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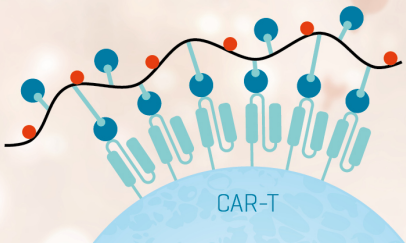

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Type I Interferons Trigger Systemic, Partial Lymphocyte Activation in Response to Viral Infection

Mohammed Alsharifi, Mario Lobigs, Matthias Regner, Eva Lee, Aulikki Koskinen, and Arno Müllbacher[1]

The vast majority of both T and B cells in mice were found to up-regulate cell surface expression of the early activation markers CD69 and CD86, but not CD25, within 24 h of infection with Semliki Forest virus. Kinetics and magnitude of activation marker expression was dependent on live virus, dose, and correlated with strain virulence. Activation marker expression declined to baseline levels over the next 96 h. This very early "activation" of such a high percentage of lymphocytes required the presence of type I IFN receptor genes, was inducible with poly(I:C), and correlated with IFN-I levels in serum. We conclude that virus-induced IFN-I release systemically affects most of the hosts T and B cells by triggering them rapidly and independently of Ag-reactivity into a semiactivated state. ***The Journal of Immunology,*** **2005, 175: 4635–4640.**

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Type I IFNs (IFN-I) were discovered on the basis of their antiviral activity (1). IFN-I (IFN-α, IFN-β) are encoded by a family of over 20 genes, (2) and can be secreted by a large spectrum of cell types (3, 4). All IFN-I subtypes bind to a common cell surface receptor (5, 6) and play an important role in host protection following infection by a variety of viruses (2, 7–9).

Virus-infected cells synthesize IFN-I within hours of infection, which can act in an autocrine and paracrine manner to limit replication and spread of virus (10). Although all virus-infected cells can produce IFN-I within hours of infection, immature dendritic cells (DCs),[2] particularly plasmacytoid DCs, produce 1000-fold higher IFN-I levels than other cell types and are thought responsible for the systemic IFN-I responses to many viruses (11, 12).

Beside their antiviral activities, IFN-I exerts regulatory effects on cellular and humoral immune responses. IFN-I stimulates Th functions in both mouse (13) and human (14), can directly support survival of activated T cells (15, 16), and induces bystander cytotoxic T cell proliferation during viral infections, particularly that of memory T cells (16). Moreover, IFN-I stimulates Ab production in vitro (17, 18), and can act as an adjuvant for vaccination against influenza virus (19). However, the role of IFN-I in immunoregulation during viral infection in vivo has received little attention.

Lymphocyte activation is conventionally measured by the expression of several cell surface molecules such as CD69, CD86, and CD25. CD69 is the first cell surface Ag expressed following activation of T, B, NK cells, monocytes, and most other cells of hemopoietic lineages (20). Recent results suggest that CD69 may have immunoregulatory functions (21). CD80 and CD86 are cell surface molecules that provide costimulation for T cell activation by interaction with CD28 (22, 23), and both molecules can be expressed on various APCs such as DCs, macrophages, and B cells (24). CD25 is the α-chain of the IL-2 receptor and can be expressed on the surface of activated B and T cells following Ag recognition (25, 26). In vitro, treatment of B cells with IFN-I upregulates the expression of CD69, CD86, and CD25 (27).

Here we investigated the magnitude and kinetics of lymphocyte activation on lymphocytes ex vivo associated with an acute cytopathic viral infection in mice. We chose the alphavirus Semliki Forest virus (SFV), a natural mouse pathogen, as a model. SFV strains can be classified into virulent (vSFV) and avirulent (aSFV) (28), and these strains differ in type I IFN induction and lethality in mice (29).

Materials and Methods

Viruses and cells

Vero (African green monkey kidney), and baby hamster kidney cells were maintained in Eagle's MEM plus nonessential amino acids and 5% FCS and incubated at 37°C in humidified condition with 5% CO_2.

vSFV, strain V13, and aSFV, strain A7, were used in this study, and were obtained from Dr. I. D. Marshal (John Curtin School of Medical Research in the late 1970s). Working stocks of vSFV and aSFV were prepared by infecting semiconfluent baby hamster kidney cell monolayers at a multiplicity of infection of 0.5 PFU per cell. Infected cells were incubated for 24 h, then culture supernatants were harvested and centrifuged at $1200 \times g$ for 4 min and stored in single-use aliquots at -70°C. Titers, determined by plaque assay on Vero cells, were 5×10^7 PFU/ml for vSFV and 1×10^8 PFU/ml for aSFV. Inactivated vSFV and aSFV were obtained by gamma irradiation (1.3 GRad) using a Cobalt-60 source (CSIRO-Canberra). Loss of virus inactivity was confirmed by plaque assay.

A volume of 150 μl/mouse was used for both i.v (administering SFV) and i.p (administering poly(I:C)) injections. PBS was used to dilute both SFV and poly(I:C) (Sigma-Aldrich) stock before injections.

Mice

Wild-type C57BL/6 (B6) and 129-2SV (129) mice, and IFN-α receptor knockout (IFN-IR$^{-/-}$) (2), IFN-γ receptor knockout (IFN-IIR$^{-/-}$) (30), and double receptor knockout (IFN-I&IIR$^{-/-}$) mice were bred under specific pathogen-free conditions and supplied by the Animal Breeding Facilities at the John Curtin School of Medical Research, Canberra. Only 10-wk-old females were used. All animal experiments were conducted with approval from the ANU Animal Ethics Committee.

Flow cytometric analysis

Spleens from infected and control mice were harvested and red cell-depleted single cell suspensions prepared. Lymphocytes (1×10^6) were stained using fluorescent-conjugated anti-CD3, -CD4, -CD8, -CD19, and

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[2] Abbreviations used in this paper: DC, dendritic cell; SFV, Semliki Forest virus; vSFV, virulent SFV; aSFV, avirulent SFV; p.i., postinfection.

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-B220 Abs (BD Pharmingen). Expression of activation markers was assessed by FACS after staining with CD25-, CD69-, and CD86-specific Abs (BD Pharmingen), and dead cells were labeled with 7-aminoactinomycin D (Sigma-Aldrich). Fc 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 vSFV- and aSFV-infected B6 mice were collected at 0.5, 1, 2, 3, 4, and 5 days p.i. (2 mice/time point) and tested for IFN-α 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 estimation of the concentration of serum IFN-α. The detection limit of IFN-α was 12.5 pg/ml.

Results

Systemic expression of activation markers on lymphocytes after viral infection

Cell surface expression of CD69, CD25, and CD86 has been widely used as an indicator of lymphocyte activation in vitro (27).

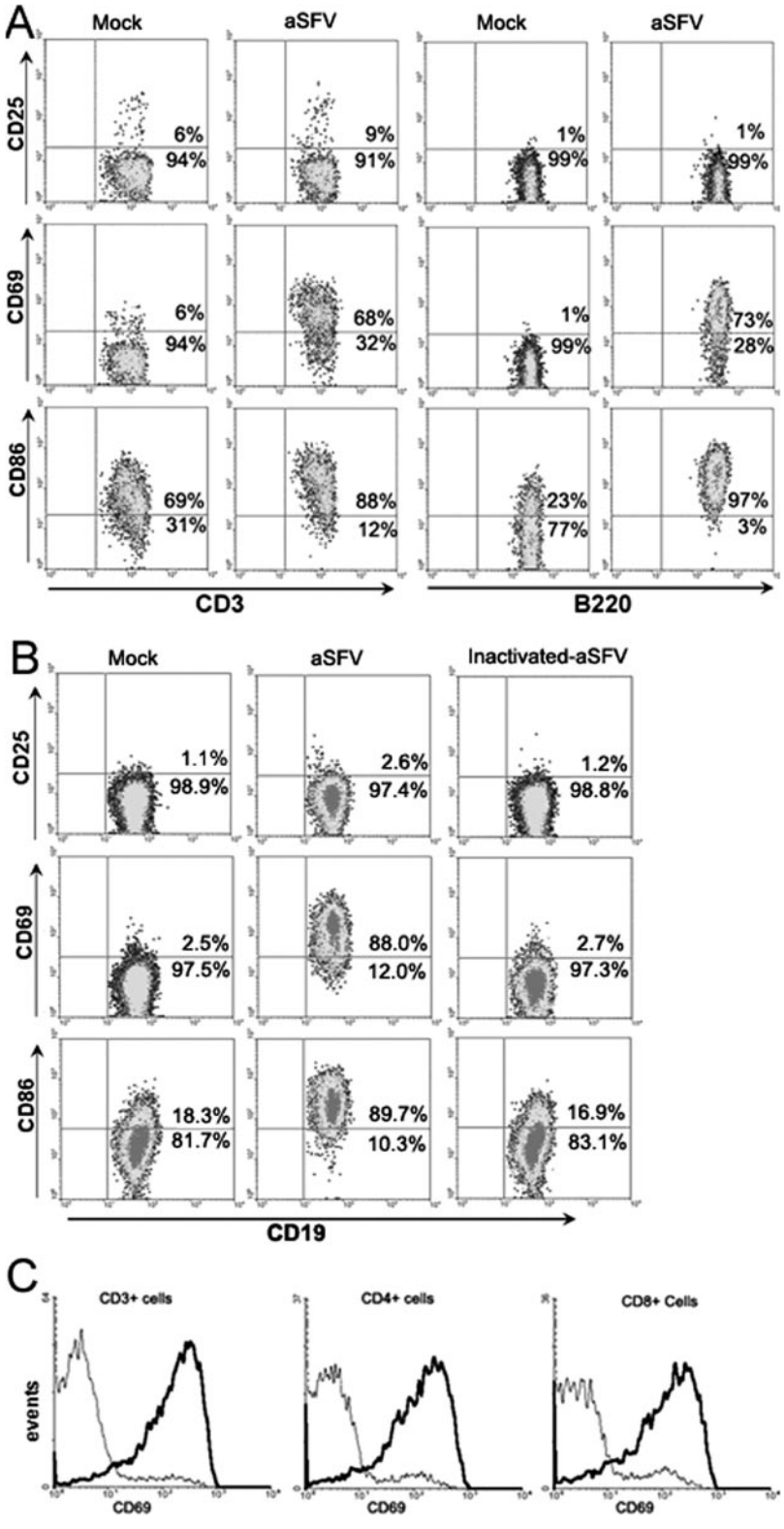

FIGURE 1. FACS analysis of lymphocyte activation marker expression on splenocytes following in vivo infection with aSFV. *A*, CD25, CD69, and CD86 expression on B220$^+$ B and CD3$^+$ T cells. *B*, CD25, CD69, and CD86 expression on CD19$^+$ B cells (*C*) CD69 expression on CD3$^+$, CD4$^+$, and CD8$^+$ T cells.

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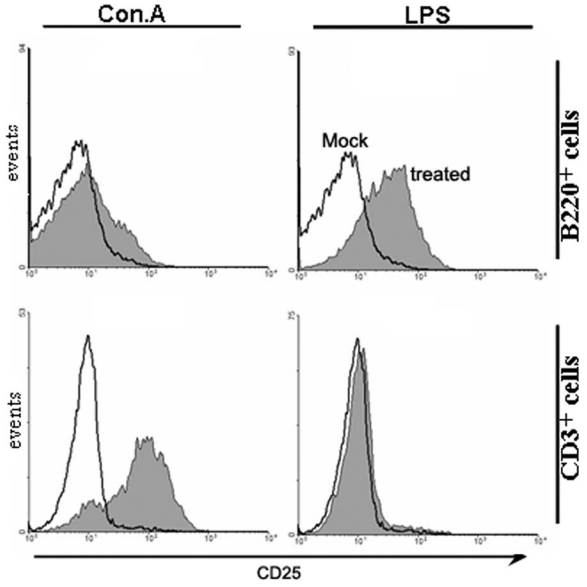

FIGURE 2. FACS analysis of CD25 expression on splenocytes after in vitro treatment with mitogens. Splenocytes, cultured for 24 h with Con A (*left panels*), LPS (*right panels*) (shaded), or mock treated (clear). Fluorescence histograms of CD25 expression on $B220^+$ B cells (*top panels*) and $CD3^+$ T cells (*bottom panels*).

To evaluate the in vivo activation status of splenocytes in response to a viral infection, B6 mice were infected with aSFV (10^7 PFU/mouse) and expression levels of the activation markers were determined 1 day later by FACS. As shown in Fig. 1*A*, CD69 and CD86 expressions are elevated on both $B220^+$ B and $CD3^+$ T cells. Because B220 is not exclusively expressed on B cells, we have verified B cell activation marker expression using CD19 as the B cell marker and show similar results (Fig. 1*B*). Both $CD4^+$ and $CD8^+$ T cell subpopulations showed a similar CD69 profile (Fig. 1*C*). Thus, results for $CD3^+$ T cells obtained throughout this study are representative for both $CD4^+$ T and $CD8^+$ T cells. Because background levels of CD86 expression were higher on $CD3^+$ T cells than on $B220^+$ B cells, we decided to use CD86 expression as the indicator of activation for B cells only. No elevation of CD25 expression was observed on any of the leukocyte subpopulations (Fig. 1*A*). This contrasts with observations in vitro (27). However, when we treated splenocytes from naive mice in vitro with B (LPS) or T (Con A) cell mitogens, we also observed induction of CD25 expression on the surface of $B220^+$ and $CD3^+$ cells, respectively (Fig. 2).

To investigate whether lymphocyte activation during SFV infection was systemic or restricted to spleen, we infected B6 mice with 10^7 pfu aSFV and assessed activation levels of lymphocytes from spleen, mesenteric lymph node, and blood at day 1 p.i. (Fig. 3). Expression of CD69 was induced on both $B220^+$ and $CD3^+$ cells to similar levels on cells from all lymphoid tissues tested. CD86 expression on B cells mirrored that of CD69 expression. Thus, the bulk of both B and T cells undergo systemic partial activation in response to viral infection.

Kinetics and virus dose dependency of activation marker expression

We then established the kinetics and dose-response of activation marker expression, and loss thereof, by infecting groups of B6 mice i.v. with titrated doses of vSFV or aSFV, and measuring the percentages of B and T cells with elevated expression of lymphocyte activation markers CD69 and CD86 over a 5-day period. Kinetics and expression levels of CD69 (Fig. 4*A*) and CD86 (Fig. 4*C*) on B, and CD69 on T cells (Fig. 4*B*) were similar. One PFU of aSFV did not result in significant up-regulation of lymphocyte activation markers. In contrast, the same dose of vSFV induced expression of activation markers on both B and T cells with peak numbers at day 3 p.i. and returning to background levels by day 5 p.i. Increasing the doses of either SFV strain increased the number of lymphocytes with activation markers and accelerated their appearance. Infection with gamma ray-inactivated SFV did not result in generalized activation markers expression even when high doses (5×10^7 PFU equivalent/mouse) were administered (Fig. 4*D*), showing that viral replication was essential. Thus, the systemic

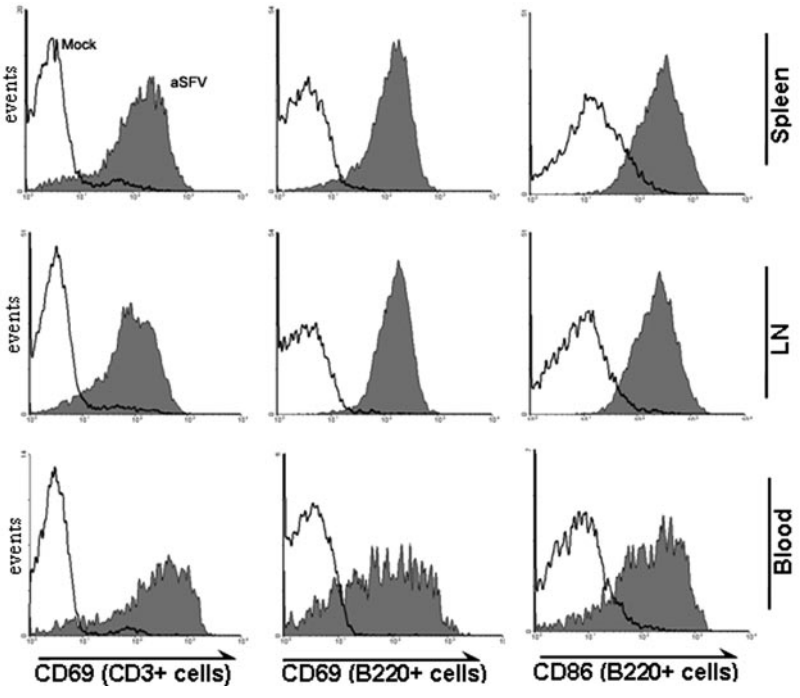

FIGURE 3. Activation marker expression on lymphocytes from different lymphatic tissues after 24 h SFV infection. CD69 expression on $CD3^+$ T cells (*left panels*), $B220^+$ B cells (*center panel*), and CD86 expression on $B220^+$ B cells (*right panels*) using splenocytes (*top*), pools of mesenteric lymph node cells (*middle*) or from blood (*bottom*). Results are from pools of three mice.

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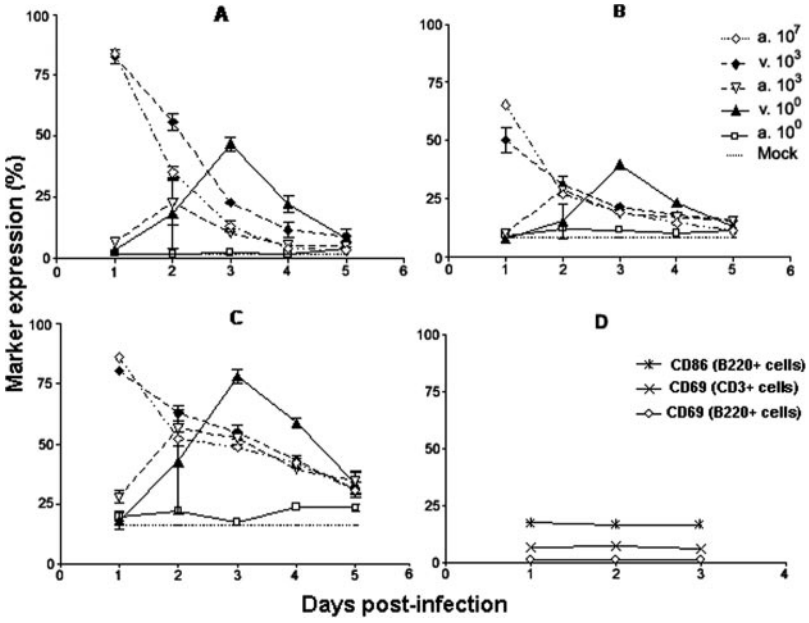

FIGURE 4. Kinetics of lymphocyte activation marker expression on splenocytes from mice infected with differing doses and strains of SFV. Percentage of activated lymphocytes detected at different times after infection with 10^3 (♦), 10^0 (▲) PFU vSFV and 10^7 (◇), 10^3 (▽) 10^0 (□) PFU aSFV. *A*, Expression of CD69 on $B220^+$ B cells. *B*, Expression of CD69 on $CD3^+$ T cells. *C*, Expression of CD86 on $B220^+$ B cells. *D*, Expression of CD69 and CD86 on T and B cells after infection with 5×10^7 PFU equivalent inactivated aSFV.

lymphocyte activation observed shortly after viral infection requires live virus, is virus-dose dependent, and correlates with viral virulence.

The role of IFN-I in systemic lymphocyte activation

The early and systemic appearance of "activated" lymphocytes after viral infection suggested the possibility that soluble host factors, such as IFN-I, produced by innate immune cells were responsible. To test this assumption, we compared serum levels of IFN-α following infection with vSFV and aSFV, studied lymphocyte activation following administration of poly(I:C) (synthetic inducer of IFN-I), and investigated lymphocyte activation in IFN receptor-deficient mice.

First, we compared serum levels of IFN-α following infection with 10^3 PFU of vSFV to that following infection with 10^3 or 10^7 PFU of aSFV. As shown in Fig. 5, elevated IFN-α levels, induced by vSFV infection, were first detected at 12 h p.i., peaked at 24 h p.i. (2900 ± 100 pg/ml), and declined to background levels by day 3 p.i. Using the same dose of aSFV, a low level of serum IFN-α was detectable later, at 24 h p.i. the serum IFN-α level peaked at 48 h p.i. (875 ± 25 pg/ml) and declined to undetectable levels on day 3 p.i. Peak IFN levels were substantially lower than those observed with vSFV. Increasing the dose of aSFV 10^4-fold produced kinetically and quantitatively similar results as that observed with 10^3 PFU vSFV. Importantly, the virus strain- and dose-dependent induction of IFN-α secretion corresponded closely in kinetics and magnitude to that of lymphocyte activation shown in Fig. 4.

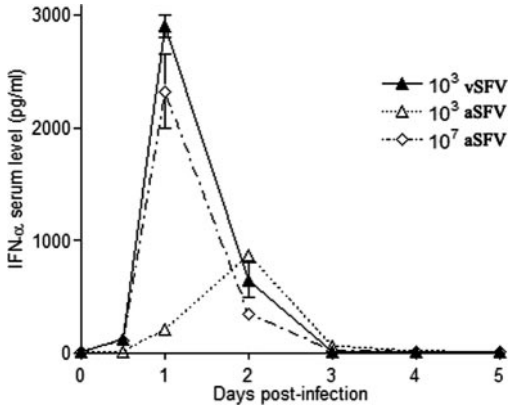

FIGURE 5. Kinetics of serum IFN-α levels following viral infection. Two mice per day (per group) were infected with either 10^3 PFU vSFV (▲) or 10^3 (△) or 10^7 (◇) PFU aSFV. Serum of individual mice were tested for IFN-α concentrations and means were expressed as picograms per milliliter. Detection limit was 12.5 pg/ml.

Second, i.p. injection with 0.5 and 1 mg/mouse of the IFN-I inducer, poly(I:C), resulted in up-regulation of activation markers CD69 and CD86 on both B and T cells, although to a slightly lower level than that seen with SFV infections (Fig. 6).

Finally, we compared the magnitude of virus-induced lymphocyte activation in mice deficient in type I and/or type II IFN receptor(s) (IFN-$IR^{-/-}$, IFN-$IIR^{-/-}$, and IFN-$I\&IIR^{-/-}$) with their wild-type 129 counterpart. IFN-$IR^{-/-}$ mice, in general, are highly sensitive to and die very early after viral infection (2, 7–9). IFN-$IR^{-/-}$ mice died at days 2 and 3 when given 10^3-10^7 PFU aSFV. Therefore, mice were infected with 10^7 PFU aSFV and lymphocyte activation was only determined at day 1 p.i. A generalized elevated expression of CD69 on T cells was observed in both 129 and IFN-$IIR^{-/-}$ mice (Fig. 7). In contrast, CD69 expression on T cells from IFN-$IR^{-/-}$ and IFN-$I\&IIR^{-/-}$ mice was only marginally elevated above mock infected (Fig. 7). A similar lack of elevated expression was also seen for CD86 and CD69 on B cells when compared with wild-type or IFN-$IIR^{-/-}$ mice (Fig. 7).

We conclude that IFN-α is a crucial factor in the induction of the generalized lymphocyte activation seen shortly after a viral infection.

Discussion

We present evidence that in vivo infection with viruses causes a generalized elevation of cell surface expression of the activation markers CD69 and CD86 on both B and T ($CD4^+$ and $CD8^+$) cells. This activation marker expression is rapid, correlates with virus dose and strain virulence, and appears to be a feature of most viral infections because it also occurs with all other viruses investigated: flavi, adeno, and orthomyxo virus infections (M. Alsharifi, M. Regner, M. Lobigs, E. Lee, A. Koskinen, R. Blanden, and A. Müllbacher, manuscript in preparation). Activation marker levels

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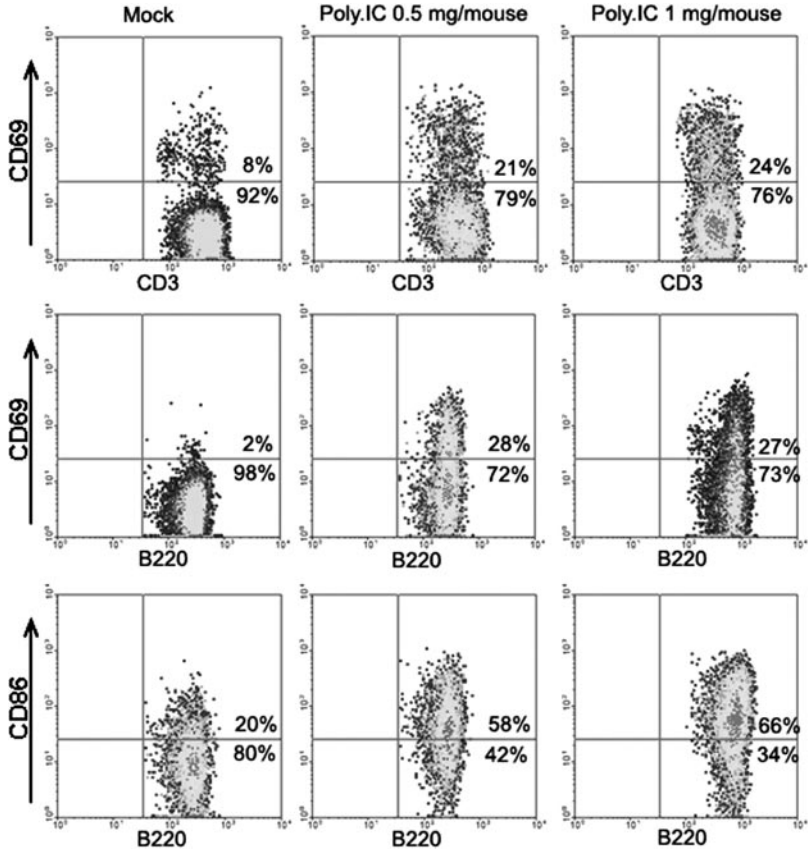

FIGURE 6. FACS analysis of CD69 and CD86 expression on splenocytes following i.p. injection with poly(I:C).

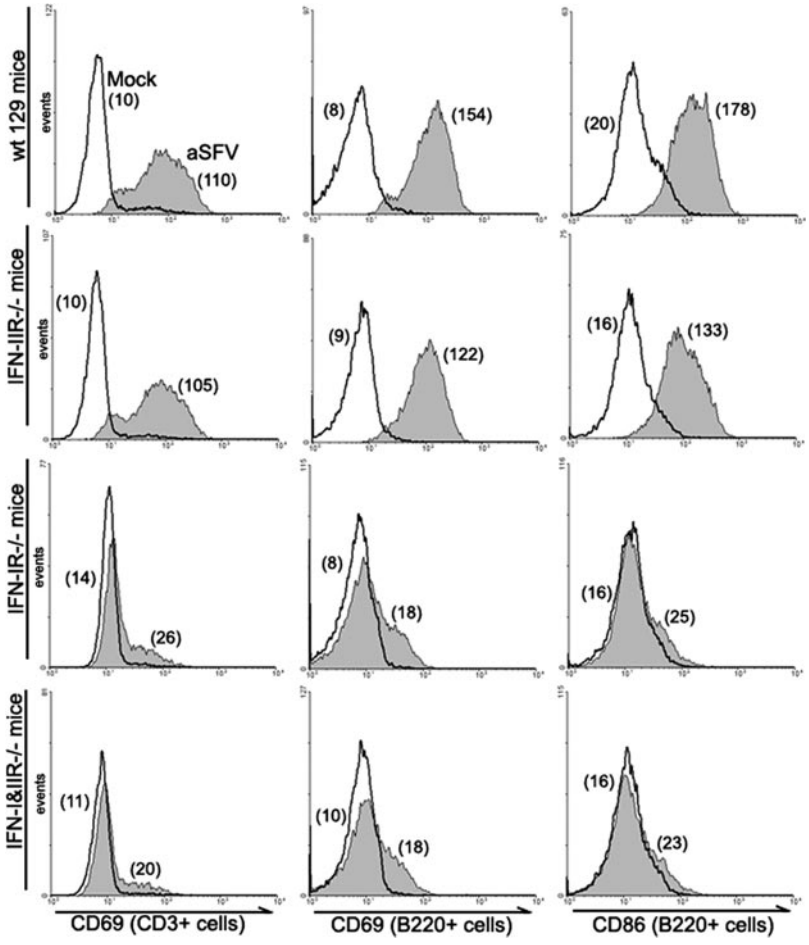

FIGURE 7. FACS analysis of CD69 and CD86 expression on splenic B and T cells of IFN receptor-deficient mice following in vivo infection with aSFV. Histograms are comparing fluorescence profiles from mock (clear) and 10^7 PFU aSFV infected (shaded) wild-type 129, IFN-IIR$^{-/-}$, IFN-IR$^{-/-}$, and IFN-I&IIR$^{-/-}$ mice. Mean fluorescence units are shown in brackets.

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decline to background levels at ~5 days p.i. This observed lymphocyte activation is clearly a consequence of viral replication because high doses (5×10^7 PFU live virus equivalent/mouse) of gamma ray-inactivated SFV did not induce detectable levels of activation over that seen in control lymphocytes. This also rules out any mitogenic activity, possibly present in the viral preparation, being responsible for the observed lymphocyte activation.

The finding that the systemic and generalized activation of lymphocytes occurred as early as 24 h p.i. suggested the involvement of early released cytokines in the process. Three pieces of evidence identify IFN-I as playing a major role in this lymphocyte activation. First, the magnitude and kinetics of activation marker expression correlated with that of IFN-I induction by the two strains of SFV. That is, vSFV is a more potent inducer of IFN-I than aSFV and consequently induced earlier and higher levels of activation marker expression at lower virus doses than aSFV. Second, poly(I:C), a classical inducer of IFN-I, produced activation marker up-regulation similar to that seen when mice were infected with SFV. Finally, no elevation of lymphocyte activation marker expression was observed when mice lacking IFN-IR (IFN-IR$^{-/-}$ and IFN-I&IIR$^{-/-}$mice), but not IFN-IIR$^{-/-}$ mice, were infected with viruses.

IFN-I exert, in addition to their important antiviral properties, pleiotropic effects including inhibition of cell growth, anti-tumor activity, involvement in homeostasis, and regulatory effects on cellular and humoral immune responses (for review, see Ref. 31). Data presented here illustrate a generalized phenomenon characterized by massive lymphocyte activation during early days following a viral infection. The biological significance of this phenomenon, however, is at present unclear. Especially, the host's benefit of having most of the lymphocytes in a partially activated state, irrespective of their Ag reactivity, is obscure. One possibility is that CD69 and CD86 expression is a by-product of lymphocytes attaining an IFN-induced antiviral state, which could bestow an advantage to the host by reducing viral dissemination. In this context, the lack of CD25 expression may be significant by not rendering the lymphocytes susceptible to IL-2-mediated proliferation. Thus, "true activation" may also require CD25 up-regulation, which may only be triggered by ligation of the clonally expressed Ag receptors. CD25 expression has been linked to TCR engagement by others (32).

An additional consequence of this partial activation state of T and B cells may be a reduced threshold of Ag-mediated activation facilitating a broader and faster adaptive immune response at the early stages of a viral infection, with selection of the most efficient clonal populations during later stages. We are at present investigating whether IFN-I-activated T cells respond to lower Ag concentrations than do non-IFN-I treated T cells, to test this hypothesis.

Disclosures

The authors have no financial conflict of interest.

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