Out-of-Sequence Signal 3 as a Mechanism for Virus-Induced Immune Suppression of CD8 T Cell Responses

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Abstract

Virus infections are known to induce a transient state of immune suppression often associated with an inhibition of T cell proliferation in response to mitogen or cognate-antigen stimulation. Recently, virus-induced immune suppression has been linked to responses to type 1 interferon (IFN), a signal 3 cytokine that normally can augment the proliferation and differentiation of T cells exposed to antigen (signal 1) and co-stimulation (signal 2). However, pre-exposure of CD8 T cells to IFN-inducers such as viruses or poly(I:C) prior to antigen signaling is inhibitory, indicating that the timing of IFN exposure is of essence. We show here that CD8 T cells pretreated with poly(I:C) down-regulated the IFN receptor, up-regulated suppressor of cytokine signaling 1 (SOCS1), and were refractory to IFN-induced signal transducers and activators of transcription (STAT) phosphorylation. When exposed to a viral infection, these CD8 T cells behaved more like 2-signal than 3-signal T cells, showing defects in short lived effector cell differentiation, reduced effector function, delayed cell division, and reduced levels of survival proteins. This suggests that IFN-pretreated CD8 T cells are unable to receive the positive effects that type 1 IFN provides as a signal 3 cytokine when delivered later in the signaling process. This desensitization mechanism may partially explain why vaccines function poorly in virus-infected individuals.

Introduction

The fact that virus infections can induce a transient state of immune suppression was first described over a century ago, as patients acutely infected with the measles virus failed to develop a recall response to tuberculin even though they had previously been immunized [1]. Since then, infection with a number of other viruses, including HIV [2], Lymphocytic choriomeningitis virus (LCMV) [3], CMV [4] and Influenza A [5] have been shown to induce a transient state of immune suppression in humans and animal models [6,7]. Although virus-induced immune suppression can affect many aspects of the immune system, it is often associated with a reduced ability of T cells to proliferate in response to mitogens or antigen-specific stimulation. Viruses may induce this suppression by directly infecting cells of the immune system, but they can also induce immune suppression without directly targeting hematopoietic cells. In vitro studies have shown that inhibition of T cell proliferation can be due to death receptor-associated activation-induced cell death (AICD) [8,9], impaired antigen presentation [10,11], exposure to immunosuppressive cytokines [12], and perhaps to competition for limited amounts of cytokine growth factors. Recent in vivo studies from our laboratory showed that type 1 IFN can be a profound and universal factor inducing suppression of T cell proliferation during viral infections if the T cells are exposed to type 1 IFN prior to encountering their cognate ligand [13].

Efficient clonal expansion and differentiation of CD8 T cells is required to develop protective memory CD8 T cells. This requires three signals: a cognate peptide MHC-TCR interaction (signal 1), co-stimulation (signal 2), and infection-induced cytokines (signal 3) [14–16]. CD8 T cells that encounter antigen and co-stimulation undergo programmed cell division, but these two signals alone are not sufficient for full effector cell differentiation and survival into memory [14,17,18]. CD8 T cells need a third signal, provided by cytokines, including IL-12 or type 1 IFN, for efficient clonal expansion, differentiation into various effector populations, acquisition of cytolytic effector functions, and memory formation [15,19]. One in vitro study showed that without IL-12, CD8 T cells did not proliferate well or develop full effector function [20]. Type 1 IFN, however, can evidently substitute for IL-12 as a signal 3 cytokine [21,22].

Signal 3 cytokines are required for efficient clonal expansion in response to antigen, and the infecting pathogen and resulting inflammatory environment determine which cytokine(s) provide signal 3 activity [23–26]. LCMV-specific CD8 T cells use type 1 IFN as the signal 3 cytokine for effective primary T cell expansion [25,27,28], whereas Listeria and VSV depend on both type 1 IFN and IL-12 [23,25,29,30]. Studies showed that IFNαβ Receptor (R) KO LCMV-specific transgenic P14 CD8 T cells divided similarly to WT P14 cells but had reduced survival, thereby limiting their overall clonal expansion [27]. In other systems, the addition of adjuvants or IL-12 to activated CD8 T cells promoted their expansion by up-regulating the IκB family member BCL3, which was found to prolong T cell survival [31–33].

Signal 3 cytokines also play an important role in CD8 T cell differentiation into various phenotypic and functional effector
Author Summary

Vaccines are used to protect individuals against infection with a number of different pathogens and depend on the formation of antigen-specific memory cells. The efficacy of vaccines can be affected by a number of different factors. It has been known for some time now that suppression of the immune system occurs during acute viral infections. Thus, receiving a vaccine during an acute illness may reduce the efficacy of the vaccine administered. We have identified a common mechanism of immune suppression that may occur with many different pathogens that induce a particular inflammatory response. Any pathogen that induces type 1 interferon could potentially suppress the immune response to a subsequent pathological insult. The mechanism of immune suppression identified here was not having a direct negative effect on lymphocytes, but rather was inhibiting the cells ability to receive positive signals that influence their differentiation, expansion and memory formation. This desensitization mechanism may partially explain why vaccines function poorly in virus-infected individuals.

populations. Differences in CD8 T cell exposure to co-stimulatory molecules and cytokines can alter their differentiation into early effector cells (EECs), short-lived effector cells (SLECs) and memory precursor effector cells (MPECs) [17,25,34]. Recent studies investigating the role for signal 3 cytokines in primary CD8 T cell responses have shown that loss of IL-12R, IFNAR, or both receptors can alter CD8 T cell differentiation, showing reduced SLEC and increased MPEC formation [25,35]. Signal 3 cytokines have also been shown to augment the acquisition of CD8 T cell effector functions, including production of cytokines (IFNγ and TNF) and CTL activity. In vitro studies showed that without type 1 IFN or IL-12, CD8 T cells had decreased lytic ability and low levels of granzyme B expression [21,27,36]. Additional in vivo studies showed reduced granzyme B expression in IFNαβR KO transgenic P14 CD8 T cells compared to WT P14 CD8 T cells [27]. However, not all infection models show the same requirements for the specific signal 3 cytokines in driving these effector functions [25,27].

Type 1 IFN signaling is complex in that it can activate multiple downstream pathways, including the JAK/STAT pathway. Engagement of the type 1 IFN receptor promotes phosphorylation of downstream STAT molecules, including STAT1, 3, 4, and 5 [37]. The combination of STAT molecule(s) that are phosphorylated and translocated into the nucleus controls the outcome of CD8 T cell activation. Activation of STAT1 downstream of type 1 IFN receptor signaling generally has anti-proliferative effects on CD8 T cells [38,39]. In contrast, type 1 IFN-mediated activation of STAT3 and/or STAT5 has anti-apoptotic and pro-mitogenic effects [38,40,41]. Type 1 IFN signaling via STAT4 promotes both the acquisition of effector function, including IFNγ production, and clonal expansion [21,42]. Recent studies showed that during LCMV infection, virus-specific CD8 T cells had decreased total STAT1 levels and increased STAT4 levels, thereby promoting effector T cell differentiation and clonal expansion over anti-proliferative effects [38,43]. Thus, type 1 IFN can have both inhibitory and stimulatory effects on CD8 T cell proliferation, and when type 1 IFN provides signal 3 cytokine activity, it has positive effects on CD8 T cell expansion.

The timing of exposure of CD8 T cells to all 3 signals is very important, as T cells exposed to virus-induced inflammatory environments prior to cognate antigen respond differently to signals 1 and 2 compared to CD8 T cells from naive environments [13,44,45]. Under circumstances when CD8 T cells see antigen and co-stimulation prior to or at the same time as inflammatory cytokines, IL-12 or type 1 IFN have positive effects on T cell differentiation and expansion. However, CD8 T cells pre-exposed to virus-induced inflammatory environments showed reduced proliferation when exposed to cognate antigen [13,44]. Virus-induced impaired proliferation could be mimicked by the type 1 IFN-inducer poly(I:C). In this study, we utilized poly(I:C) to study the mechanism of IFN-mediated virus-induced T cell immune suppression. We sought to investigate whether the IFN-mediated suppression of CD8 T cells is due to type 1 IFN having direct suppressive effects on CD8 T cells or if it inhibits the positive effects IFN has on CD8 T cells. We show here that poly(I:C)-pretreated CD8 T cells are refractory to IFNβ signaling in terms of downstream STAT phosphorylation, suggesting that they are unable to receive positive effects that signal 3 cytokines normally provide during acute infections. Indeed, these out-of-sequence signal 3 CD8 T cells were found to behave more similar to 2-signal-only CD8 T cells rather than T cells that receive all 3 signals in the proper order. Therefore, the inability to respond to signal 3 cytokines limits CD8 T cell expansion and suggests a causative mechanism for reduced vaccine efficacy when administered during acute infections.

Results

Poly(I:C)-induced sensitization to impaired proliferation is transient and requires direct effects of type 1 IFN

Previously, we had shown that CD8 T cells exposed to exogenous cognate antigen 3–9 days, but not 12 days, after initiation of a virus infection proliferated poorly in response to a cognate antigen stimulus, and viruses that induced a strong type 1 IFN response had the greatest suppressive effects [13]. To investigate the mechanism of this virus-induced suppression of T cell proliferation, the IFN-inducer poly(I:C) was used to prime CD8 T cells. Congenic transgenic LCMV-specific P14 CD8 T cells were used here to study virus-specific T cells exposed to the IFN-inducer poly(I:C) prior to infection. As demonstrated in Figure 1A, Ly5.1 P14 cells were transferred into Ly5.2 B6 hosts that were inoculated with either HBSS or poly(I:C). One to three days later, splenocytes were isolated, and equal numbers of P14 cells (enumerated by flow cytometry staining) were transferred into recipients that were immediately infected with LCMV. Spleens from recipient mice were harvested at the peak of transgenic T cell expansion, day 7 post infection, and the proportion (Figure 1B and 1C) and total number (Figure 1D) of transgenic P14 cells were then determined. Suppression of proliferation of poly(I:C)-pretreated P14 cells (black bars) was greatest at 1 and 2 days of treatment compared to control treated cells (open bars). After 3 days of pre-treatment, clonal expansion of poly(I:C)-pretreated P14 cells was comparable to that of the HBSS-pretreated control P14 cells, indicating that poly(I:C)-mediated suppression of proliferation is a transient effect.

Because type 1 IFN is required for efficient clonal expansion of LCMV-specific CD8 T cells, we investigated its role in the reduced proliferation seen in poly(I:C)-pretreated CD8 T cells. We previously showed that impaired proliferation required direct effects of type 1 IFN acting on the T cell [13], and this is illustrated in Figure 1E, using a similar experimental set up as that described in Figure 1A. Congenic Thy1.1 IFNαβR KO P14 CD8 T cells were transferred into Thy1.2 mice that were inoculated with either HBSS or poly(I:C). One day later, equal numbers of transgenic T cells were transferred into mice prior to
LCMV infection. The percentage (Figure 1E) and total number (Figure 1F) of donor IFNαβR KO P14 cells in recipient host mice were determined at various times after LCMV infection. Poly(I:C)-pretreated IFNαβR KO P14 cells proliferated to similar numbers as the control-treated counterparts at both day 7 and 8 post LCMV infection. The fact that these CD8 T cells lacked expression of the IFNαβR and showed no difference in proliferation between HBSS- and poly(I:C)-primed groups suggested that there was a direct role for type 1 IFN on the CD8 T cells in this model of immune suppression.

Poly(I:C)-pretreated CD8 T cells are transiently refractory to IFNβ stimulation in terms of STAT phosphorylation

Knowing that type 1 IFN delivered at an optimal time can provide a positive signal 3 to CD8 T cells and enhance their proliferation, we questioned whether an out of sequence early exposure to IFN would interfere with later attempts at IFN signaling. Type 1 IFN can activate multiple downstream STAT molecules including STAT1, 3, 4, and 5. Because type 1 IFN can have both positive and negative effects on T cell expansion, recent studies have shown that the specific STAT(s) activated dictate the outcome, all of the aforementioned STAT molecules were tested. The phosphorylation of STAT molecules downstream of the type 1 IFN receptor was thus examined in CD8 T cells from mice pretreated with either HBSS or poly(I:C). Mice were inoculated with either HBSS or poly(I:C) for 1 day, and their splenocytes were isolated and stimulated 

Out-of-Sequence Signal 3 and Immune Suppression

To make sure that STAT molecules were available to be phosphorylated, total STAT protein levels in naive CD8 T cells after different days post HBSS or poly(I:C) inoculation were determined (Figure 3E–H). After 1, 2, and 3 days of treatment, total STAT1, 3, 4 and 5 levels in poly(I:C)-pretreated naive CD8 T cells were similar to, if not higher than, the control-treated cells. Total STAT1 expression was higher in poly(I:C)-pretreated naive CD8 T cells after 1 day and stayed high through day 3 of treatment, as compared to STAT1 levels in HBSS-treated CD8 T cells. Since STAT1 is an IFN-inducible gene [46], higher STAT1 protein expression in poly(I:C)-pretreated CD8 T cells was expected. These data indicate that the reduced phosphoSTAT staining found in IFNβ-stimulated poly(I:C)-pretreated CD8 T cells was not due to lower levels of total STAT protein.

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downstream phosphoSTATs. We tested IL-2, IL-6, IL-7, IL-12, and IL-15, because these cytokines have positive effects on T cell survival or proliferation. IL-2, IL-7, and IL-12 stimulation did not elicit positive phosphoSTAT staining in the control- or poly(I:C)-treated naive CD8 T cells (Figure S2). Of these cytokines tested, IL-6 and IL-15 were found to elicit positive phosphoSTAT staining in HBSS-treated naive CD8 T cells (Figure S2). However, unlike poly(I:C)-pretreated naive CD8 T cells stimulated with IFNβ, poly(I:C)-pretreated naive CD8 T cells stimulated with IL-6 (black bars, Figure 4A) or IL-15 (black bars, Figure 4B) responded just as well, in terms of phosphorylating downstream STAT3 and STAT5, respectively, as their control-treated counterparts. Similar to naive T cells, CD44hi CD8 T cells also phosphorylated downstream STAT3 and STAT5 in response to IL-6 and IL-15 stimulation (respectively) from both the HBSS- and poly(I:C)-treated groups (Figure S3). Together, these data indicate that poly(I:C) treatment did not make P14 CD8 T cells universally unresponsive to all cytokines; rather, the impairment was instead more specific to type 1 IFN. These data suggest that poly(I:C)-pretreated CD8 T cells, when put into hosts subsequently infected with LCMV, are not able to respond to the type 1 IFN induced by the virus, and thus are unable to receive positive signal 3 cytokine signals.

Figure 2. Poly(I:C)-pretreated CD8 T cells are refractory to IFNβ stimulation in terms of STAT phosphorylation. Mice were HBSS- or poly(I:C)-treated for 1 day. Splenocytes were isolated and either unstimulated, or stimulated ex vivo with IFNβ for 30 min and then stained for (B) pSTAT1, (C) pSTAT3, (D) pSTAT4, and (E) pSTAT5. (A) shows representative histograms gated on CD44lo CD8α+ (naïve) T cells showing pSTAT1, 3, 4 or 5 staining in HBSS- or poly(I:C)-treated naive CD8 T cells unstimulated (shaded histograms) or IFNβ stimulated (open histograms). HBSS-pretreated naive CD8 T cells stimulated with IFNβ shown as solid line histograms, and poly(I:C)-pretreated naive CD8 T cells stimulated with IFNβ shown as dotted line histogram. (B–E) show pSTAT MFI of naive T cells from unstimulated vs. IFNβ stimulated cells HBSS (open bars) or poly(I:C) (black bars) pretreated for 1 day. Data are representative of at least 4 independent experiments with n of 3 mice per group.
Poly(I:C)-pretreated CD8 T cells have decreased IFNAR1 and increased SOCS1 expression

Cytokine signaling must be tightly regulated in order to prevent over-active and prolonged immune activation. A number of different mechanisms are in place to limit cytokine signaling, including reducing cytokine receptor expression, down-regulating expression of signaling protein components, and up-regulating the expression of suppressors of cytokine signaling proteins (SOCS) [47,48]. Reduced STAT protein levels did not account for the refractoriness to IFNβ stimulation seen in the poly(I:C)-pretreated CD8 T cells (Figure 3). To investigate why naive CD8 T cells pre-exposed to poly(I:C) were unresponsive to type 1 IFN, but not all cytokines, cytokine receptor expression was determined. At various days post HBSS or poly(I:C) treatment, naive CD8 T cells were assessed for cytokine receptor signaling components, including portions of the IL-2, IL-6, IL-7, and IL-15 complexes, and these are represented in Figure 4A. The type 1 IFN receptor is comprised of two components, IFNAR1 and IFNAR2 [37]. Naive CD8 T cells from mice inoculated with poly(I:C) for one day had much lower expression levels of IFNAR1, compared to the HBSS-treated cells (Figure 4A–4B). However, IFNAR1 expression levels returned to control-treated levels by 2 days post treatment. The CD44hi CD8 T cells had similar kinetics of IFNAR1 expression as the naive CD8 T cells, showing slightly reduced receptor expression with 1 day treatment of poly(I:C) but not 2 or 3 days of treatment (Figure 4B). Thus, unresponsiveness to type 1 IFN at day 1 correlated with the lack of expression of the IFN receptor.

Since poly(I:C)-pretreated CD8 T cells were still less responsive to IFNβ stimulation, as measured by STAT phosphorylation, 2 days after poly(I:C) treatment (Figure 3), there were likely other suppressive mechanisms to limit IFNβ responsiveness, in addition to the reduced receptor expression shown in Figure 4A and B. SOCS proteins are known to inhibit cytokine receptor signaling by acting at many different steps in the JAK/STAT signaling pathway [47]. SOCS1 inhibits type 1 IFN signaling by binding to the receptor-associated JAK protein TYK2, thus blunting IFN receptor signaling [49]. Naive CD44lo CD8 T cells that were sorted from mice inoculated with HBSS or poly(I:C) for 1 day showed a 7-fold relative increase in SOCS1 message expression after poly(I:C) treatment. We next utilized the well-established protocol for staining for phosphorylated proteins to identify intracellular levels of SOCS1. SOCS1 expression was determined in naive CD8 T cells from poly(I:C)- or HBSS-inoculated mice 1, 2, or 3 days after treatment (Figure 4C–4D). Indeed, at both 1 and 2 days after poly(I:C) treatment, naive CD8 T cells had higher expression of SOCS1 compared to the control-treated cells. However, by 3 days of pretreatment, there was no longer a significant difference in SOCS1 expression between control and poly(I:C)-treated CD8 T cells. CD44hi CD8 T cells also showed increased SOCS1 protein levels (Figure 4E). This suggests that a combination of a decrease in IFNβR expression and an increase in SOCS1 expression may account for the observed refractoriness to IFNβ stimulation. These results correlated kinetically with refractoriness to IFNβ stimulation (Figure 3) and to the suppressed proliferation seen in poly(I:C)-pretreated CD8 T cells (Figure 1). These experiments do not definitively parse out the relative contributions of decreased receptor expression vs. inhibitory molecule contribution to T cell refractoriness to IFN stimulation, but the results are very consistent with previous work in more tractable systems studying the mechanism of unresponsiveness to IFN [49–51]. Further, they clearly show that this unresponsiveness is not due to decreases in overall STAT protein expression.

Poly(I:C)-pretreated CD8 T cells behave similarly to 2-signal-stimulated rather than 3-signal-stimulated CD8 T cells

Because the suppression of proliferation of poly(I:C)-pretreated CD8 T cells correlated with refractoriness to IFNβ stimulation, we...
hypothesized that poly(I:C)-pretreated CD8 T cells were unable to receive the positive effects that type 1 IFN exerts as a signal 3 cytokine when delivered in the proper sequence. This hypothesis would suggest that poly(I:C)-pretreated P14 cells would behave similarly to 2-signal only CD8 T cells, rather than 3-signal CD8 T cells. Thus, we examined their effector phenotype and their abilities to divide, produce cytokines, degranulate, and express the survival protein BCL3 in response to antigen exposure.

Studies have shown that 2-signal CD8 T cells divide similarly to 3-signal CD8 T cells but have defects in survival [27]. We therefore tested whether the impairment in proliferation seen in poly(I:C)-pretreated CD8 T cells was due to division or survival defects. A similar experimental setup as shown in Figure 1A was used, but after inoculation with HBSS or poly(I:C), congenic P14 CD8 T cells were labeled with CellTrace violet and transferred together into the same recipients to track cell division during LCMV infection. Cells were harvested at days 3 and 4 post infection, and CellTrace violet dilution was measured. Because we were looking at early days post infection, a larger number of transgenic cells was transferred than what would normally be considered physiologically relevant in order to quantify early cell division. Neither the poly(I:C)- nor the HBSS-pretreated P14 cells diluted CellTrace violet, indicating that they did not divide (Figure 6A). At day 3 post infection, the control-treated P14 cells diluted more CellTrace violet, indicating they had undergone more cell divisions compared to the poly(I:C)-pretreated P14 cells (Figure 6A–C). However, by day 4 post LCMV infection, the division profiles of both HBSS- and poly(I:C)-pretreated P14 cells appeared similar. Although the percentage of cells that divided was statistically similar between the two groups (Figure 6B), the proliferation index of poly(I:C)-pretreated P14 cells was lower compared to HBSS-treated cells (Figure 6C). The proliferation index represents the average number of divisions of the cells that have undergone at least one division. These results show that poly(I:C)-pretreated CD8 T cells have a delay in the number of cell divisions.

If a delay in cell division were the only thing contributing to the suppression of proliferation, at later time points post infection the expansion of poly(I:C)-pretreated CD8 T cells might eventually reach the same level as the control-treated cells. Therefore, a time course of HBSS- or poly(I:C)-pretreated P14 CD8 T cell expansion in response to LCMV infection was performed. The peak of expansion of poly(I:C)-pretreated P14 CD8 T cells was delayed (day 9) compared to HBSS-pretreated P14 CD8 T cells (day 7), but the magnitude of the response was still reduced in the poly(I:C)-pretreated cells (Figure 6D). In addition, out-of-sequence P14 cells showed decreased memory frequencies (Figure 6E) and number (data not shown) compared to their control

Figure 5. Poly(I:C)-pretreated CD8 T cells have decreased type 1 IFN receptor expression and increased SOCS1 expression. Mice were HBSS- (open bars) or poly(I:C)- (black bars) treated for 1, 2, or 3 days. Splenocytes were isolated and stained for type 1 IFN receptor expression (A–B) or SOCS1 expression (C–D). (A) Representative histograms showing IFNAR1, gating on CD44lo CD8a+ T cells and IFNAR1 MFI (B). (C) Representative histograms of SOCS1 expression, gating on CD44lo CD8a+ T cells. (D) SOCS1 MFI in poly(I:C)- or HBSS-treated cells after 1, 2, or 3 days of pretreatment. Data are representative of 2 independent experiments with n of 3 mice per group.

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treated counterparts at multiple time points tested. This suggests that the defects in clonal expansion were not solely due to a delay in cell division but may also be due to other factors such as defects in cell survival. In vitro and in vivo studies by others found that survival of activated T cells in response to signal 3 cytokines and adjuvants was in part due to an increase in the IκB family member BCL3 and that cells lacking signal 3 cytokines have reduced expression of BCL3 [31–33]. To determine if the poly(I:C)-treated virus-stimulated T cells resembled two signal only T cells in this respect, the expression of BCL3 was thus determined in HBSS-control or poly(I:C)-pretreated P14 CD8 T cells after LCMV infection. Indeed, a lower percent of poly(I:C)-pretreated P14 cells compared to the HBSS-pretreated P14 T cells was lower than in HBSS-control or poly(I:C)-pretreated P14 CD8 T cells after LCMV infection. Additionally, a lower percent of poly(I:C)-pretreated P14 cells up-regulated BCL3 than HBSS-pretreated P14 cells at days 4, 5 and 6 post LCMV infection (Figure 6F). Combined, these results showed that similar to 2-signal CD8 T cells, out-of-sequence signal 3-stimulated P14 CD8 T cells had a delay in cell division when compared to CD8 T cells that receive all 3 signals in the correct order, and that they had defects in a survival protein that may limit the ability of these cells to clonally expand.

To further support the hypothesis that out-of-sequence signal 3 CD8 T cells do not receive the positive effects that type 1 IFN can have as a signal 3 cytokine during acute virus infection, and thereby contribute to suppression of proliferation, we determined the frequency of poly(I:C)- or HBSS-pretreated P14 cells after cognate peptide stimulation. Cogenic P14 mice were directly stimulated with HBSS or poly(I:C) for 1 day, and their splenocytes were transferred together into the same recipients that remained uninfected, that received 5 μg 13mer GP33.45 peptide, or that were infected with LCMV. The appropriate naive control for was used to calculate the fold expansion. Data are representative of 2 individual experiments with n of 4–6 per group (A–C), one experiment with n of 5 per group (D), 3 individual experiments with n of 4–5 per group (E), and one experiment with n of 3–5 per group (F–G). Data were combined from 3 individual experiments, each with 2 time points tested with n of 5 mice per group (H). doi:10.1371/journal.ppat.1004357.g006
Out-of-Sequence Signal 3 and Immune Suppression

Discussion

Transient states of immune suppression occur during many acute viral infections, and it has long been known that individuals should not get vaccinated when they are sick. Virus-induced immune suppression was first noted over 100 years ago [1], and more recent studies have shown it to be a common element of many viral infections and often be associated with suppressed T cell proliferation in response to antigens and mitogens. In vitro studies had suggested that AICD contributed to this inhibition of T cell proliferation [2,9,37], and other studies implicated impaired antigen-presenting cell function [10,11,30], induction of immunosuppressive cytokines like IL-10, [12], and perhaps the competition for T cell growth factors could play a role. While studying viral infection models, we recently found that a general mechanism of virus-induced immune suppression could be linked to type 1 IFN, normally induced at high quantities in most viral infections [13]. This was somewhat surprising, given that type 1 IFN has been described as a signal 3 cytokine, which drives the expansion and differentiation of T cells after they have encountered cognate ligand (signal 1) and co-stimulation (signal 2). The primary observation of the present report is that if T cells are exposed to type 1 IFN inducers before exposure to cognate ligand, they lose their sensitivity to further IFN stimulation and do not receive the benefits of a signal 3 cytokine. Instead, they behave like T cells receiving only two signals, with defects in effector cell differentiation, reduced effector function, lower expression of a pro-survival protein, and limited clonal expansion.

Dating back to the early days of IFN therapy in humans, it has long been known that lymphocytes like NK cells become hyporesponsive to treatment, and IFN, like many other cytokines, can render treated cells resistant to further IFN stimulation by down regulating the IFN receptor and by inducing factors like SOCS1 that impair IFN-induced signal transduction [47,48]. We show here that this is the case with virus-specific T cells, and that these T cells pre-exposed to IFN fail to drive the benefit of the positive signal 3 effects of IFN signaling. The implications of this phenomenon are widespread. Because IFN is induced so rapidly during viral infections, one can deduce that the T cells that encounter antigen in the first day or two of infection would respond more impressively than “late-comer” virus-specific T cells stimulated later in infection. Thus, the dynamics of how much antigen is synthesized and presented vs. how much and how quickly IFN is induced may dictate the efficacy of the host response. Secondly, the T cell response to many acute infections, at least in mouse models, is relatively ordered and undergoes a rather synchronized contraction from 6-9 days post-infection. How can this occur when the amount of T cell proliferation is a programmed event [59-62] and when different T cells should encounter antigen at different time periods? We would argue that the late-comer T cells, because of their previous exposure to IFN, would not undergo as many divisions and possibly have lower

cells stained to a similar extent, if not slightly more, than HBSS-treated cells in response to GP33 peptide stimulation (Figure 7E–7F). However, poly(I:C)-pretreated P14 CD8 T cells had substantially lower levels of granzyme B expression than control-treated cells at day 5 post infection (Figure 7E). Reduced granzyme B expression in the out-of-sequence CD8 T cells was seen as early as day 4 post infection and lasted at least until day 6 post infection (Figure 7G). KLRL1 expression in CD8 T cells is considered a marker for effector function, and there was a positive correlation between KLRL1 expression and granzyme B expression (R square = 0.8563, p<0.0001) (Figure 7H). Since granzyme B expression has been used as a correlative marker for cytotoxic capability [36], this suggests that poly(I:C)-pretreated CD8 T cells have reduced cytolytic function compared to HBSS-treated CD8 T cells. These data showing similar cytotoxic production but reduced granzyme B expression are consistent with published phenotypes for CD8 T cells that only receive 2 signals [25,27].

A new method to study effector cell function is the trogocytosis assay, whereby target cells are labeled with a membrane dye, mixed with cytotoxic effector cells for 1 hr, and then examined for the transfer of dye to a flow cytometry-defined effector cell population [53,54]. This is an indicator of how aggressively the effector cells are attacking the targets. Figure S6 shows that the poly(I:C)-pretreated P14 cells, 5 days after LCMV infection, had modest but statistically significant reduced ability to acquire the dye from GP33-pulsed RMA cells, when compared to the HBSS-pretreated donor T cells. Reduced incorporation of the lipid dye is an indicator of reduced effector cell function [55,56].

We tested whether these donor poly(I:C)-pretreated T cell responses, which were dramatically reduced in number and modestly reduced in effector function, would affect viral load differently than that in mice receiving HBSS-treated cells. Mice receiving either HBSS-pretreated or poly(I:C)-pretreated P14 CD8 T cells were subsequently infected with LCMV, and viral titers were examined at different time points post infection. As early as day 4 post infection, mice receiving poly(I:C)-pretreated P14 cells had modest but statistically significant increased viral titers compared to mice receiving control treated P14 cells in the fat pad (4.7±0.1 vs. 4.3±0.08 log pfu, two independent experiments combined for n = 10 per group) respectively (p = 0.0082). In addition, at day 6 post infection, there was a modest but significant increase in viral load in the spleen and liver in mice receiving poly(I:C)-pretreated P14 CD8 T cells (4.2±0.07 log pfu in the spleen, 4.2±0.08 log pfu in the liver) compared to mice receiving HBSS-pretreated P14 cells (3.8±0.12 log pfu in the spleen, 3.8±0.08 log pfu in the liver) (3 independent experiments combined for n = 14–15, p = 0.0197 (spleen) and p = 0.0074 (liver)). These differences in viral titer are admitted small, but they are statistically significant and occur in environments where normal endogenous host T cell responses are simultaneously occurring.

Figure 7. Poly(I:C)-pretreated P14 CD8 T cells have reduced effector function. (A–B, E–H) WT or IFNαR KO P14 cells were transferred into congenic mice that were inoculated with HBSS or poly(I:C), and 1 day later equal numbers of P14 cells were transferred into separate congenic mice subsequently infected with LCMV. (C–D) P14 mice were directly treated with poly(I:C) or HBSS for 1 day prior to transfer into congenic mice that were infected with LCMV. Cells were harvested at various days post infection (day 4, 5, 6, or 7). (A–C) Cells were unstimulated or (H–I) stimulated ex vivo with GP33 peptide. (A) Representative flow cytometry plots gating on IFNαR KO P14 cells (Vv2+Thy1.1+ CD8+) or WT P14 cells (Vv2+Ly5.1+CD8+) showing KLRL1 and CD127 expression at day 7 p.i. (gates drawn using naive CD8 T cells). (B) Percent of WT donor P14 cells that express the SLEC phenotype (KLRL1hi/CD127lo) at different days post infection. (C) Representative flow cytometry plots gating on P14 cells (Vv2+Ly5.1+CD8+) showing TNF and IFNγ production at day 5 p.i. (D) Frequency of HBSS- (open bars) or poly(I:C)- (black bars) pretreated P14 CD8 T cells that produce both TNF and IFNγ. (E) Representative histograms for either CD107a/b or granzyme B staining in poly(I:C)- (open solid lines) or HBSS- (shaded dashed lines) pretreated P14 CD8 T cells. Total frequency of HBSS- (open bars) or poly(I:C)- (black bars) P14 cells producing CD107a/b (F) or MFp of granzyme B at different time points post infection. (G) Correlation between frequency of granzyme B+ P14 cells and KLRL1+ P14 cells at day 5 post infection. Data are representative of at least 2 individual experiments with n of 3-5 mice per group.

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survival properties, thereby enabling them to contract when the rest of the T cells do. Third, we would argue that naïve or memory T cells specific to third party antigens would not respond well to a cognate antigen stimulus if they were first exposed to the IFN milieu of a viral infection and then stimulated with antigen. This failure to respond to recall antigens, such as tetanus toxoid or tuberculin, is a common feature of virus-induced immune suppression in humans, and the weak efficacy of vaccines in already infected individuals may well be a function of the same problem [63–65]. Finally, under conditions when a host develops a persistent viral infection there would be a chronic stimulation of the type 1 IFN response, and such hosts would probably not immunologically respond well to either the antigens of the infecting virus or to third party antigens on challenge. This weak response to third party antigens is not only seen during persistent viral infections but also during chronic autoimmune diseases, such as lupus erythematosus, where signal 3 cytokines may be chronically produced [66,67]. We therefore suggest that the elimination of signal 3 stimulation by out-of-sequence exposure to the signal 3 stimulant, in this case IFN, would be a common factor disrupting T cell responses in the context of acute or persistent viral infections.

This generalized IFN-induced impairment of proliferation is one example of how out-of-sequence signaling can alter responses to cognate antigen exposure. On the other hand, virus-induced inflammatory environments can alter the response of bystander CD8 T cells not specific for the infecting virus to third party cognate antigens by driving the T cells down a different differentiation pathway [45]. Our previous studies showed that transgenic CD8 T cells exposed to virus-induced inflammatory environments were sensitized to undergo rapid effector function such that upon stimulation with cognate antigen they produced cytokines including granzyme B and IFNγ within a few hours and without a need for cell division. The sensitization to rapid effector function was most dramatic with viruses that induced a strong type 1 IFN response, and this event could also be induced by poly(I:C). We do not know if the CD8 T cells sensitized to rapid effector function are in fact the same cells that ultimately are suppressed in proliferation. However, we do know that these two changes in T cell response to cognate antigen stimulation occur by very distinct mechanisms and occur in virus-induced inflammatory environments; consequently, the impairment of proliferation may contribute to generalized IFN-induced immune suppression, even though there may be an initial transient activation of the T cells.

Type 1 IFN can have both stimulatory and inhibitory effects on CD8 T cell proliferation, but here it was initially unclear if poly(I:C)-pretreated CD8 T cells were receiving direct inhibitory signals or fewer stimulatory signals from IFN. Since type 1 IFN response, and this event could also be induced by poly(I:C). We do not know if the CD8 T cells sensitized to rapid effector function are in fact the same cells that ultimately are suppressed in proliferation. However, we do know that these two changes in T cell response to cognate antigen stimulation occur by very distinct mechanisms and occur in virus-induced inflammatory environments; consequently, the impairment of proliferation may contribute to generalized IFN-induced immune suppression, even though there may be an initial transient activation of the T cells.

Type 1 IFN can have both stimulatory and inhibitory effects on CD8 T cell proliferation, but here it was initially unclear if poly(I:C)-pretreated CD8 T cells were receiving direct inhibitory signals or fewer stimulatory signals from IFN. Since type 1 IFN signaling can act through multiple STATs, each capable of altering cell fate, it might have been expected that poly(I:C)-pretreated CD8 T cells would have different STAT phosphorylation in response to IFNβ stimulation. Recent work has shown that virus-specific CD8 T cells down-regulate type 1 STATs and up-regulate STAT4, so that when IFN signals through cognate antigen they produced cytokines including granzyme B and IFNγ within a few hours and without a need for cell division. The sensitization to rapid effector function was most dramatic with viruses that induced a strong type 1 IFN response, and this event could also be induced by poly(I:C). We do not know if the CD8 T cells sensitized to rapid effector function are in fact the same cells that ultimately are suppressed in proliferation. However, we do know that these two changes in T cell response to cognate antigen stimulation occur by very distinct mechanisms and occur in virus-induced inflammatory environments; consequently, the impairment of proliferation may contribute to generalized IFN-induced immune suppression, even though there may be an initial transient activation of the T cells.

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Out of Sequence Signal 3 and Immune Suppression

To summarize, if CD8 T cells see signal 3 first, they become refractory to further IFN stimulation and are unable to receive the positive signals that type 1 IFN can provide when delivered at the proper time after antigen and co-stimulation. This limits their ability to clonally expand, to sustain cytolytic capabilities, and form memory. Our studies show lower proportions and numbers of out-of-sequence CD8 T cells at different stages of memory formation (Figure 6E), including as late as 11 weeks post infection. Preliminary data show that the out-of-sequence CD8 T cells that do form memory are able produce similar proportions of cytokines, when stimulated ex vivo, compared to memory cells from the control environment (data not shown), but the effectiveness of these memory cells has not been further investigated. The efficacy of an out-of-sequence CD8 T cell memory response to secondary challenge is important to study but is beyond the scope of the paper, whose focus was to examine why IFN causes naive T cells to function poorly during the context of an acute viral infection. Thus, under circumstances when CD8 T cells can receive positive signals, such as during an infection or vaccination with adjuvants, out-of-sequence signals can have a profound effect on CD8 T cell expansion and activation. This out-of-sequence inhibition of T cell proliferation may account for the more general immune suppression seen in many acute virus infections known to induce type 1 IFN. This mechanism of CD8 T cell suppression would be expected to contribute to the reduced efficacy of vaccines when they are administered during an acute infection.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee, Docket # A-305, Animal Welfare Assurance Number A-3306-01.

Mice

C57BL/6 [Ly5.2+] male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Ly5.1 and Thy1.1 P14 [73] TCR-transgenic mice were bred in the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS). The P14 transgenic mice were bred onto the B6. IFNβ−/− KO background to generate P14 CD8 T cells that lacked IFNβ [13,74].

Virus stocks and peptides

Lymphocytic choriomeningitis virus (LCMV), strain Armstrong, was propagated in baby hamster kidney (BHK21) cells, as previously described [75,76]. Mice were injected intraperitoneally (i.p.) with 5 × 10^4 pfu of LCMV. Organ homogenate viral titers were determined by plaque assay using Vero cells. To activate P14 virus stocks and peptides

Mice

B6.IFNβ−/− [176], Ly5.1 and Thy1.1 P14 [13,74] were propagated in baby hamster kidney (BHK21) cells, as previously described [75,76]. Mice were injected intraperitoneally (i.p.) with 5 × 10^4 pfu of LCMV. Organ homogenate viral titers were determined by plaque assay using Vero cells. To activate P14

out-of-sequence CD8 T cells

P14 transgenic mice were inoculated with HBSS or poly(I:C) i.p. for various times, after which, the same protocol was used as the dual transfer method to transfer in equal numbers of P14 transgenic T cells. In experiments where control- and poly(I:C)-treated transgenic P14 cells were transferred into the same recipients subsequently infected with LCMV, a total of 10,000 P14 cells were transferred. In experiments where HBSS- and poly(I:C)-treated P14 cells were transferred into the same recipients receiving 13-mer GP 33–45 peptide, −2–4 × 10^5 P14 cells were transferred i.v. This higher amount was necessary for the peptide-stimulated cells to be detected. Prior to any adoptive transfer, single cell suspensions were prepared by lysing red blood cells with 0.84% NH_4Cl solution and washing with HBSS.

Where described, cells were labeled with 5 μM CellTrace Violet (Invitrogen) by incubating at 37°C for 15 min. Cells were then washed with HBSS at least 2 times prior to adoptive transfer. A larger number of transgenic P14 cells (~1 × 10^6 per group) were transferred into hosts to identify virus-specific cells early after LCMV infection.

Surface and intracellular cytokine staining

Spleen leukocytes were stained with a combination of fluorescently labeled monoclonal antibodies (MAb) specific for CD8b (53-6.7), Vα2 TCR (B20.1), Ly5.1 (A20), Thy1.1 (HS51), CD44 (IM7) KLRG1 (2F1), CD127 (A7R34), and IFNAR1 (MAR1-5A3) for 20 min at 4°C. Intracellular cytokine staining was performed as described previously [45]. Briefly, spleen leukocytes (2–4 × 10^6) were plated with or without 5 μM synthetic peptide stimulation in the presence of GolgiPlug (BD Pharmingen) and human rIL-2 for 4 to 5 hours at 37°C. After stimulation, cells were washed in Flow Cytometry Buffer (2% FCS in HBSS), blocked with r-FeR (2.4G2) and stained with a combination of fluorencently labeled mAbs listed above. After surface staining, spleen leukocytes were fixed and permeabilized with Cytofix/Cytoperm (BD Bioscience) for 20 min at 4°C and then stained with a combination of fluorescently labeled MAbs specific for TNF (MP6-XT22), IFNγ (XMG1.2), and Granzyme B (GB11, Invitrogen). To identify cells undergoing Ag-induced degranulation, splenocytes were stimulated as stated above with addition of CD107a (1D4B) and CD107b (ABL-93). All MAbs were purchased from eBioscience, SanDiego, CA, BioLegend, San Diego, CA, or BD Bioscience, San Diego, CA, unless otherwise noted.

Freshly stained and previously fixed samples were acquired using a BD Bioscience LSR II flow cytometer with FACS Diva software. Data were analyzed with FlowJo software (Tree Star Technologies, Ashland, OR).
irrelevant peptide, K3L (YSLPNAGDVI) or pulsed with a specific peptide, GP33 (KAVYNFATC), at 1 μM for ~90–120 minutes at 37°C. After incubation, target cells were labeled with fluorescent lipids SP-DiIC6(3) (Molecular probes) and diluted in Diluent C (Sigma Aldrich) using the protocol adapted from Daubeuf S, et al [54]. Target cells (~7×10^6) were co-cultured with effector cells (1.5×10^6 total splenocytes) per well for 1 hour at 37°C. Cells were stained with surface antibodies of interest, and samples were acquired using a BD Bioscience LSR II flow cytometry with FACS Diva software. Data were analyzed with FlowJo software.

Statistical analyses
Where appropriate, Students t test and linear regression were calculated using GraphPad InStat software. Significance was set at a P value of 0.05; * indicates a P of <0.05, ** a P of <0.01, *** a P of <0.001, and **** a P of <0.0001. All results are expressed as means of +/- standard deviations.

Supporting Information
Figure S1 Poly(I:C)-pretreated memory phenotype CD8 T cells are refractory to IFNβ-induced STAT phosphorylation. As described in Figure 2 and Materials and Methods, mice were HBSS (open bars) or poly(I:C) (black bars) treated for 1 day. Splenocytes were isolated and were either unstimulated, or stimulated ex vivo with IFNβ for 30 min and then stained for (A) pSTAT1, (B) pSTAT3, (C), pSTAT4, and (D) pSTAT5. Cells were gated on CD44hi CD8+ lymphocytes, and the MFI of each respective pSTAT is graphed. Data are representative of at least 4 independent experiments with n of 3 mice per group. Statistical analysis is described in Materials and Methods section.

EPS

Figure S2 Naïve CD44lo CD8 T cells do not phosphorylate downstream STATs in response to other cytokines. As described in Figure 4 and Materials and Methods, mice were HBSS (open bars) or poly(I:C) (black bars) treated for 1 day. Splenocytes were isolated and were unstimulated, stimulated with IFNβ, IL-2, IL-7, or IL-12 for 30 min and stained for appropriate downstream STAT molecules (A–B) pSTAT3 MFI, and (C) pSTAT4 MFI. Splenocytes were gated on CD44hi CD8+ lymphocytes. (A) responsiveness to IFNβ and IL-2, (B) responsiveness to IFNβ and IL-7, and (C) responsiveness to IFNβ and IL-12. Data are representative of at least 2 independent experiments with n of 3 mice per group.

EPS

Figure S3 CD44hi CD8 T cells respond to some cytokines after 1 day of poly(I:C) treatment. As described in Figure 4, mice were inoculated with HBSS (open bars) or poly(I:C) (black bars) for 1 day. Splenocytes were isolated and either unstimulated or stimulated with IL-6 (A), or IL-15 (B) and stained for downstream pSTAT3 (A) or pSTAT5 (B). Splenocytes were gated on CD44hi CD8+ lymphocytes and plotted for pSTAT MFI. Data are representative of at least 2 independent experiments with n of 3 mice per group.

EPS

Figure S4 Cytokine receptor expression after 1, 2, or 3 days of poly(I:C)-pretreatment. As described in Figure 5, mice were given one dose of HBSS (open bars) or poly(I:C) (black bars) and harvested at 1, 2, or 3 days after the inoculation. Cytokine receptor expression was determined on the CD44lo CD8+ T cells. The MFI is plotted for (A) CD25, (B) CD122, (C) CD126, (D) CD127, and (E) CD132. Cytokine receptors tested include IL-2 (A–B, E), IL-6 (C), IL-7 (D–E) and IL-15 (B, E). Data
are representative of 2 independent experiments with n of 3 mice per group. (EPS)

Figure S5 Memory phenotype CD8 T cells increase SOCS1 expression after poly(I:C) treatment. As described in Figure 5, mice were given one dose of HBSS (open bars) or poly(I:C) (black bars) and harvested at 1, 2, or 3 days after the inoculation. Splenocytes were gated on CD44hi CD8α+ T cells showing MFI of (A) IFNAR1 and (B) SOCS1. Data are representative of 2 independent experiments with n of 3 mice per group. (EPS)

Figure S6 Trogocytosis capability of HBSS- and poly(I:C)-pretreated P14 cells co-cultured with GP33 pulsed RMA cells. As described in the Materials and Methods section, a trogocytosis assay was performed using day 5 HBSS- or poly(I:C)-pretreated P14 CD8 T cells as effectors and RMA cells pulsed with peptides as targets. Effectors were developed by poly(I:C) or HBSS treating a P14 transgenic mouse, transferring ~10,000 P14 cells from each group into separate mice 1 day after treatment and infecting the recipient mice with LCMV. At day 5 post infection, splenocytes containing the donor P14 CD8 T cells were isolated and used as effectors. Target cells were RMA cells that were not pulsed with peptide (no peptide), pulsed with an irrelevant peptide (K3L), or pulsed with the specific peptide (GP33). Target cells were labeled with fluorescent lipid molecule SP-DiIC18(3) that can be detected if it is transferred to a different cell through trogocytosis. Target cells were in excess and were co-incubated with effectors for 1 hour, stained with surface antibodies and ran on a flow cytometer. (A) shows representative FACs plots gated on donor P14 cells that were HBSS or poly(I:C) pretreated co-incubated with 1. No targets, 2. No peptide pulsed targets, 3. K3L pulsed targets, or 4. GP33 pulsed targets, looking at P14 cell incorporation of SP-DiIC18(3). Data are representative of 2 independent experiments with n of 3–5 mice per group. (B) MFI of SP-DiIC18(3) gated on donor P14 cells, normalized to HBSS control for K3L and GP33 pulsed targets. HBSS pretreated P14 cells are in the open bars and poly(I:C)-pretreated P14 cells represented as black bars. Data are combined from 2 independent experiments with a total n of 6 mice per group. (EPS)

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Author Contributions
Conceived and designed the experiments: SLU RMW. Performed the experiments: SLU. Analyzed the data: SLU RMW. Contributed reagents/materials/analysis tools: RMW. Wrote the paper: SLU RMW.

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