rVV-HA, or Ad-HA.

Abs and flow cytometry

Monoclonal Abs used for staining were CyChrome-conjugated anti-CD8; FITC-conjugated anti-CD62 ligand, anti-CD69, and anti-IFN-γ; PE-conjugated anti-Thy1.1, anti-CD44, anti-CD25, and Annexin V; allophycocyanin-conjugated streptavidin; and biotinylated anti-Thy1.1 (all from BD Biosciences). Collection of flow cytometry data was conducted using a FACSCanto (BD Biosciences) and events were analyzed using FACSDiva software (BD Biosciences).

Intracellular IFN-γ and Annexin V staining

For intracellular staining of clone 4 CD8 T cells, splenocytes were cultured in 6 h in the presence of 5 μg/ml GolgiPlug and 2 μg/ml of the K⁺-HA peptide (518IYSTVASSL526). After culture, cells were surface-stained with anti-CD8 and anti-Thy-1.1, and permeabilized for intracellular staining with anti-IFN-γ as described (16–18).

For intracellular IFN-γ staining on polyclonal CD8+ T cells, splenocytes were stimulated for 6 h in the presence of 5 μg/ml GolgiPlug and 2 μg/ml of the previously described K⁺-restricted VV B8R epitope (6TYSKFESV⁵) (20), and stained for IFN-γ intracellularly as described.

Annexin V staining of clone 4 CD8 T cells was conducted according to the manufacturer’s instructions as described (18). Briefly, splenocytes were harvested and stained with anti-CD8 and anti-Thy-1.1 Abs. Cells were then washed twice with ice-cold PBS, followed by staining for 15 min at room temperature with PE-conjugated Annexin V in a total volume of 50 μl.

Statistical analysis

Results were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student t test.

Results

STAT1 mediates antiproliferative effect of type I IFN on CD8 T cells in vitro

Previous studies have shown that STAT1 plays an important role in type I IFN-mediated inhibition of T cell proliferation in vitro (4, 15). To confirm this role, purified WT, STAT1−/−, or IFN-αβR−/− CD8 T cells were stimulated in vitro with plate-bound anti-CD3 and anti-CD28 Abs for 72 h in the presence or absence of IFN-α. Following culture, cell division was analyzed via the CFSE dilution assay. CD8 T cells derived from all mice underwent multiple cycles of cell division (Fig. 1, left column). However, the addition of exogenous IFN-α during culture strongly inhibited the proliferation of WT, but not STAT1−/− or IFN-αβR−/− CD8 T cells (Fig. 1, right column), indicating that indeed the antiproliferative activity of type I IFN on CD8 T cells is mediated by STAT1.

Efficient expansion of VV-specific CD8 T cells in vivo is dependent on type I IFN-STAT1 signaling

The observations that type I IFN-STAT1 signaling exerts an antiproliferative effect on T cells in vitro raised the question whether STAT1 mediates the direct effect of type I IFN on CD8 T cell expansion in response to viral infections in vivo. It has been hypothesized that down-regulation of STAT1 selectively in Ag-specific CD8 T cells may contribute to their efficient expansion in the presence of type I IFNs (15). However, direct evidence to support this model has been lacking. To directly address the role of STAT1 in type I IFN-mediated expansion of Ag-specific CD8 T cells in vivo, we infected WT, STAT1−/−, or IFN-αβR−/− mice with 1 × 10⁶ PFU of VV i.p. VV was chosen for this study because it has been shown recently that VV infection elicits potent type I IFN production (19), and that direct type I IFN signaling is required for efficient expansion of VV-specific CD8 T cells (10). Seven days after infection, splenocytes were analyzed for CD8 T cell response to a VV-specific epitope as measured by intracellular IFN-γ production upon restimulation with the epitope peptide. A significant (p < 0.001) expansion of VV-specific CD8 T cells was detected in WT mice infected with VV compared with the uninfected naive mice (Fig. 2). In contrast, the expansion of Ag-specific CD8 T cells in STAT1−/− or IFN-αβR−/− mice was diminished compared with WT controls (Fig. 2). The reduced expansion of VV-specific CD8 T cells in STAT1−/− or IFN-αβR−/− mice was not due to differential homing or trafficking of the cells as a similar degree of decrease was observed in other lymphoid as well as nonlymphoid organs (data not shown). Taken together, these results suggest that type I IFN-STAT1 signaling is critical for efficient expansion of Ag-specific CD8 T cells in response to VV infection in vivo.
Type I IFN-STAT1 signaling on CD8 T cells is required for their clonal expansion upon VV infection in vivo

Type I IFN-STAT1 signaling has multiple effects on both innate and adaptive immune responses. STAT1−/− mice are highly susceptible to viral infections due to a defect in direct antiviral defense mediated by type I IFNs (11, 12). STAT1 signaling is also critical for type I IFN-dependent activation of NK cells (13, 14). Consequently, type I IFN-STAT1 signaling may influence pathogen clearance and alter the antigenic load, leading to defective CD8 T cell response in vivo. In addition, mice lacking STAT1 display increased susceptibility to autoimmune disease due to a lack of functional CD4+CD25+ regulatory T cells (21). Thus, the comparison of CD8 T cell responses in WT, STAT1−/− and IFN-αβR−/− mice cannot specifically address the direct role of type I IFN-STAT1 signaling on CD8 T cells during infection.

To address this question, we used a transgenic T cell system in which we assessed HA-specific CD8 T cell response to recombinant VV encoding HA (rVV-HA) in vivo. We first intercrossed the clone 4 HA-TCR transgenic mice with STAT1−/− or IFN-αβR−/− mice to generate STAT1−/− or IFN-αβR−/− mice that were subsequently infected with 5 × 10^5 PFU of rVV-HA i.p. Seven days after infection, massive clonal expansion and effector differentiation as measured by the production of IFN-γ were detected in the spleen of mice infected with WT clone 4 T cells (Fig. 3). By contrast, the extent of expansion for STAT1−/− or IFN-αβR−/− clone 4 T cells was significantly (p < 0.001) diminished compared with WT clone 4 T cells (Fig. 3). These results indicate that type I IFN-STAT1 signaling in CD8 T cells is required for their clonal expansion in response to VV infection in vivo.

As recent studies have highlighted the need to most closely mimic the endogenous CD8 T cell response when using a TCR transgenic adoptive transfer system (22, 23), we repeated this experiment using 500 WT, STAT1−/−, or IFN-αβR−/− clone 4 CD8 T cells. This replication would allow us to both add validity to our conclusions and to make certain that we were not simply observing an artifact brought on by our initial CD8 T cell frequency. Following the transfer of 500 clone 4 T cells, mice were i.p. infected with 5 × 10^5 PFU rVV-HA, and both the transgenic and endogenous HA-specific CD8 T cell responses were assessed 7 days later. We found that the endogenous HA-specific CD8 T cell responses were similar among all groups, including those receiving no transgenic T cells (Fig. 4A), suggesting that transfer of 500 clone 4 T cells did not suppress the endogenous HA-specific CD8 T cell response. Under this condition, we demonstrated once again that the clonal expansion and effector function of STAT1−/− and IFN-αβR−/− clone 4 T cells was significantly (p < 0.001) reduced compared with the WT controls (Fig. 4). These data confirm that STAT1 signaling in CD8 T cells is required for their clonal expansion in response to VV infection in vivo.
FIGURE 4. Initial CD8 T cell frequency does not affect defective clonal expansion of CD8 T cells in the absence of type I IFN-STAT1. A total of 500 naive WT, STAT1−/−, or IFN-αβR−/− HA-specific CD8 T cells (Thy1.1+) were transferred into Thy1.2+ recipient mice that were i.p. infected with rVV-HA. Recipient mice receiving no transgenic T cells (No transfer) infected with rVV-HA were used as a control. A. Splenocytes were harvested at day 7 postinfection and stained with anti-CD8 and anti-Thy1.1 Abs to determine the percentage of clonotypic T cells among total lymphocytes (top row). Cells were also subjected to intracellular staining to determine the percentage of IFN-γ-producing HA-specific T cells among total CD8 T cells (bottom row), with the percentage of IFN-γ Thy1.1+ CD8+ (Transgenic) T cells indicated in the top right quadrant and the percentage of IFN-γ Thy1.1+ CD8+ (endogenous) T cells indicated in the bottom right quadrant. B, The absolute cell number of Thy1.1+ CD8+ cells (left), IFN-γ Thy1.1+ CD8+ cells (middle), and IFN-γ Thy1.1+ CD8+ cells (right) per spleen for each respective group are shown with SD indicated. Data shown are representative of three independent experiments.

**Diminished expansion of STAT1−/− CD8 T cells is due to increased cell death following activation**

We next investigated what contributed to the defective clonal expansion of STAT1−/− CD8 T cells. One possibility could be that STAT1−/− CD8 T cells were not fully activated. To investigate, we transferred 1 × 10⁶ naive WT, STAT1−/−, or IFN-αβR−/− clone 4 CD8 T cells (Thy1.1+) into congenic recipients (Thy1.2+) that were subsequently i.p. infected with 5 × 10⁶ PFU rVV-HA. Higher clone 4 T cell numbers were used in this experiment because transfer of 1 × 10⁶ cells was below the limit of detection at early time points. At 24 h after infection, WT, STAT1−/−, and IFN-αβR−/− clone 4 T cells all displayed a similarly activated phenotype of CD25high and CD69high compared with the naive T cell phenotype of CD25low and CD69low (Fig. 5). In addition, the degree of CD62 ligand down-regulation was similar among them (Fig. 5). These results suggest that initial activation of T cells was not affected in the absence of type I IFN-STAT1 signaling.

Three days after infection, all three groups of clone 4 T cells underwent extensive rounds of cell division by CFSE dilution (Fig. 6A). Although STAT1−/− and IFN-αβR−/− clone 4 T cells seemed to be slightly delayed in each round of their division profiles as compared with WT clone 4 T cells (Fig. 6A), over 73% of each respective population had undergone at least five rounds of division by this time, suggesting clone 4 T cell proliferation was not grossly affected by a lack of type I IFN-STAT1 signaling. Furthermore, the degree of reduction in IFN-γ-producing STAT1−/− and IFN-αβR−/− clone 4 T cells was proportionate to the decrease in total STAT1−/− and IFN-αβR−/− clone 4 T cells, respectively (Figs. 3 and 4), suggesting that the effector differentiation of STAT1−/− and IFN-αβR−/− clone 4 T cells on per cell basis appeared to be intact as compared with the WT controls. These data suggest that CD8 T cell activation and effector differentiation in response to VV infection in vivo is not affected by a lack of type I IFN-STAT1 signaling.

Despite similar degrees of activation, STAT1−/− and IFN-αβR−/− clone 4 T cells accumulated poorly (Fig. 6B). We then investigated whether direct type I IFN-STAT1 signaling was needed for the survival of activated CD8 T cells. Three days after infection with rVV-HA, clone 4 CD8 T cells were stained with Annexin V as an indicator of those cells undergoing the early stages of apoptosis. Indeed, the activated STAT1−/− and IFN-αβR−/− clone 4 T cells displayed a significant (p < 0.01) increase in Annexin V positivity (48.0% and 51.7%, respectively) compared with their WT counterparts (25.3%) (Fig. 6C). Taken together, these results suggest that the diminished clonal expansion...
of STAT1−/− clone 4 CD8 T cells in response to VV infection is not caused by a defect in T cell activation, but by poor survival of the activated T cells.

Defective CD8 memory formation in the absence of type I IFN-STAT1 signaling

The observation that the activated STAT1−/− and IFN-αβR−/− CD8 T cells survived poorly prompted us to study their ability to develop into stable memory cells. After the peak of clonal expansion at day 7, WT clone 4 effector CD8 T cells underwent marked contraction between days 7 and 14, and those that survived developed into stable memory CD8 T cells (Fig. 7A). This result is consistent with previous observations in other models of viral infection (24). However, the smaller pool of STAT1−/− and IFN-αβR−/− clone 4 effector CD8 T cells could not survive the contraction phase to develop into memory cells (Fig. 7A). To insure
that the lack of memory formation from STAT1−/− or IFN-αβR−/− clone 4 T cells was not due to a limit of detection in our system, we boosted mice at day 42 with recombinant adenovirus encoding HA (Ad-HA) to assess the recall response. We observed a robust recall expansion of WT clone 4 memory CD8 T cells (Fig. 7B). However, there were still no detectable STAT1−/− or IFN-αβR−/− clone 4 CD8 T cells (Fig. 7B). To rule out the possibility that the observed lack of memory cell formation from STAT1−/− clone 4 T cells was due to host-mediated rejection following VV infection, 2 × 10^7 naive WT or STAT1−/− clone 4 T cells were transferred into uninfected recipients or recipient mice that have been infected 42 days earlier with 5 × 10^5 rVV-HA. Splenocytes were harvested at 24 h or 7 days following transfer and were stained with anti-CD8 and anti-Thy1.1 Abs to determine the absolute number of CD8+Thy1.1+ cells among total lymphocytes, which is indicated. Data shown are representative of three independent experiments.

**Discussion**

In this study, we have demonstrated that although STAT1 mediates the antiproliferative effect of type I IFN on T cells in vitro, type I IFN-STAT1 signaling in CD8 T cells is critical for their clonal expansion in response to VV infection in vivo. This role is achieved by promoting the survival of activated CD8 T cells. Furthermore, effector CD8 T cells that lack type I IFN-STAT1 signaling did not survive the contraction phase to differentiate into long-lived memory cells. These results identify a critical role for direct type I IFN-STAT1 signaling in multiple stages of CD8 T cell responses in vivo.

STAT1 has been shown to mediate the antiproliferative effect of type I IFNs on T cells in vitro (4, 15). Our results confirmed this observation. However, upon viral infections that elicit high levels of type IFNs, virus-specific CD8 T cells often expand vigorously in vivo (7). To explain this paradoxical effect of type I IFN on T cells in vitro vs in vivo, it has been proposed that Ag-specific CD8 T cells down-regulate the levels of STAT1 selectively to counter the antiproliferative effect of type I IFN during viral infection and allow expansion of Ag-specific T cells in the presence of type I IFNs (15). However, direct evidence to support this model is lacking and the precise role of STAT1 in CD8 T cell response remains unknown. Recent studies in a variety of models of viral infection in vivo have demonstrated that direct type I IFN signaling is, in fact, required for efficient expansion of virus-specific CD8 T cells in vivo (8–10). In this study, we provided evidence that similar to IFN-αβR-deficient CD8 T cells, the expansion of STAT1-deficient CD8 T cells upon VV infection is also impaired, suggesting that STAT1 signaling is critical for CD8 T cell response and that the direct effect of type I IFNs on CD8 T cell clonal expansion in vivo is mediated by STAT1. Because type II IFN (IFN-γ) also mediates its effect through STAT1, our data also support the previous observations that the direct action of IFN-γ on T cells promotes their responses to viral infections in vivo (25, 26).

It is not clear what contributes to the paradoxical effect of type I IFN-STAT1 signaling on T cell responses in vitro vs in vivo. One possible explanation is viral infection in vivo often induces an array of cytokines and factors that may offset the antiproliferative effect of type I IFN. It might be related to the activation status of T cells (naive vs activated), the timing, or the source (exogenously administered vs endogenously induced upon VV infection) of type I IFN. Interestingly, type II IFN has also been shown to inhibit T cell proliferation in vitro, but to promote T cell expansion upon viral infections in vivo (25, 26).

Our observation that the diminished clonal expansion of CD8 T cells that lack STAT1 signaling is due to their poor survival following activation in vivo is in line with previous studies that report activated T cells survive better in the presence of type I IFNs (27, 28). Furthermore, STAT1-deficient effector CD8 T cells could not survive the contraction phase to develop into long-lived memory CD8 T cells. How does type I IFN-STAT1 signaling promote the survival of activated T cells? We observed that the proliferation of STAT1−/− and IFN-αβR−/− clone 4 T cells was slightly delayed in each round of their cell division as compared with WT clone 4 T cells. It is not clear whether this delay impacts on their survival as the effector differentiation of these cells seemed to be intact. Also unknown is whether the enhanced survival of CD8 T cells by direct type I IFN-STAT1 signaling is mediated by up-regulation of receptors for other survival cytokines such as IL-15, a cytokine known for self-renewal of memory CD8 T cells (29). Thus, the mechanisms by which type I IFN-STAT1 signaling promotes the survival of activated CD8 T cells in vivo requires further investigation.

In summary, we have shown that the direct role of type I IFN signaling in CD8 T cell clonal expansion is mediated by STAT1, and that the reduced clonal expansion in the absence of STAT1 signaling is due to poor survival of the activated CD8 T cells. We have further demonstrated that STAT1 signaling is also critical for effector CD8 T cells to survive the contraction phase and develop into long-lived memory cells. Our findings may have important implications in the design of new vaccine strategies to promote CD8 T responses based on activating the type I IFN-STAT1 signaling pathway.

**Disclosures**

The authors have no financial conflict of interest.

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