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Exhaustion of Type I Interferon Response following an Acute Viral Infection

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Viral infections often cause a period of heightened susceptibility to a secondary infection but the cause of this phenomenon is unknown. We found that a primary viral infection in mice rapidly triggers an IFN-I-dependent partial activation state in the majority of B and T lymphocytes, which reverts to a resting phenotype within 5 days. When a secondary infection with an unrelated virus occurred 5 to 9 days after the primary infection, no recurrence of marked activation of lymphocytes was observed. This was not due to an inherent inability of the previously activated cells to undergo renewed partial activation, because they responded when challenged with virus after transfer into “naive” recipients. Instead, the failure to respond optimally resided in the original host’s incapacity to mount an IFN-I response to the secondary infection during this time period. Thus, transient immunosuppression through exhaustion of IFN-I production during an acute viral infection creates a time period of enhanced susceptibility to secondary infection. The Journal of Immunology, 2006, 177: 3235–3241.

The mammalian host responds to viral infections with a rapid nonspecific innate followed by the specific adaptive immune response. One of the first reactions to a viral infection is the induction of type I IFN (IFN-I) to provide the first line of defense by inducing an anti-viral state to prevent viral replication and to provide signals to modulate the adaptive immune response (1). Mice lacking functional IFN-I receptors are extremely susceptible to a wide variety of viral infections (2–5). These mice are still able to produce and secrete IFN-I following a viral infection; however, they are devoid of auto and paracrine IFN-I signaling due to the lack of a functional IFN-I receptor, a property of the IFN response that appears to be crucial for recovery from most viral infections. Although all virus infected cells can produce IFN-I within hours of infection, immature dendritic cells (DCs),2 particularly plasmacytoid DCs (pDCs), produce 1000-fold higher IFN-I levels than other cell types and are thought to be responsible for systemic IFN-I responses to many viruses (6). Besides the induction of the anti-viral state, IFN-I influences a plethora of anti-viral immune responses, from Ag presentation, induction of cytokines, T cell activation, clonal expansion, and T cell memory formation (7–13).

We have previously reported (14) massive generalized lymphocyte activation marker CD69 and CD86, but not CD25, expression on the vast majority of B and T lymphocytes during infection with the alphavirus, Semliki Forest virus (SFV). This up-regulation of CD69 and CD86 was IFN-I mediated. The percentages of lymphocytes expressing activation markers decreased with time to reach background levels at 5 days after infection (14). The precise function of CD69 is at present not known with recent data suggesting an immune regulatory function (15). CD86 is a cell surface molecule with costimulatory activity for T cells by its interaction with CD28 (16, 17). The biological relevance and function of such a systemic, partial activation of most of the host’s lymphocytes, irrespective of Ag reactivity, is at present not known.

In this study, we report our investigations on the fate of these activated lymphocytes and their responsiveness to subsequent heterologous viral challenges.
Flow cytometric analysis

Spleens from infected and control mice were harvested and RBC-depleted single-cell suspensions were prepared. Lymphocytes (1 × 10^7) were stained using fluorescent-conjugated anti-CD3, -CD4, -CD8, -B220, and CD11c Abs (BD Pharmingen). Expression of activation markers was assessed by FACS after staining with CD69- and CD86-specific Abs (BD Pharmingen), and dead cells were labeled with 7-aminocoumarin D; Sigma-Aldrich) to be excluded from the analyses. For receptors were blocked by the addition of mouse CD16/CD32 (Fcγ III/II receptor) Ab (BD Pharmingen). This Ab and 7-aminoactinomycin D were added before the addition of cell subpopulation- and activation marker-reactive Abs.

Serum IFN-α levels

Serum samples from SFV- and/or Ad2-infected B6 mice were collected (3 mice/group) and tested for IFN-I levels using a sandwich ELISA kit according to the manufacturer's instructions (US Biological). In each experiment, a standard curve in the range of 0–500 pg/ml IFN-α was generated for the estimation of the concentration of serum IFN-α. The detection limit of IFN-α was 12.5 pg/ml.

Irradiation, reconstitution, and adoptive transfer

Splenocytes at a concentration of 5 × 10^6 cell/ml in PBS were labeled with the cell tracker CFSE (Invitrogen Life Technologies) at a final concentration of 100 μM. After 5 min of incubation at room temperature, 10 volumes of PBS containing 2% FCS were added, and cells were centrifuged and washed three times with PBS/FCS. CFSE-labeled lymphocytes were resuspended in PBS at a concentration of 2.5 × 10^6 cell/ml; 10-wk-old irradiated (650 rad) B6 mice, 24 h after irradiation, were reconstituted with 5 × 10^7 CFSE-labeled cells/mouse. Similarly, 5 × 10^7 CFSE-labeled cells/mouse were adoptively transferred into nonirradiated, naive, B6 mice.

Results

Viral infection results in generalized activation marker expression on lymphocytes

We have shown previously (14) that infection of mice with SFV results in rapid and systemic up-regulation of two early activation markers, CD69 and CD86, on a large proportion of CD3^+ T and B220^+ B cells. This activation marker expression is mediated by the IFN-I response to the infection and is transient, returning to baseline levels around 5 days p.i. In this study, we expand this investigation by examining whether other virus infections trigger a similar response; we compared activation marker expression after virulent and avirulent SFV infection with that observed after infection with three heterologous viruses, the human Ad2, the flavivirus, WNV, and the orthomyxovirus, influenza virus (A/WSN) (Table I). All viruses investigated induced systemic up-regulation of CD69 on B and T cells and CD86 on B cells (also seen on T cells, data not shown) at 24 h p.i., with activation marker expression returning to that of naive lymphocytes by day 5. By the same time, viremia determined for SFV-infected mice was undetectable (Fig. 1), and virus had been cleared or reduced to low levels in brain and spleen (data not shown). Expression of CD25 was not or was only very marginally augmented by the different viral infections, similar to that documented for SFV (Ref. 14 and data not shown). Thus, generalized and transient lymphocyte activation is a general feature of acute viral infections.

IFN-I-activated lymphocytes survive and revert to a nonactivated state

The return to baseline activation marker expression on lymphocytes by day 5 p.i. may be the result of two processes. One is the loss of activated cells due to activation-induced cell death (21, 22) and/or attrition caused by IFN-I exposure (11) and replenishment by hematopoiesis; the other is a reversion of the activated lymphocytes to a quiescent state. To differentiate between these two possibilities, we used sublethally irradiated mice reconstituted with CFSE-labeled lymphocytes and monitored the fate of activated lymphocytes following viral infections.

B6 mice were reconstituted with CFSE-labeled syngeneic donor splenocytes 24 h after sublethal irradiation and infected with aSFV or mock-infected, and the activation marker profiles of these donor cells were monitored (Fig. 2A). The CFSE profiles at days 1 and 5 p.i. were similar in both mock- and aSFV-infected mice. As expected, CD69 expression on donor cells was markedly elevated at day 1 p.i. in aSFV-infected mice and had mostly returned to background levels by day 5 p.i.

Some CFSE-labeled splenocytes divided following transfer in both uninfected and infected recipients (Fig. 2A, top right), which probably represents homeostatic proliferation in the lymphopenic recipients. Fig. 2B shows the CFSE profile of individual subpopulations of splenocytes at 1 and 5 days p.i. B cells underwent minimal cell division in the 5-day interval. A fraction of CD4^+ T cells underwent at least one division by day 5 p.i. CD8^+ T cells constituted the main dividing splenocyte population with 2 and 4 divisions on days 1 and 5 p.i., respectively.

Table I. Percentage of T and B cells from spleen expressing activation markers after in vivo viral infection

<table>
<thead>
<tr>
<th>Marker</th>
<th>Day p.i.</th>
<th>aSFV</th>
<th>Ad2</th>
<th>WNV</th>
<th>A/WSN</th>
<th>mock</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD69 on CD3^+ cells</td>
<td>1</td>
<td>50.2 (±8.9)</td>
<td>65.4 (±2.4)</td>
<td>62.4 (±4.4)</td>
<td>39.3 (±8.1)</td>
<td>83.3 (±2.8)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>15.5 (±1.2)</td>
<td>11.1 (±0.2)</td>
<td>10.5 (±0.8)</td>
<td>9.9 (±0.4)</td>
<td>NT</td>
</tr>
<tr>
<td>CD69 on B220^+ cells</td>
<td>1</td>
<td>82.7 (±4.9)</td>
<td>83.9 (±1.5)</td>
<td>72.3 (±9.3)</td>
<td>48.4 (±1.8)</td>
<td>91.7 (±0.6)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.0 (±2.1)</td>
<td>3.3 (±0.5)</td>
<td>3.5 (±0.4)</td>
<td>3.5 (±0.7)</td>
<td>NT</td>
</tr>
<tr>
<td>CD86 on B220^+ cells</td>
<td>1</td>
<td>80.3 (±2.1)</td>
<td>85.9 (±1.8)</td>
<td>74.9 (±4.5)</td>
<td>68.9 (±2.0)</td>
<td>89.6 (±4.9)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>21.0 (±5.7)</td>
<td>24.6 (±2.6)</td>
<td>17.1 (±4.2)</td>
<td>22.5 (±3.5)</td>
<td>NT</td>
</tr>
</tbody>
</table>

a Activation marker expression is given as a percentage of cells with higher Ag-specific fluorescence intensity relative to that on cells from uninfected mice; three mice per group per day were used.

FIGURE 1. Kinetics of viremia after aSFV infection. Groups of three mice were sacrificed at 1, 3, 5, and 7 days p.i. with 10^7 PFU aSFV. Virus titers in serum were estimated by plaque assay.
Two hours after transfer of donor splenocytes into B6 mice and evaluated their activation marker up-regulation, we adoptively transferred naive CFSE-labeled donor splenocytes into B6 mice and enumerated their percentages after mock and aSFV infections.

To assess whether previously activated lymphocytes respond to a secondary viral infection with comparable cell surface activation marker up-regulation, we adoptively transferred naive CFSE-labeled donor splenocytes into B6 mice and evaluated their activation pattern after infection with an unrelated virus at different time intervals after a primary infection. Two hours after transfer of donor cells, recipients were infected with aSFV or mock treated, and the total number of splenocytes recovered from spleens of infected mice at day 7 and 10 postinfection was quantitated at days 1, 3, and 6 p.i. (Fig. 3). There was a general decrease of the total number of transferred CFSE-labeled splenocytes, including CD4+ and CD8+ T cells over a 6-day period after infection. However, the loss observed was independent of aSFV infection. Thus, clearly, lymphocytes that had expressed elevated levels of activation markers, as a result of the viral infection, did not undergo apoptosis and were not replenished by hematopoiesis but reverted to a nonactivated state.

**Induction of activation markers during a secondary, unrelated, viral infection is impaired**

To test the possibility of lymphocyte proliferation compensating for lymphocyte attrition, particularly of T cells, we adoptively transferred naive CFSE-labeled donor splenocytes into B6 mice and enumerated their percentages after mock and aSFV infections. Two hours after transfer of donor cells, recipients were infected with aSFV or mock treated, and the total number of splenocytes (CFSE + donor cells), T cells subpopulation (both CD4+ and CD8+) within the transferred CFSE-labeled splenocytes were quantitated at days 1, 3, and 6 p.i. (Fig. 3). There was a general decrease of the total number of transferred CFSE-labeled splenocytes, including CD4+ and CD8+ T cells over a 6-day period after infection. However, the loss observed was independent of aSFV infection. Thus, clearly, lymphocytes that had expressed elevated levels of activation markers, as a result of the viral infection, did not undergo apoptosis and were not replenished by hematopoiesis but reverted to a nonactivated state.

**Duration of antiviral state induced by primary infection**

One possible explanation for the observed lack of activation at early times after primary infection by a secondary viral infection is that IFN-I, or other innate immune responses, induced by the initial infection generated a long-lasting antiviral state that may have prevented a subsequent infection from becoming established, removing the trigger for IFN-I secretion necessary for re-expression of activation markers on lymphocytes. If this were true, an increase in resistance of mice to a second viral infection would be expected.
However, mice that had received Ad2 6 days before a challenge with a lethal dose of vSFV surprisingly died faster, rather than slower or not at all, than mice that had not been infected previously (Fig. 6). This indicates that the inability of the second viral infection to activate lymphocytes was not due to a prolonged antiviral state induced by the primary infection. To the contrary, it suggests that a major component of the antiviral immune response is absent or deficient for a certain period of time after primary viral infection.

Reduced resistance to the natural mouse pathogen ECTV

We decided to more closely investigate the higher susceptibility to a secondary infection after primary infection. Infection of mice with ECTV, the causative agent of mousepox, provides one of the few laboratory animal models of a biologically relevant virus infection (23, 24). We thus asked whether an infection with aSFV affects the viral load in spleen of a subsequent infection with ECTV 5 days after the primary infection. Avirulent SFV was used, given that this virus elicits a high IFN-I response and activation marker expression on B and T cells without causing significant signs of disease. Five days after mice were infected with aSFV or mock-infected, they were reinfected with ECTV into the hind footpad, the route mimicking natural infection (25). Four days later, spleens were removed and assayed for virus titers and histology.

Spleens from three mice, which had not been infected with aSFV, had low or undetectable ($\leq 10^3$ PFU/spleen) ECTV titers with one spleen yielding $2 \times 10^4$ PFU. This was consistent with previous studies on ECTV replication in B6 mice (26). In contrast, spleens from mice preinfected with aSFV all contained detectable virus, with titers ranging from $1 \times 10^5$ to $6 \times 10^7$ PFU/spleen (Fig. 7). Histological damage of spleens reflected the viral burden, with splenic necrosis in all mice infected with aSFV before ECTV infection but not in spleens from mice only infected with ECTV (data not shown). Spleens of mice only infected with aSFV for 5–9 days showed no histological abnormalities (data not shown).

Lymphocytes are not refractive to reactivation

To investigate whether the refractory period for lymphocytes to undergo a renewed activation process is caused by impaired IFN-I responsiveness of the previously activated lymphocytes, mice were infected with aSFV and splenocytes harvested 5 days p.i., labeled with CFSE, and transferred into naive mice. Two hours later, recipients were infected with Ad2. Expression of activation markers on CFSE-labeled (transferred) and unlabeled (resident) lymphocytes was determined 1 day after Ad2 infection. Fig. 8 shows that Ad2 infection induced cell surface expression of activation markers, CD69 and CD86, on both recipient (naive) and donor (previously activated) cells. Thus, previously activated lymphocytes are inherently able to respond to IFN-I as a result of a second viral infection, but the milieu at a particular time interval after primary infection in the donor mouse does not provide the required trigger(s) for reactivation upon a secondary viral infection. Indeed, we found that secreted IFN-I, the critical requirement for partial lymphocyte activation (14), was not detectable at 1 day after a secondary infection with Ad2 when mice had previously been infected for 6 days with aSFV (Table II). However, when a
secondary Ad2 infection occurred 9 or more days after a primary infection, IFN-I activity was apparent as lymphocyte activation re-occurred. This suggests that transient exhaustion of the IFN-I response in an infected animal underlies the inability to respond to a secondary infection with partial lymphocyte activation. So far it remains elusive whether the increased susceptibility of mice to secondary viral infection during a period of 5 to 9 days after a primary infection is the result of an impaired IFN-I response, lack of systemic, partial, activation of lymphocytes, or both.

Primary viral infections do not affect quantity of DCs

To address the possibility that the transient deficiency of the antiviral IFN-I response was the result of a loss of DCs due to the primary infection, we analyzed the percentages of both pDCs (CD11c+ B220+) and conventional DCs (CD11c− B220−) in the spleens of aSFV-infected B6 mice at 5 days p.i. (Fig. 9). No decrease in numbers of DCs was detected 5 days p.i. In fact, although statistically not significant, both splenic pDCs and conventional DCs increased in numbers as a result of aSFV infection. The numbers of splenocytes at day 5 p.i. from aSFV-infected mice were not significantly different than those from mock-infected mice (Fig. 9). Thus, the transient lack of IFN-I response to a second infection is not due to an attrition of DCs.

Discussion

Although it has been a long-standing clinical observation and problem that patients suffering from a microbial infection are often more susceptible to a secondary infection, the physiological basis of this phenomenon remains to be elucidated. In this study, we provide evidence in mice, that the infected host undergoes a transient period of partial immunosuppression between days 5 and 9 after the primary viral infection. During the height of the adaptive immune response to the primary infection, the host is unable to respond to a secondary infection with one of the most important innate responses, the secretion of IFN-I.

We have previously shown that IFN-I trigger a global partial lymphocyte activation that resolves by 5 days p.i. These data are consistent with the observation of Jiang et al. (27), who showed that most T cells, regardless of their specificity, express early activation markers soon after an infection with Listeria monocytogenes or lymphocytic choriomeningitis virus. Based on in vitro studies of B cell activation, this IFN-I effect has been suggested to result in a lowering of the activation threshold for Ag-specific full activation (28). Thus, IFN-I might increase the pool of low-affinity

Table II. Serum IFN-α concentration (pg/ml) induced by aSFV and/or Ad2 infection

<table>
<thead>
<tr>
<th>Serum Samples</th>
<th>Days p.i.</th>
<th>IFN-α Concentration ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>1</td>
<td>&lt;12.5</td>
</tr>
<tr>
<td>Ad2</td>
<td>1</td>
<td>1975 ± 389</td>
</tr>
<tr>
<td>aSFV</td>
<td>1</td>
<td>2325 ± 459</td>
</tr>
<tr>
<td>aSFV</td>
<td>3</td>
<td>125 ± 70</td>
</tr>
<tr>
<td>aSFV</td>
<td>6</td>
<td>&lt;12.5</td>
</tr>
<tr>
<td>aSFV</td>
<td>7</td>
<td>&lt;12.5</td>
</tr>
<tr>
<td>+ Ad2</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*Three mice per group were used.*
lymphocytes able to be triggered by “cognate” Ag, with subsequent selection of the higher avidity clones (29) or fine-tuning of functional avidity (30). The inducible and transient nature of this phenomenon suggests that it would be disadvantageous to constitutively lower the lymphocyte activation threshold, presumably because of increased risk of autoimmunity.

In contrast to Jiang et al. (27), however, we did not observe massive lymphocyte death accompanying the partial activation. Instead, there was no difference in survival of adoptively transferred splenocytes in naive hosts challenged with virus or left untreated. This difference is not surprising in light of past and recent evidence that during infections with lymphotropic viruses such as lymphocytic choriomeningitis virus (31) also used by Jiang et al. (27), or human T cell leukemia virus-I (32), CD8 T cells commit massive suicide and/or fratricide. This does not occur with most other virus infections, especially not with SFV because B6 mice are cytolytic CD8 T cell nonresponders (33, 34). It is noteworthy that IFN-I actually results in proliferation of lymphocytes, mainly those of a memory phenotype (35), and that IFN-I increases the viability of activated T cells (36). Thus, partially activated lymphocytes did not die, but underwent a deprogramming of activation to revert to a state with CD69 and CD86 expression at basal levels.

Currently, we do not know whether cells can undergo several rounds of de-activation or will eventually die, or become refractive to IFN-I stimuli; nor is it clear whether the cells that respond to IFN-I are phenotypically distinguishable from the 20 to 30% that fail to be triggered into partial activation.

It remains to be established why a renewed IFN-I response is not mounted in mice at 5 to 9 days after a primary viral infection. The potent inflammatory capacity of IFN-I might not be tolerable for longer than a few days lest autoimmunity ensues (37) such that negative feedback systems assure down-regulation or prevention of renewed IFN-I production. A refractory phase to IFN-I secretion by pDC has been reported recently in response to viral stimulation (38). It is tempting to speculate that infected pDC, the predominant producers of IFN-I (6, 39), are subject to immune attack by CTLs. Consistent with this idea is the temporal correlation of the peak of the cytotoxic T cell response with the deficiency in IFN-I production. pDC might also be depleted, irrespective of infection status, by NK cells (40, 41) and may need to be replenished by homeostatic processes. However, our data indicate that numbers of both pDCs and conventional DCs slightly increased in spleens of aSFV-infected mice at 5 days p.i. This is consistent with previously reported increases in pDC numbers in the spleen of HSV-infected mice (42). Alternatively, given the maturation stimulus of IFN-I on pDC (9), a strong IFN-I signal might induce them to differentiate into a non-IFN-secreting phenotype. Previous studies have shown that DCs from HSV-infected mice fail to produce IFN-I when re-stimulated in vitro (42), and that murine cytomegalovirus infection of DCs results in their paralysis and deficiency in IL-12 and IL-2 production (43). It is therefore possible that DCs were paralyzed as a result of the primary infection, either by direct viral infection or as the consequence of a secondary event.

It would be of great clinical importance to minimize the duration and extent of this heightened susceptibility to secondary infections after a primary viral episode. Prophylactic administration of rIFN-I would appear to provide the logical solution. However, when tested rIFN-I administration did not reduce the severity of a secondary viral infection (as measured by ECTV replication) and, importantly, did not induce activation marker expression on lymphocytes in vivo compared with that seen in vitro (data not shown). In addition, administration of rIFN-β simultaneously with a lethal primary vSFV infection did not affect the outcome of the infection (unpublished data). This suggests that presently available rIFN-I preparations exert limited efficacy in vivo in mice, possibly due to the recently suggested IFN-I subtype specificity of function (44).

We are in the process of screening a variety of IFN-I products, recombinant and produced by mammalian cells for their biological activity. Clearly, the inability to respond to a viral infection with a potent IFN-I-mediated immune response carries high risks for the host, as shown in the high susceptibility of IFN-I receptor-deficient mice to viral infections (2–5). Consistent with this, we show that the lack of IFN-I during several days after a primary infection is associated with a striking failure to control a subsequent infection, as shown by an early mortality due to secondary vSFV and massively increased virus titers after secondary ECTV infection compared with primary infection with the viruses. In conclusion, we have demonstrated that, for several days during a viral infection, the host undergoes a transient immunosuppression that is characterized by an inability to respond to a subsequent infection with one of the most important innate immune mediators.

Disclosures

The authors have no financial conflict of interest.

References


