Superantigen-Activated T Cells Redirected by a Bispecific Antibody Inhibit Vesicular Stomatitis Virus Replication In Vitro and In Vivo

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Superantigen-Activated T Cells Redirected by a Bispecific Antibody Inhibit Vesicular Stomatitis Virus Replication In Vitro and In Vivo

Ana Fernandez-Sesma,* Richard W. Peluso,† Xu Bai,‡ Jerome L. Schulman,* David E. Levy,§ and Thomas M. Moran2*

A bispecific Ab (BsAb) that binds the TCR on T cells and the G protein of the vesicular stomatitis virus (VSV) can redirect staphylococcal enterotoxin B (SEB)-activated T cells to kill VSV-infected cells and to inhibit VSV replication in vitro. Inhibition of virus replication in our system is dependent upon the specificity of the Ab for the viral protein. IFN-γ does not play a very important role in this phenomenon, which is mainly mediated by the release of Pfp from CD8+ T cells. We have used a Stat1 knockout mouse model in which VSV infection is lethal. Infusion of staphylococcal enterotoxin B T cells and bispecific Ab significantly slowed virus progression and prolonged the survival of VSV-infected Stat1 knockout mice in vivo.


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3 Abbreviations used in this paper: VSV, vesicular stomatitis virus; KO, knockout; BsAb, bispecific Ab; SEB, staphylococcal enterotoxin B; NaPy, sodium pyruvate; BHK, baby hamster kidney; Iscove’s-HT, Iscove’s medium with 100 mM hypoxanthine and 1 mM thymidine; Pfp, perform; MOI, multiplicity of infection; SEB T cells, SEB-activated T cells; WT, wild-type; MDCK, Madin-Darby canine kidney; PBS-BSA-azide, PBS-BSA (1%) and sodium azide (0.02%); CPE, cytopathic effect.

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in the presence of SEB-activated T cells. In addition, it was extremely potent in inhibiting multicycle replication of VSV in vitro. The Ab alone was incapable of neutralizing virus in the absence of activated CD8 \(^+\) T cells. Interestingly, inhibition of virus replication was dependent on perforin (Pfp) mediated lysis and IFN-γ did not seem to play an important role in it.

To test our system in vivo, we used VSV-infected Stat1 KO mice as a model. Infusion of BsAb and SEB T cells prolonged the life of animals that were given a lethal infection of virus. This is the first evidence that BsAb can be used in vivo as an immunotherapeutic agent to fight virus infection.

**Materials and Methods**

**Viruses and cells**

VSV (Indiana and New Jersey) were expanded on baby hamster kidney (BHK) cells. Supernatants were collected and stored at −80°C. BHK P815 cells were grown in DMEM (Bio-Whittaker, Walkersville, MD), 10% FCS (HyClone, Logan, UT), 1 mM sodium pyruvate (NaPy), 2 mM l-glutamine (Life Technologies, Grand Island, NY), and 50 μg/ml gentamicin (Boehringer Mannheim, Indianapolis, IN) (tissue culture medium). EL4 cells were grown in RPMI 1640 (Life Technologies), 10% FCS (Life Technologies), 100 μg/ml penicillin/streptomycin (Life Technologies), 2 mM l-glutamine (Life Technologies), 1 mM NaPy, 20 mM nonessential amino acids (Life Technologies), and 20 mM HEPES (Life Technologies) (T cell medium). All cells were grown at 37°C, 7% CO₂. Infectivity titers were determined by CPE on crystal violet-stained monolayers of BHK cells using triplicates of supernatants from VSV-infected P815 cells 48 h after infection with the virus. Endpoint virus titers were determined by the interpolation of the dilution that infected 50% of the wells by the method of Reed and Muench (10).

**Mice**

BALB/c mice and CD1 mice were purchased from Charles River Laboratories (Wilmington, MA). Stat1 KO mice were bred in the New York University Animal Facility (New York, NY) as described (3), Pfp KO C57BL/6 and C57BL/6 mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

**BsAb generation**

BALB/c mice were immunized with purified VSV (Indiana) and boosted with the same virus 3 wk later. Splenocytes from these mice were used for the fusion to generate the BsAbs.

F23.1 hybridomas (11) were grown and cloned in the presence of 0.33 M 8-azaguaine (Life Technologies) to select cells that cannot use the salvage pathway for DNA synthesis. F23.1 cells were grown in Iscove’s medium (Life Technologies), 10% FCS (HyClone), 1 mM NaPy, 2 mM l-glutamine (Life Technologies), 20 mM nonessential amino acids (Life Technologies), and 50 μg/ml gentamicin (Boehringer Mannheim) (Iscove’s medium).

Splenocytes from BALB/c mice immunized with VSV (Indiana) were removed and a single cell suspension was prepared. RBC were lysed with 0.17 M tris ammonium chloride (2-ME (Sigma), (tissue culture medium). EL4 (mouse T cell line) were added. Cultures were incubated at 37°C, 7% CO₂ for 36 h. After incubation, cells were resuspended in fresh medium (50 μg/ml) and seeded on a 96-well plate (Falcon). Plates were spun down and supernatants harvested to measure the \(^{51}\text{Cr}\) release in a gamma counter. Target cells incubated with 0.5% Nonidet P-40 (Sigma) were used to determine maximum release, and cells incubated with SEB-activated T cells and medium (repetitively less than 10% of the maximum release) were used to calculate spontaneous release. The percentage of cytotoxicity was calculated by the formula: % cytotoxicity = test release − spontaneous release/maximum release − spontaneous release × 100.

**Inhibition of multicycle virus replication**

A total of 3 × 10⁶ P815 cells were infected in suspension with VSV at an MOI = 0.1 using DMEM maintenance medium. One hour later, 3 × 10⁴ VSV-infected P815 cells with either BsAb 526 (1 μg/ml), SEB-activated T cells (different E:T ratios), or a combination of both were added to each of triplicate wells. The cultures were incubated for 48 h in DMEM maintenance medium. Supernatants from each well were titrated on BHK cell monolayers plated in 96-well plates. BHK cells were incubated with the supernatants for 24 h and then stained with crystal violet for determination of CPE of the monolayers.

**CD4-CD8 depletions**

SEB-activated T cells were prepared as described above. At day 3 of incubation, cells were resuspended in fresh medium (5 × 10⁶ cells/ml) and incubated with either anti-CD4 Ab (GK1.5), anti-CD8 Ab (2.43), both at 10 μg/ml, or medium alone for 30 min at 4°C. Abs were then washed away and cells were incubated with freshly washed anti-IgG-linked BioMag magnetic beads (PerSeptive Diagnostics, Cambridge, MA) (50 beads/cell) for 30 min at 4°C. The depletion of Ab-bearing cells was done by placing the flasks on a magnet (Advanced Magnetics) and collecting unbound cells after washing several times. CD8-depleted, CD4-depleted, and unseparated cells were then used for cytotoxicity and inhibition of virus replication.
cell cultures for inhibition of virus replication (see Fig. 3).

that we had a BsAb that could bind to V

b

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ing of supernatant to purified VSV (Indiana)-coated plates in a

with spleen cells from a BALB/c mouse immunized with VSV

LD 50 was used) diluted in PBS to a final volume of 1 ml/mouse. One hour

b

aminopterin-thymidine)-sensitive F23.1 (anti-V

Production of BsAb 526

Results

Inhibition of multicycle virus replication by SEB and BsAb 526

To determine whether 526 could inhibit multicycle replication of VSV, we added it to VSV-infected P815 cells at concentrations ranging from 2.5 μg/ml to 150 ng/ml with or without SEB T cells. The results in Figure 1B demonstrate that in the presence of 526, SEB T cells can be redirected to inhibit virus replication at concentrations of Abs as low as 600 ng/ml. An irrelevant BsAb (3F12), 526 alone, or SEB alone has no effect on virus replication at any concentration tested.

In the absence of inhibition, slightly higher titers of virus are observed when SEB T cells are present. This possibly results from infection of SEB T cells by VSV.

Cytotoxicity and inhibition of virus replication against an irrelevant VSV subtype

To verify the specificity of 526, we tested it for its ability to lyse target cells infected with another VSV subtype. Cells were infected with either VSV (Indiana) or VSV (New Jersey) and used in a cytotoxicity assay as targets. 526 was only able to mediate lysis of VSV (Indiana)-infected cells (data not shown). These results were confirmed by the demonstration that 526 was able to inhibit multicycle replication of P815 cells infected with VSV (Indiana) but not when the cells were infected with VSV (New Jersey) (Fig. 2).

Is IFN-γ release from SEB T cells responsible for inhibition of multicycle virus replication?

IFN-γ has been reported to play an important role in inhibition of virus replication in some studies [12, 13]. Our SEB T cell cultures secrete very high levels of IFN-γ (data not shown), thus we performed an anti-IFN-γ ELISA (see Materials and Methods) to check how much of the Ab XMG1.2, which has been shown to inhibit the in vivo function of IFN-γ (12), was needed in our assay to bind all the IFN-γ present in our SEB T cell culture. Thus we analyzed what role, if any, IFN-γ might play in inhibition of virus replication by SEB T cells and BsAb. Cultures of SEB T cells, BsAb, and infected P815 were set up in the absence or presence of a neutralizing concentration of anti-IFN-γ Ab (XMG1.2 at 1 mg/ml, threefold in excess of that needed to bind all the IFN-γ present in our SEB T cell culture). The results shown in Figure 3 indicate that inhibition of IFN-γ had no effect on the ability of SEB T cells and BsAb to inhibit virus replication in P815 cells.

Are CD8+ T cells responsible for inhibition of virus replication?

SEB activation leads to maturation of both CD4+ and CD8+ T cells, either of which can be targeted by our BsAb. However, there is evidence that both CD4+ and CD8+ T cells are capable of inhibiting virus replication in vivo (14–19). Thus, either CD4+ or CD8+ T cells or both could be responsible for inhibition of virus replication. Therefore, we depleted SEB T cell cultures of either

Table I. Survival of VSV-infected STAT1 KO mice after different treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Alive/Total†</th>
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<tr>
<td></td>
<td>24 h</td>
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<td>Mock§</td>
<td>18/18</td>
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<tr>
<td>SEB T Cells</td>
<td>16/16</td>
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<td>BsAb§</td>
<td>16/16</td>
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<tr>
<td>SEB T Cells + BsAb</td>
<td>20/20</td>
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† Values are the sum of several experiments.
§ Treatments given i.p. 1 h after infection.
¶ Mice infected i.p. with 1 to 5 LD50 of VSV (Indiana).
¶¶ BsAb used was 526 (anti-TCR + anti-VSV) at 50 μg/mouse.
# The differences in the values are statistically significant (χ² with p < 0.005).

FACS analysis

Samples from CD4-depleted, CD8-depleted and unseparated SEB T cells (3 × 10⁶ cells/sample) were incubated with 100 ng/ml of anti-CD3-FITC (Life Technologies), anti-CD8-PE (Boehringer Mannheim) (100 ng/ml each), anti-IgG2a isotype standard (PharMingen, San Diego, CA) (100 ng/ml), or medium alone for 30 min at 4°C. Abs were then washed away with T cell medium and each pelleted sample was incubated for 5 min at room temperature with 50 ml of 1% paraformaldehyde. Volume of each sample was raised to 0.5 ml with PBS-BSA-azide. Samples were kept at 4°C overnight and FACS analysis was performed in an EPICS Profile Analyzer (Coulter Corporation, Hialeah, FL). On average, 89% of the SEB-activated unseparated spleen cells were CD3⁺, out of which 44% were CD8⁺ and 53% were CD4⁺. After each depletion there were less than 2% residual cells in each case (i.e., after CD8 depletion less than 2% of the cells left were CD8⁺ and after CD4 depletion less than 2% of the remaining cells were CD4⁺).

Anti-IFN-γ ELISA

Immunon-ELISA plates (Dynatech, Alexandria, VA) were coated with XMG1.5 at 5 μg/ml. Control IFN-γ (Boehringer Mannheim) was incubated at 50 ng/ml together with different concentrations of XMG1.5 (0.01–30 μg/ml). XMG1.5 competed the binding of 50 ng/ml IFN-γ to the coating plate at 0.3 μg/ml. Based on these results we used a concentration of XMG1.2 three times higher than needed to block IFN-γ in the SEB T cell cultures for inhibition of virus replication (see Fig. 3).

In vivo infections and treatment of Stat1 KO mice

Stat1 KO mice (generated in David Levy’s laboratory) were infected by i.p. injection with 1 to 5 LD50 of VSV (for each experiment a single LD50 was used) diluted in PBS to a final volume of 1 ml/mouse. One hour after infection, mice were injected i.p. again with either 95 × 10⁶ SEB T cells/mouse (prepared from CD1 mice) only, BsAb 526 only (50 μg/mouse), SEB T cells (95 × 10⁶ cells/mouse) + BsAb 526 (50 μg/mouse), or PBS, all injections in a final volume of 1 ml/mouse. Survival times were recorded in hours postinfection and results are shown in Table I.

Results

Production of BsAb 526

The BsAb 526 was produced by fusion of the HAT (hypoxanthine-aminopterin-thymidine)-sensitive P23.1 (anti-Vβ8) hybridoma with spleen cells from a BALB/c mouse immunized with VSV (Indiana). Hybridomas producing BsAbs were identified by binding of supernatant to purified VSV (Indiana)-coated plates in a RIA. All bispecific hybridomas that bound the virus should also produce anti-Vβ8 since F23.1 was the fusion partner. To ensure that we had a BsAb that could bind to Vβ8 with one arm and to a VSV protein on the surface of infected cells with the other arm, we tested it for its ability to redirect SEB-activated T cells (SEB T cells) to kill VSV-infected cells in a 4-h chromium release assay. This insured that the VSV protein recognized by the BsAb 526 was expressed on the surface of the infected cells. The hybridoma 526 was positive in both RIA and chromium release assays and was cloned. After purification of culture supernatants through protein A, 526 was used for all subsequent assays. BsAb 526 was also tested by immunoprecipitation using lysates from VSV-infected cells and shown to bind to the G protein from VSV (Indiana) (data not shown).

Redirected lysis of virus-infected cells by BsAb 526

Cloned and purified BsAb 526 was then tested for its ability to redirect SEB T cells to kill VSV-infected cells in a 4-h chromium release (cytotoxicity) assay. Figure 1A shows a typical cytotoxicity assay with E:T ratios from 40:1 to 10:1. SEB T cells alone or in the presence of another BsAb, 3F12, specific for Vβ8 and influenza A M2 protein (5), failed to lyse VSV-infected target cells.
CD4\(^+\) cells or CD8\(^+\) cells and tested them in assays of both cytotoxicity and inhibition of virus replication. Figure 4 shows that significant cytotoxicity is only observed with unseparated cells or CD8\(^+\) T cells and BsAb 526. When these different cell populations were mixed with 526 and VSV-infected P815 cells and the release of virus progeny was measured 48 h later, only unseparated and CD8\(^+\) T cells (CD4 depleted) were able to inhibit multicycle virus replication as shown in Figure 4 Inset.

Analysis of the ability of SEB from Pfp KO mice to inhibit multicycle replication

Inhibition of virus replication did not seem to be mediated by IFN-γ release and was dependent on the presence of CD8\(^+\) T cells. Thus, we set out to determine whether Pfp-mediated cytotoxicity was essential for inhibition of virus replication. Spleen cells from Pfp KO mice were cultured for 3 days with SEB and tested for their ability to lyse target cells infected with VSV as well as to inhibit multicycle virus replication. Figure 5A shows that, as expected, Pfp KO SEB T cells were unable to lyse the target cells in the presence of BsAb at an E:T ratio of 20:1 together with BsAbs 526, 3F12, or medium. Supernatants were then collected and the VSV titers determined by inoculation of BHK cell monolayers for 48 h with log 10 dilutions of the supernatants. CPE was observed following crystal violet staining and end points were determined by the method of Reed and Muench (10).
Test of the ability of SEB and BsAb to inhibit virus replication in vivo

VSV is not able to successfully infect normal mice unless inoculated directly into the central nervous system (20). Recently, it has been demonstrated that Stat1 KO mice, which are unable to react to IFN, are extremely sensitive to VSV. These mice, given only a few hundred infectious particles, die within 48 h (3). Therefore, Stat1 KO mice were tested as a model to evaluate the ability of our BsAb and SEB T cells to prolong the survival of mice infected with VSV. Animals were infected by i.p. injection with VSV. Independent experiments were performed using either 1, 2.5, or 5 LD₅₀ per mouse. Mice received SEB T cells only, BsAb only, SEB T cells + BsAb, or PBS and survival was monitored. SEB T cells were prepared from CD1 mice, which is the background strain for the Stat1 KO mice. These effectors were tested in our standard assays and shown to be efficient in inhibition of virus replication as other strains. The results shown in Table I demonstrate that virtually all infected animals given SEB T cells or nothing died within 24 to 48 h after infection. In contrast, infected animals receiving SEB T cells and BsAb began to die only after 48 h. A χ² test showed that the differences in survival rates were statistically significant (p < 0.005). Some prolongation was observed with BsAb

FIGURE 2. Inhibition of VSV replication by SEB T cells (20:1) and 526 (1 μg/ml) using either the Indiana or New Jersey strains of VSV to infect P815 cells. Both VSV strains were used to infect P815 cells at an MOI = 0.1 for 48 h. Virus titers in the supernatants were calculated by performing an infectivity assay on BHK cells for 48 h and then testing for CPE on the BHK monolayers using crystal violet.

FIGURE 3. Inhibition of VSV replication by SEB T cells and 526 in the absence of IFN-γ. SEB T cell cultures (20:1) + 526 (1 μg/ml) were incubated with either XMG1.2 (anti-IFN-γ) at 1 μg/ml or medium and then the mixtures were added to VSV-infected P815 cells (MOI = 0.1). After 48 h, supernatants were used to infect BHK cells for another 48 h and infectivity titers were calculated by staining BHK monolayers with crystal violet.
alone, which is quite surprising since in no in vitro experiments could we demonstrate any neutralizing ability by 526 itself. Thus, SEB T cells and 526 were able to prolong survival of mice infected with a lethal dose of VSV, probably by reducing virus titers and preventing spread to other tissues of the mouse.

Discussion

In this paper we describe and analyze the properties of the monoclonal BsAb 526, which binds the Vβ8 molecule on the TCR of T cells and the G protein of VSV. We have previously generated similar Abs in our laboratory that differ with respect to the virus protein they bind. Two of these BsAbs, HHA6 and 3F12, which have specificity for the influenza virus proteins HA and M2, respectively, were tested for their ability to redirect SEB-activated T cells to kill virus-infected cells and to inhibit influenza virus replication (5–7). Our results with the influenza virus model demonstrated that BsAbs and SEB-activated T cells were capable of inhibiting multicycle virus replication in vitro. Both HHA6 and 3F12 could redirect SEB T cells to inhibit influenza virus replication in tissue culture (5–7). One of the drawbacks of the influenza system was that multicycle virus replication could only be achieved using xenogeneic cells (MDCK or Madin-Darby bovine cells). Thus, cytokine and/or adhesion molecules with species specificity might not be functional in this system leading to suboptimal inhibition. In addition, this might not accurately simulate the functioning of BsAb in vivo, the ultimate goal of these studies. Nevertheless, we observed that the addition of BsAb and SEB T cells to influenza virus infected...
MDCK cells reduced virus titers by two log_{10} units (3F12) throughout the 48-h infection. These results encouraged us to develop a syngeneic system to further study this inhibition of virus replication by non-virus-specific T cells and BsAbs.

P815, a mouse mastocytoma (DBA/2) was used for VSV infection. Thus, we now had a completely syngeneic system to study inhibition of virus replication in vitro. The BsAb 526, which recognizes the TCR and the G protein of VSV, was tested for its ability to redirect SEB T cells to kill VSV-infected cells and to inhibit VSV replication in P815 cells. As expected, 526 could efficiently do both and, in fact, the inhibition of virus replication was greater in this model than with influenza virus. We proceeded to use this model to test the requirements for inhibition of virus replication in vitro and in vivo.

Many groups, using various systems, have shown that both CD4^{+} and CD8^{+} T cells are able to clear virus infections in vivo (14–19). Since SEB activates both subsets of T cells we were able to compare the ability of each to inhibit virus replication directly. This was particularly interesting since both CD4^{+} and CD8^{+} T cells inhibited multicycle replication of influenza virus in our system, and cytotoxicity did not correlate with inhibition (6). Thus, we tried to manipulate the system to test which T cell population

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** A, Cytotoxicity assay by SEB T cells from either C57BL/6 (WT) mice or Pfp-deficient mice (Pfp KO) redirected by 526 (1 μg/ml). EL4 cells infected with VSV (MOI = 40) for 1 h and chromium labeled for another hour were incubated 4 h with 526 (1 μg/ml) and SEB T cells from either C57BL/6 (WT) mice or Pfp KO mice. The percentage of cytotoxicity was calculated from the supernatants. When medium was used instead of BsAb, the percentage of cytotoxicity ranged between 0 and 6%. Effector cells were used at 20:1 and 40:1 E:T ratios. B, Inhibition of VSV replication in P815 cells by SEB T cells from WT mice or Pfp KO mice (E:T 20:1) or no SEB T cells redirected by 526 (1 μg/ml). P815 cells infected with VSV (MOI = 0.1) were incubated 48 h with SEB T cells from C57BL/6 (WT) mice or Pfp KO mice, or medium together with 526 (1 μg/ml), or medium. Supernatants were used to infect BHK cells for 48 h and virus titers were calculated by CPE.
was capable of inhibiting VSV replication. In contrast to the influenza virus model, with VSV we observed that inhibition of virus replication was mediated by CD8⁺ T cells, dependent on Pp-mediated cytotoxicity, and IFN-γ independent.

When we infected Stat1 KO mice with a lethal dose of VSV and treated them with a single injection of SEB T cells and 526, we prolonged the life of the mice for at least 48 h compared with controls. Although mice were not cured from our single injection (data not shown), the fact that we observed such a difference in survival suggests that BsAbs are able to inhibit virus replication in vivo. The fact that we were unable to completely cure the mice may result from the failure of Stat1 KO mice to generate a normal cell-mediated immune response. Even very small numbers of residual virus particles could lead to death since Stat1 KO mice infected with a nonlethal influenza virus fail to adequately generate a proinflammatory response, and demonstrate impaired virus clearance. In these Stat1 KO mice as little as 100 VSV infectious particles can be lethal.

It should be noted that BsAbs alone also prolonged the lives of Stat1 KO mice infected with VSV, although to a lower extent than in combination with SEB T cells. As can be clearly seen in Figures 1B, 3, 4B, and 5B, 526 alone, or when bound to CD8⁺ depleted effector cells, is not able to inhibit virus replication. Yet, in vivo it has some inhibitory activity. This may result from the ability of the bifunctional Ab to activate the complement cascade and its opsonizing and ADCC activity due to its isotype (IgG2a). Effects of BsAbs alone have been observed by others in an anti-tumor model (21, 22). Also, Stat1 KO mice infected with influenza virus had good levels of Ab redirected cytotoxicity in P815 cells with an anti-TCR Ab, which may suggest that self T cells from Stat1 KO mice could be bound by 526 and be responsible for the inhibition of VSV replication in vivo. We are presently attempting to determine which of these mechanisms may be responsible for this effect.

Our view of BsAbs + SEB T cells is that they can efficiently inhibit virus replication, but probably will not remain active for a prolonged period. This may result from modulation of BsAb from the surface of the T cells or apoptosis of the SEB-activated cells (23). It is possible that repeated injections of cells might function to reduce the virus load and, in the presence of an intact immune system, protect animals from consequences of virus infection. It is also possible that BsAbs could redirect preexisting CTLs of different specificities (i.e., specific for existing latent viruses such as herpes viruses) to be used as effectors for redirected lysis of virus-infected cells. Fast spreading viruses like VSV will clearly kill infected animals in the absence of innate immunity. Thankfully, such viruses also seem exquisitely sensitive to the effects of innate immunity.

Another important aspect of this work is that it demonstrates that non-virus-specific T cells can inhibit virus replication when redirected by BsAbs in vitro and in vivo. It has been suggested that neither cytotoxic T cells, Pp, nor fas ligand plays a role in recovery from VSV in normal animals (24). The fact that the virus-bearing costimulatory molecules or inhibition of the generation of immunity by elaboration of factors as has been shown for a number of herpes viruses or inhibition of MHC presentation (27, 28). The use of BsAbs in virus infections has been more limited, but there have been successful results as well against HIV (25, 26, 37, 38) and in targeted delivery of adenoviruses (39). Our in vivo results encourage further pursuit of their therapeutic use in experimental models of virus infection.

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References


