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ANALYSIS OF THE IMMUNE RESPONSES AGAINST INFLUENZA AND VESICULAR STOMATITIS VIRUS (VSV) USING BISPECIFIC ANTIBODIES (BsAbs) AND CYTOKINES.

by

Ana Fernandez-Sesma.

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

ANALYSIS OF THE IMMUNE RESPONSES AGAINST INFLUENZA AND VESICULAR STOMATITIS VIRUS (VSV) USING BISPECIFIC ANTIBODIES (BsAbs) AND CYTOKINES.

by

Advisor: Thomas Moran Ana Fernandez-Sesma.

The immune response against virus infections is dictated by the way viral antigens are presented to the cells of the immune system and by the cytokine context in which the infection takes place. Th1 or inflammatory immune responses in which infected cells are eliminated by T cells are believed to be optimal to clear virus infections, while Th2 immune responses are not believed to be responsible of fighting viruses. BsAbs which bind some molecule on the T cell receptor of T cells with one arm and a viral protein on the other arm can be used to redirect T cells not specific for that particular virus to kill virus infected cells.

In this thesis I show results using bispecific antibodies that bind the T cell receptor on T cells and proteins of either influenza virus or VSV to inhibit virus replication. This system allowed me to study inhibition of virus replication by non virus specific T cells which can clear virus infections without standard antigen presentation. Both BsAbs worked *in vitro*, and one of them, 526, could redirect superantigen activated T cells to prolong the life of mice lethally infected with VSV.

The study of the immune response in Stat1 KO mice after influenza virus infection, showed a mixed response which was slightly biased towards Th2. These

mice were unable to clear virus infection.

I also studied the generation of immunity by mice infected with influenza virus under different cytokine treatments. When IL-4 was added at the time of infection, mice could not generate cytotoxic T cells against influenza virus and the cytokine profile in supernatants from spleen cultures was clearly of the Th2 type.

When different cytokines were used in combination with either live or inactivated influenza viruses for vaccinations the Th1 response generated by live virus could be reversed to Th2 by the addition of IL-4, unprotective upon challenge with live virus. The addition of IL-12 to inactivated virus switched the response of vaccinated mice from a clear Th2 to a more Th1 type, protective after challenge.

This work proves the importance of generation of an adequate immune response against viruses for efficient clearance of viruses, and how limitations that may exist in some organisms can be overcame to efficiently fight these infections.

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I also have to thank another collaborator: Dr. David Levy, who provided us with an *in vivo* model for looking at the immunity to virus infections as well as to test our bispecific antibodies.

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Finally, I would like to dedicate my thesis to my parents, who always encouraged me to study hard and learn English and whose sacrifices have finally been rewarded, and to my husband, Adolfo Garcia Sastre, who is the most patient listener and best advisor and companion I could ever have.

Format of thesis

This thesis was prepared in accordance with guidelines of City University of New York.

Chapter 1 contains general methods and materials used in this thesis. Each one of the chapters containing results also contain specific materials and methods for that particular chapter.

Chapter 2 shows part of the results published as Fernandez-Sesma et al., 1996.

Chapter 3 contains results from Fernandez-Sesma et al., 1997, in press (Journal of Immunology).

Chapter 4 shows results published as part of Moran et al., 1996.

Chapter 6 contains results which are part of Durbin et al.,1997, submitted for publication. **Chapter 7** contains part of the results of Moran et al., 1997, manuscript in preparation.

In **chapters 2, 3, 4, 5, 6**, and **7** there is a brief introduction and discussion. There is a general introduction at the beginning of the thesis and a general discussion at the end. "References" have been consolidated and put in the respective chapter. Figures and tables are shown at the end of each chapter.

List of Manuscripts and publications.

Piccone, M. E., Fernandez-Sesma, A. and P. Palese. 1993. Mutational analysis of the influenza virus VRNA promoter. *Virus Res.*, 28(2):99-112.

Fernandez-Sesma, A., Schulman, J. L. and T. M. Moran. 1996. A bispecific antibodie recognizing influenza A virus M2 protein redirects effector cells to inhibit virus replication *in vitro*. *J. Virol.*, 70(7):4800-4804.

Moran, T. M., Isobe, H., Fernandez-Sesma, A. and J. L.Schulman. 1996. Interleukin-4 causes delayed virus clearance in influenza virus-infected mice. *J. Virol.*, 70)8):5230-5235.

Fernandez-Sesma, A., Peluso, R. W., Bai, X., Schulman, J. L., Levy, D. E. and T. M. Moran. 1997. Superantigen activated T cells redirected by a bispecific antibody inhibit VSV replication *in vitro* and *in vivo*. In press (*J. Immunol.*).

Durbin, J. E., Fernandez-Sesma, A., Garcia-Sastre, A., Rao, T. D., Frey, A. B., Moran, T. M. and D. E. Levy. 1997. Essential role of Stat1 in repression of Th2-mediated response to influenza virus infection.. (Submitted for publication).

Moran, T. M., Park, H., Fernandez-Sesma, A. and J. L.Schulman. 1997. Inactivated influenza virus elicits a Th2 immune response. (Manuscript in preparation).

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Chapter 1

INTRODUCTION

IMMUNITY TO VIRUS INFECTION

1. Innate Immunity

The specific immune response initiated following virus infection is comprised of both antibody formation and cellular immunity (Doherty and Zinkernagel, 1974, Cambridge et al., 1976, Doherty et al., 1992, Berke, 1995, Abbas et al., 1996, Zinkernagel, 1996). In the interval prior to maturation of the specific immune response, innate immunity plays an important role in slowing or preventing virus replication. Among the components of innate immunity which function against virus infection are interferon and NK or natural killer cells (Staeheli, 1990, Brutkiewicz and Welsh, 1995). Type I interferon is released by cells following virus infection and binds to specific receptors on most cells. Interferon acts on other cells inducing them to synthesize "antiviral proteins" such as the Mx protein, P1/eIF-2α protein kinase and 2'-5'oligo(A) that serve to inhibit replication of viral proteins (Staeheli, 1990). Other important elements of the innate immune response are the NK cells, which kill virus infected cells and release cytokines such as gamma interferon prior to the appearance of specific cytotoxic T cells (Brutkiewicz and Welsh, 1995). These two elements of the innate immune response probably function to slow virus progression until specific immunity develops. The activation of the alternative pathway of complement is very

important for immunity to bacterial infections, and it may play some role in inhibiting enveloped viruses (Lefrancois, 1984).

2. Acquired Immunity

After infection by a pathogen, antigens are presented by antigen presenting cells (APCs) to the T cells in the context of major histocompatibility complexes (MHC) class I and class II molecules. Antigens presented by APC using MHC class I are recognized by the T cell receptor (TCR) on $CD8^+$ cytotoxic T cells and antigens presented through MHC class II are recognized by the TCR on $CD4^+$ T cells (Morrison et al., 1986). Evidence suggests that professional APCs probably are needed to present to both $CD4^+$ and $CD8^+$ cells since activation requires co-stimulation which is provided only by such cells (Schwartz, 1992, Bluestone, 1995). Yet the requirements for presentation by MHC class I are different from those for MHC class II. Class II presentation occurs when antigen is taken up by an endosomal pathway, broken into peptides which bind to empty class II molecules which are returned to the cell surface to interact with the TCR of a $CD4^+$ T cell. This pathway is known as the exogenous pathway (Morrison et al., 1986). Class I presentation occurs primarily by endogenous processing. This pathway presents only proteins found in the cytoplasm of a cell. Cytoplasmic proteins, both self and those produced by invading organisms are broken down by an enzymatic organelle known as the proteasome into short peptides, transported to the endoplasmic reticulum where they bind to nascent class I molecules and proceed to the surface (Morrison et al., 1986, Long and Jacobson, 1989, Rock et al., 1994). The MHC class

I-peptide complex interacts with $CD8^+$ T cells that mature into cytotoxic cells. $CD8^+$ cytotoxic T cells (CTLs) once activated gain the ability to lyse virus infected cells expressing the viral peptide in the MHC class I context (Morrison et al., 1986, Townsend, 1986, Long and Jacobson, 1989, Abbas et al., 1996, Zinkernagel, 1996).

CTLs lyse virus infected cells by the release of granules containing complement like molecules known as perforin. Alternatively, they bind factors expressed on the surface of the infected cell such as Fas through their membrane bound Fas-ligand, which trigger the infected cell to programmed cell death (reviewed in Berke, 1995, Kägi et al., 1995, Nagata and Suda, 1995, Atkinson and Bleackly, 1997). CTLs can sometimes be responsible for the pathology associated with virus infection (Doherty and Zinkernagel, 1974). Non-cytotoxic mechanisms for clearance of virus by $CD8^+$ T cells have been documented. In these cases the release of cytokines such as IFNγ and TNFα by $CD8^+$ cells has been implicated (Guidotti et al., 1996)

Although CTLs cells are believed to be primarily responsible for clearing virus infections, in some experimental cases such as the use of mice lacking $CD8^+$ T cells, $CD4^+$ T cells have been shown to do it, but the mechanism is not clear (Lukacher et al., 1984, Eichelberger et al., 1991, Sarawar et al., 1994). The restriction of $CD4^+$ cells for MHC class II makes it difficult to understand how they are able to clear virus from class II negative epithelial cells. Evidence suggests that their effector function is dependent on bone marrow derived class II bearing cells (Topham et al., 1996).

Antibodies are important for virus neutralization, but their effectiveness is limited to preventing primary cell infection or following release from infected cells (Wilson and Cox, 1990, Liang et al., 1993, Palladino et al., 1995). Some viruses which spread directly from cell to cell avoid even this effect. Once viruses infect, only $CD8^+$ cytotoxic T cells are able to fight them by killing the infected cells (reviewed in Koup, 1994, Berke, 1995 and Zinkernagel, 1996, Welsh et al., 1997, Zajac et al., 1997). The isotypes of antibodies determine their function, such as the capability to activated the complement cascade, function as opsonins, in anti-parasitic immunity, or antibody dependent cellular cytotoxicity (ADCC), etc. (Konterman et al., 1997). The isotype of antibodies produced following exposure to immunogen is directly determined by the cytokine environment created by antigen specific $CD4^+$ T cells (Spriggs et al., 1992, Taylor, 1995, reviewed in Abbas et al., 1996).

CD4 subsets and their functions.

When $CD4^+$ T cells are activated, they secrete soluble factors that act to influence and direct other cells of the immune system. $CD4^+$ T cells or helper cells (Th) are the central element in the immune system, since all specific immune reactions are mediated by the cytokines they release. Naïve $CD4^+$ cells (Th0) can be directed to become either Th1 or Th2 cells. The commitment into Th1 or Th2 is dictated by the cytokine milieu at the time of antigen presentation to the common precursor Th0 cell. This cell type secretes a mixture of Th1 and Th2 cytokines including IL-2 (Salgame et al., 1991, Swain et al., 1991, Paul and Seder, 1994, Seder and Paul, 1994, Mosmann

and Sad, 1996). Cytokines such as IFNγ and IL-2 are released by Th1 cells and promote the generation of CTLs, activate NK cells, and direct B cells to class switch to isotypes of antibodies that activate the complement cascade, such as IgG2a in mice(Zinkernagel, 1996, Coutelier et al.,1987, Bossie and Vitteta, 1991). Th2 cells secrete cytokines such as IL-4 and IL-5. Immune responses generated by these cells lead to the release of non-complement activating antibody isotypes such as IgG1 and IgE and proliferation and migration of eosinophils (Finkelman et al., 1990, Mosmann and Sad, 1996). Cytokines secreted by Th1 cells inhibit the secretion of cytokines by Th2 cells and Th2 cytokines act on Th1 cells to inhibit their cytokine release (Swain et al., 1991). Evidence suggests that release of IL-12 directs Th0 cells to become Th1 while the presence of IL-4 in the milieu during APC-T cell interaction induces Th0 cells to become Th2 (reviewed in Mosmann and Sad, 1996).

Dendritic Cells and Th1/Th2 decision

Dendritic cells are thought to be the primary APCs in virus infections (reviewed in Pennisi, 1996, Ridge et al., 1996). They express both MHC class I and class II proteins and also express so called costimulatory molecules, namely B7.1, B7.2. These costimulatory molecules bind their counterparts on T cells (CD28) and provide a second signal for T cell activation. The first signal for T cell activation is the binding of the TCR on the T cell to MHC molecules containing antigenic peptide. Both signals start a cascade of events in the T cell that culminate with the expression of genes

encoding cytokines ($CD4^+$ T cells) or pore forming soluble factors such as perforin or granzymes ($CD8^+$ T cells) (Schwartz, 1992).

Recent work has shown that dendritic cells have a unique maturational program which seems to be triggered by a number of factors (Heufler et al., 1996, Winzler et al., 1997). Stage 1 dendritic cells take up antigen aggressively, are immobile, poor T cell stimulators, and do not release IL-12. Interaction with a number of factors such as LPS, TNFα, CD40 ligand and probably live virus, induce stage 2 of the maturation pathway. The stage 2 cells no longer take up antigens, are mobile, probably migrate to lymph nodes, strongly increase expression of costimulatory molecules, and are excellent stimulators of T cells. Most importantly, the cells now spontaneously release IL-12. The final stage is programmed cell death.

It is not clear what determines the cytokine context at the time of antigen presentation by APCs. Any pathogen which infects aggressively should alert the Th1 immune response and promote an inflammatory environment (Doherty et al., 1992, Coutelier et al., 1994, Kägi et al., 1995, reviewed in Abbas et al., 1996, Welsh et al., 1997, Zajac et al., 1997). The fact that bacteria and bacterial substances, inflammatory cytokines, and live viruses have all been shown to induce IL-12 release from dendritic cells suggests that this may be the key event in driving Th1 immune response(Heufler et al., 1996, Ohshima and Delespesse, 1997, Winzler et al., 1997). This is supported by the recent discovery that the high affinity receptor for IL-12 found only on Th1 cells stimulates their proliferation and the subsequent release of IFNγ. The high affinity

receptor for IFNγ is found only on Th2 cells and induces apoptosis (Welsh et al., 1997). In general Th2 immune responses do not promote inflammation (Swain et al., 1991, Seder and Paul, 1994, Mosmann and Sad, 1996, reviewed in Abbas et al., 1996). It is not known what cell is responsible for secretion of IL-4 but it is not the dendritic cell.

Release of IL-12 from dendritic cells has been shown to be mediated by binding of NFkB in the IL-12 promoter region (Murphy et al., 1995). This pathway is known to be activated by bacterial substances such as LPS and cytokines such as TNFα (Baeuerle and Henkel, 1994, Winzler et al., 1997). IL-12 release following virus infection is likely to be mediated by similar events. This was illustrated by results showing that synthesis of influenza virus hemagglutinin (HA) activates NFkB even when the gene was transfected by itself into 293 cells (Pahl and Baeuerle, 1995). The fact that no viral proteins are produced when inactivated virus is used as an immunogen may explain its failure to elicit a Th1 response.

3. Bispecific antibodies and redirected lysis

There is a time lag from the point that the immune system detects an infectious agent to the generation of effective specific immunity. In the case of virus infections, specific antibodies and CTLs begin to appear after 3 days. Innate immune mechanisms such as interferon production and the activation of NK cells function during this interval to slow the progress of the infection. These innate mechanisms are easily evaded and many viruses primarily replicate during this interval (Koup, 1994, Gilbert

et al., 1996, Zinkernagel, 1996). One theoretical way to use cellular immunity to attack viruses during this early period is with the use of bispecific antibodies (BsAbs). Bispecific antibodies are antibodies with two different binding sites. They can be produced either by chemically linking two antibodies together or by hybridoma fusion. Bispecific antibodies with specificity for both the T cell receptor (TCR) and for a viral protein expressed on the surface of infected cells (Staerz and Bevan, 1986, Lanzavecchia and Scheidegger, 1987, Fanger et al., 1991, Fanger et al., 1992), are able to trigger T cell effector functions and unleash them on the infected cell. Bispecific antibody mediated killing of infected cells by non virus-specific T cells is called redirected lysis (Fanger et al., 1991, Fanger et al., 1992) and when successful can prevent the spread of the virus and allow the immune system time to mount a proper immune response.

In this thesis I report work done with such antibodies, which were generated to bind proteins of influenza and vesicular stomatitis (VSV) viruses with one arm and Vβ8 on the TCR of T cells with the other arm. In both cases the antibodies were able to redirect superantigen activated T cells to kill virus-infected cells and to inhibit virus replication. This allowed me to study different aspects of inhibition of virus replication by T cells, especially in the VSV system, in which we had a syngeneic system. 3F12, which binds the Vβ8 molecule of the TCR on T cells and the highly conserved influenza virus M2 protein was able to redirect staphylococcal enterotoxin (SEB) activated T cells to inhibit influenza virus replication in tissue culture regardless of the

influenza A virus strain used to infect. Bispecific antibody 526, which binds the Vβ8 chain with one arm and the G protein of VSV (Indiana) with the other arm, was able to redirect SEB activated T cells to totally inhibit VSV replication in tissue culture. It could also redirect SEB T cells to prolong the life of mice lethally infected with VSV. With the use of BsAbs we were able not only to successfully fight virus infections with non-virus specific T cells, but also to study the mechanism by which T cells clear virus from infected cells.

4. Stat1 knockout mice

Stat1 KO mice (Durbin et al., 1996) were used as an *in vivo* model to test our BsAbs. These mice are unable to fight virus due to the lack of the Stat1 protein. Stat1 is a signal transducer and activator of transcription. It is associated with the receptors for interferon and becomes phosphorylated upon binding of their specific ligand. Mice deficient in Stat1 have been shown to be very sensitive to virus infection (Durbin et al, 1996, Meraz et al, 1996). We used these mice as a model system for virus infection and demonstrated that we could prolong the life of mice lethally infected with VSV with the use of BsAb 526 and SEB-activated T cells. Also, when Stat1 KO mice were infected with an attenuated strain of influenza virus and their spleen cells cultured, we observed that their cytokine profile and their serum antibody isotypes were mainly of the Th2 type. On the other hand, Stat1 KO mice were able to generate CTLs to levels comparable with WT mice, which is an indicator of Th1 responses. These mice had a

mixed Th1-Th2 immune response after influenza virus infection as they did develop CTL but they could not effectively clear the virus.

5. Effect of Cytokines on Anti-viral Immunity and Immunization

Another aspect of this thesis was to investigate the immune response generated following virus infection in the presence of the Th2 inducing cytokine, IL-4. We created a Th2 environment by the injection of IL-4 into influenza virus infected mice, and monitored the immune response generated by these mice. These results show that the proper immune response to clear virus infection is of the Th1 type, as well as the importance of the cytokine context during infection and vaccination.

In most instances, the ideal vaccination protocol should mimic real infection without being detrimental to the organism. We analyzed the type of immune response elicited by live and inactivated influenza virus immunization. Live virus primed for a Th1 response and provided solid cellular immunity which speeds recovery upon infection with a virus of a different subtype, a phenomenon known as heterosubtypic immunity (Schulman and Kilbourne, 1965, Liang et al., 1993). Inactivated virus resulted in Th2 immunity and provides no such protection. The addition of IL-12 to inactivated virus led to the generation of Th1 immunity and heterosubtypic immunity when the mice were infected with a serologically distinct virus subtype.

This thesis is a compilation of different results which stress the importance of the generation of the correct type of immune response and its dependence on the proper

cytokine environment. We also show that BsAbs can be utilized as immunotherapy to inhibit the replication of virus *in vivo*.

Chapter 2

GENERAL MATERIALS AND METHODS.

Viruses.

Influenza viruses A/PR8/34 (PR8), H1N1 subtype, A/UDORN/72 (Udorn), H3N2 subtype, A/JAPAN/305/57 (Japan), H2N2 subtype, A/HK X-31 (X31) H3N2 subtype and B/Panama were grown for 48 hours in 9 day-embryonated eggs at 37^{0}C, (A/BEIJING/93 (Beijing), H3N2 subtype, was grown at 35^{0}C). Allantoic fluid was harvested and stored at -80^{0}C. A/WSN/33 (WSN),H1N1 subtype, viruses were grown in Madin-Darby bovine kidney cells (MDBK) for 48 hours at 37^{0}C. Supernatants were collected and stored at -80^{0}C.

Vesicular Stomatitis viruses (VSV) (Indiana and New Jersey) were expanded on Baby Hamster Kidney (BHK) cells. Supernatants were collected and stored at -80^{0}C. Infectivity titers were determined by CPE on crystal violet stained monolayers of BHK cells using different dilutions of supernatants from VSV-infected P815 cells 48 hours 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.

Cells.

MDBK cells were grown in REM (Bio Whitaker), 10% fetal calf serum (FCS) (Hyclone), 5% $NaHCO_3$, 0.2 M NaH_2PO_4, and 100 μg/ml penicillin/streptomycin (Gibco). Madin-Darby canine kidney cells (MDCK), P815 and Bneo cells were grown in tissue culture medium = DMEM (Bio Whitaker), 10% FCS (Hyclone), 1mM Na

pyruvate (NaPy), 2mM L-glutamine (Gibco) and 50 μg/ml gentamicin (Boehringer Mannheim). EL4 cells were grown in T cell medium = RPMI (Gibco), 10% FCS (Gibco), 100 μg/ml penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco), 1mM NaPy, 20mM non essential amino acids (Gibco), and 20mM HEPES, (Gibco). All cells were grown at 37°C, 7% CO_2.

Monoclonal antibodies (mAb).

F23.1 is an antibody specific for the Vβ8 molecule in the TCR of T cells (Staerz and Bevan, 1986), XMG1.2 (American Type Culture Collection Rockville, MD) (ATCC), is a neutralizing anti- IFNγ antibody (Ozmen et al., 1995), BVD4-1D11.2, BVD6-24G2.3 are anti-IL-4 monoclonal antibodies (DNAX), 11B11 is an anti-IL-4 mAb from ATCC. GK1.5 (ATCC) binds mouse CD4 and 2.43 (ATCC) recognizes mouse CD8. 3F12 is an anti-influenza virus M2 protein + anti-Vβ8 (TCR) BsAb previously generated in our lab (Fernandez-Sesma et al., 1996). The BsAb 526, as described in this publication, is specific for the G protein of VSV and the Vβ8. 526 is not neutralizing by standard methods.

Cytokines.

IL-12 was kindly supplied by Dr. M.K. Gately, (Hoffmann-La Roche, Nutley, NJ) and the IL-4 was purchased from Intergen, Purchase, NY or provided by Dr. S. Narula, Schering Plough, Kenilworth, NJ

Mice.

BALB/c mice and CD1 mice were purchased from Charles River Laboratories. Stat1 KO mice were bred in the New York University Animal Facility as described (Durbin et al., 1996), Pfp (perforin) KO C57Bl/6 and C57Bl/6 mice were purchased from Jackson Laboratories, Bar Harbor, ME. Mice were used after 8 weeks of age.

Immunization of mice.

BALB/c mice (Charles River Laboratories) were immunized with the KLH-linked M2 peptide SLLTEVETPIRNEWGCRCND, (Lamb et al 1985), or purified VSV (Indiana) and boosted with the same peptide or purified virus 3 weeks later. Splenocytes from these mice were used for the fusion to generate the BsAbs.

Bispecific antibody generation.

F23.1 hybridomas (Staerz and Bevan, 1986) were grown and cloned in the presence of 0.33 M 8-azaguanine (Gibco) to select cells that cannot use the salvage pathway for DNA synthesis. F23.1 cells were grown in Iscove's medium = Iscove's (Gibco), 10% FCS (Hyclone), 1mM NaPy, 2mM L-glutamine (Gibco), 20mM non-essential amino acids (Gibco) and 50 μg/ml gentamicin (Boehringer Mannheim).

Splenocytes from a BALB/c mice immunized with either the M2 peptide (generation of 3F12 hybridoma), or purified VSV (generation of 526 hybridoma) were removed and a single cell suspension prepared. Red blood cells were lysed with 0.17M tris ammonium chloride. $100x10^6$ spleen cells were fused to $10x10^6$ F23.1 cells by adding 1 ml of 50% poly ethylene glycol (PEG) (Sigma) in serum free DMEM dropwise during 1 minute while gently agitating the tube. Cells were allowed to sit for

1 minute and then 10 ml of serum free DMEM were added dropwise for 1 minute. Cells were spun down and resuspended in Iscove's+HT medium = Iscove's medium with 100mM hypoxanthine (Sigma) and 1mM thymidine (Sigma), (HT) and were plated in 96 flat bottom well plates (Falcon) at a concentration of $2.2x10^5$ cells/well (in 100 µl) and incubated at 37^0C, 7% CO_2 . 24 hours after the fusion 100 µl/well of 0.1% azaserine (Sigma) in Iscove's + HT were added. Cultures were incubated for 12-14 days before the supernatants were screened.

Radioimmunoassay (RIA).

For screening the hybridomas, 96 well flat-bottom micro Test III™ plates (Falcon) were coated with either the M2 peptide SLLTEVETPIRNEWGCRCND at 2.5 µg/ml or purified VSV at 10 mg/ml for 90 minutes at room temperature or overnight at 4^0C. After washing, plates were blocked for 30 minutes with PBS-1% BSA (PBS-BSA), sodium azide (0.02%),(PBS-BSA+AZIDE). Supernatants from hybridomas or purified antibodies (5 µg/ml) were added and incubated at room temperature for 90 minutes. NaI^{125} (NEN) labeled goat anti-mouse IgG Ab (50,000 CPM in 50 µl) was used for detection after washing away unbound antibodies. After 90 minutes of incubation, plates were washed, dried and counted in a γ-counter.

SEB activation of T cell cultures.

Spleens from naive BALB/c, C57Bl/6 , perforin (Pfp) KO C57Bl/6 or CD1 mice were removed, red blood cells were lysed with 0.17M tris ammonium chloride and single cell suspensions were prepared in T cell medium. $90x10^6$ cells were treated with γ-

irradiation for 5 minutes (1200 rads). The irradiated cells (stimulators) were exposed to 125 μg of staphylococcal enterotoxin B (SEB), (Toxin Technology, Sarasota, FL) for 30 minutes at 37^{0}C ,7% CO_2. Then 60x10^6 non-irradiated, (responder) cells were added to the stimulator cells + SEB and the culture was brought up to 25 ml with T cell medium containing 50 mM 2-mercaptoethanol (2ME) (Sigma), (final concentration of SEB 6 μg/ml) and incubated at 37^{0}C, 7% CO_2 for 36 hours.

Cytotoxicity assay with BsAbs and SEB activated T cells.

For experiments shown in chapter 3 Bneo cells were infected with PR8, WSN, UDORN, or BEIJING viruses at a MOI of 5 for 6 hours in a 37^{0}C water bath using maintenance medium = DMEM with 0.35% bovine albumin (ICN), 0.12% $NaCO_3$, 100 μg/ml penicillin/streptomycin . If only PR8 virus was used, the time of infection was 60 minutes. For experiments shown in chapter 4, EL4 cells were infected with either VSV (Indiana) or VSV (New Jersey) in maintenance medium for 1h at 37^{0}C at an MOI=40. In either case infected cells were washed and each 10^6 cells were labeled with 100 mCi (^{51}Cr) sodium chromate (NEN) for 60 minutes in a 37^{0}C water bath. After washing, cells were incubated for 4 hours in the presence of SEB activated T cells at various E:T ratios and bispecific antibodies at different concentrations, hybridoma supernatants or medium. DMEM with 5% FCS was used as incubation medium (assay medium) in 96 V-bottom microtiter plates (Dynatech laboratories). Plates were spun down and supernatants were harvested to measure the ^{51}Cr release in a γ-counter. Target cells incubated with 0.5% NP-40 (Sigma) were used to determine

maximum release, and cells incubated with SEB activated T cells and medium (routinely less than 10% of the maximum release) were used to calculate spontaneous release. The % of cytotoxicity was calculated by the formula [%cytotoxicity=test release-spontaneous release/maximum release-spontaneous release x 100].

PR8 virus infectivity titrations in tissue culture.

Influenza A/PR/8/34 (H1N1) virus was grown in the allantoic cavity of embryonated hen eggs and stored at -80^0 C. Viruses were titrated by infection of MDCK as described (Moran et al., 1991). Briefly, flat bottom 96 well plates were seeded with 3 X 10^4 cells per well and 24 hours later, the serum containing medium was washed away and 25µl of seed virus or lung homogenate, diluted from 10^{-1} to 10^{-8} were inoculated into triplicate wells. After a 45 minute incubation, 175µl of maintenance medium with 2.5ug/ml of trypsin (Worthington Chemical, NJ) were added to each well. Following a 48 hour incubation at 37^0 C, the presence or absence of virus was determined by hemagglutination of chicken red blood cells. The virus titers were determined by the interpolation of the dilution endpoint that infected 50% of wells by the method of Reed and Muench. In some cases results were confirmed by injection of lung dilutions into 9 day embryonated eggs, incubating 40 hours and checking allantoic fluid for hemagglutinating activity.

Aerosol infections of mice.

For *in vivo* experiments, eight to ten week old BALB/c mice were infected in an aerosol chamber as previously described (Schulman, 1967). Briefly, a 1:1000 dilution

of PR8 seed virus was diluted in normal saline and added to a glass nebulizer (Tri-R Instruments) connected to an aerosol chamber. For each infection 10 ml of the virus mixture were added to the nebulizer and the mice were left in the chamber for thirty minutes. Each mouse is calculated to have been exposed to 7500 $TCID_{50}$ during the thirty minute exposure. The virus is pretitered to be certain that 100% of the mice are infected. This leads to lung titers between 10^5 and 10^6 in 25 μl on day 3 following infection.

Cytotoxicity assays with spleen cells from influenza virus infected mice.

For this assay, effectors from secondary *in vitro* cultures were mixed in round bottom 96 well plates with ^{51}Chromium labeled P815 target cells. One million cells were infected with PR8 virus by incubation with 50μl of allantoic fluid virus(2.8 X 10^9 $TCID_{50}$/ml) diluted 1:2 with maintenance medium (MOI of 5). Cells were incubated 45 minutes in a 37° C water bath, washed and resuspended in 100 μl (1mCi/ml) sodium chromate (NEN) for 1 hour. Cells were washed and added to the wells. The T cells were added at various E/T ratios 20:1,40:1 and 80:1 after which the plates were incubated for 4 hours, harvested and counted. In all cytotoxicity assays, 5 X 10^3 target cells were added to each well. Maximum release was measured from wells incubated with 0.5% NP40, and spontaneous release from wells with medium alone. Percent cytotoxicity was calculated using the formula % cytotoxicity = test release-spontaneous release/maximum release-spontaneous release X 100. In all cases uninfected P815 cells were Cr^{51}- labeled and incubated with effector cells. Cytotoxicity

was routinely below 10% and was subtracted from cytotoxicity observed with infected targets. This is reported as specific immune lysis.

Chapter 3

A BISPECIFIC ANTIBODY RECOGNIZING INFLUENZA A VIRUS M2 PROTEIN REDIRECTS EFFECTOR CELLS TO INHIBIT VIRUS REPLICATION *IN VITRO*.

(Parts of this chapter published as Fernandez-Sesma et al. 1996)

ABSTRACT.

Bispecific antibodies (BsAbs) can be used to redirect cytotoxic T cells to kill virus infected cells. To do so they must bind the T cell receptor (TCR) on T cells with one arm and a viral protein with the other arm. The viral protein should 1) be expressed on the surface of infected cells, 2) ideally not be subtype specific, thus it can be used as a target for multiple virus isolates. The M2 protein of Influenza A virus fits these criteria. Therefore we produced a BsAb specific for influenza virus M2 protein and for the TCR. This Ab, named 3F12, was capable of redirecting staphylococcal enterotoxin B activated T cells to kill influenza virus infected cells. 3F12 was cross-reactive for different strains of influenza A virus, regardless of subtype, as determined by RIA, immunostaining or cytotoxicity. 3F12 could be greatly enriched by pH fractionation, and when added to MDCK cells infected with influenza virus along with SEB activated T cells, it could redirect those CTLs to inhibit influenza virus replication in tissue culture. This antibody represents a prototypical bispecific antibody which can

redirect superantigen activated T cells to attack virus infected cells overriding the need for MHC restriction and avoiding problems arising from viral subtype variation.

INTRODUCTION.

Recovery from many viral infections depends on T cell immunity, especially cytotoxic $CD8^+$ T cells (reviewed in Doherty et al 1992, Abbas et al., 1996, Zinkernagel, 1996). These $CD8^+$ T cells can kill cells in their immediate vicinity following stimulation through their T cell receptor (TCR) and this cytotoxicity is MHC restricted. Also, infusion of MHC-restricted antigen-specific T cells into infected animals has been demonstrated to speed recovery and reduce symptoms of virus infections (Cambridge et al 1976, Doherty et al 1974, Andrew et al., 1987). But, it is difficult to take advantage of this phenomenon, because of the need for large numbers of MHC-restricted antigen-specific T cells.

Some groups have shown that anti-influenza antibodies, when given very early during the infection and/or in high concentrations can also clear the virus infection in mice (Palladino et al., 1995). But in general antibodies alone do not lead to recovery from influenza virus infections.

In our laboratory we have been exploring the use of BsAbs to bypass the need for virus specific-MHC restricted T cells to inhibit experimental influenza virus infections (Moran et al., 1991, Moran et al., 1995).

Bispecific antibodies that bind to the TCR with one arm can bring into intimate contact cytotoxic T cells (CTL) and cells bearing the antigen for which the other arm is specific (reviewed in Fanger et al 1992, Lanzavechia and Scheidegger, 1987). If the antigen is a viral protein which is expressed on the surface of infected cells, BsAbs can

be used as antiviral tools (Berg et al., 1991, Howell et al., 1994, Paya et al., 1989). Once both cells are brought into intimate contact by the bispecific antibody the cytotoxic cell is triggered to kill (Staerz et al., 1986, Fanger et al., 1991). Thus, BsAbs can combine the specificity of the antibody response with the efficacy of the cellular immune response. Antibodies such as these have been previously generated in our lab (Moran et al 1991, Moran et al., 1995). Since we used influenza virus infection in mice as a model to test BsAbs as antiviral agents, our first BsAb, HHA6 was constructed to bind the TCR and the hemagglutinin (HA) of influenza virus PR8, (H1 subtype). HHA6 was shown to be capable of redirecting killing of PR8-infected Bneo cells by SEB-activated CTLs (SEB T cells). It could also redirect SEB-activated CTLS to inhibit replication of PR8 virus in MDCK cells (Moran et al 1995).

Since $CD8^+$ T cells can be strongly stimulated by superantigens such as staphylococcal enterotoxin B, and this activation has been shown to generate cytotoxic cells efficiently, (Fleischer and Schrezenmeier, 1988, White et al., 1989, Alber et al., 1990, reviewed in Herman et al., 1991), we used SEB T cells as effector cells for our experiments.

Our next goal was to generate a bispecific antibody that was crossreactive for different strains of influenza A virus. We found that the M2 protein was an ideal candidate for the generation of the bispecific antibodies due to its highly conserved N-terminal amino acids and, also because this protein is expressed on the surface of influenza virus-infected cells (Pinto et al., 1992, Wang et al., 1993, Holsinger et

al.,1994). M2 is a type III membrane protein and is capable of inducing the production of non neutralizing as well as neutralizing antibodies (Lamb et al., 1985, Zebedee and Lamb, 1988).

In this chapter we describe the generation of a bispecific antibody which binds the TCR and the M2 protein of influenza A viruses. This antibody, named 3F12, redirects SEB-activated CTLs to kill influenza A virus infected cells and, can also redirect SEB T cells to inhibit replication of influenza virus in MDCK cells. Thus, 3F12 is crossreactive for different strains of influenza virus carrying different HA subtypes.

MATERIALS AND METHODS.

Antibodies.

MD11 is a non neutralizing mAb with specificity for a conserved region of the influenza virus M2 protein (Rao and Moran, unpublished results). PY206 is a neutralizing mAb that binds the H3 subtype of influenza virus A/HK/68 hemagglutinin (HA); PY211 is a non-neutralizing mAb that binds the HA protein of influenza virus (H1 subtype) present in PR8 virus (Moran et al., 1991); PY102 is neutralizing mAb that binds the HA of influenza virus PR8. HHA6 is a bispecific antibody which binds the Vβ8 on the TCR of T cells and the HA protein of PR8 influenza virus (Moran et al., 1991, Moran et al., 1995). 3F12 is a bispecific Ab which binds Vβ8 TCR and the conserved extracellular region of the M2 protein of influenza virus. Hybridomas were grown at 37^{0}C ,7% CO_2 in Iscove's + HT medium.

Purification of BsAb.

3F12 was purified by passing the supernatant from the hybridoma through a protein A-agarose column (Boehringer Mannheim) using PBS at pH 7 as buffer for washing. A pH step gradient (pH 7-3.5) 0.1M citrate buffer was used for elution. Fractions were tested for their absorbance at 280 nm and peak fractions were dialyzed against PBS pH 7 for 24 hours.

Immunostaining.

MDCK cells were infected with either PR8, WSN, Beijing, or Udorn viruses at a MOI of 10 in maintenance medium and incubated overnight at 37°C, (Beijing virus

was incubated at 35°C). After 2 PBS washes the infected cells were fixed with 1% paraformaldehyde (25μl/well) for 5 minutes and then blocked with PBS-BSA (no azide) for 15 minutes. Antibodies (5μg/ml) or medium were incubated at room temperature for 1 hour. Plates were then washed with PBS-BSA (no azide) and a 1/500 of rabbit-anti mouse IgG-peroxidase linked (DAKO Corporation) was added to the wells and incubated for 1 hour at room temperature. Plates were washed with PBS-BSA and 30 μl/well of AEC substrate (DAKO Corporation) were added and incubated for 10 minutes at room temperature. Plates then were observed under the microscope for detection of staining.

Inhibition of multicycle virus replication.

MDCK cells were plated in flat bottom 96 well microtiter plates at a concentration of $3X10^4$ cells per well and incubated overnight in tissue culture medium. The monolayers were washed free of FCS and $2x10^3$ tissue culture infectious units 50 ($TCID_{50}$) of PR8 virus in maintenance medium were added to each well in 25μl of volume. Plates then were incubated at 37°C, 7% CO_2. Four hours later, 3F12 (4μg/ml) and SEB activated T cells (different E:T ratios) were added to each of triplicate wells. The cultures were incubated for 48 hours in maintenance medium (final volume of 200μl/well), and samples were taken every 12 hours. Virus titers in the supernatants were calculated by hemagglutination of chicken red blood cells.

RESULTS.

Generation of 3F12 hybridoma. F23.1 is a hybridoma which produces an IgG2a antibody with specificity for Vβ8 T cell receptor proteins which are found on approximately 20% of BALB/c T cells (Staerz et al., 1986). We were able to make F23.1 cells HAT sensitive and use them as a fusion partner with spleen cells from animals immunized with the M2 peptide (SLLTEVETPIRNEWGCRCND) representing the extracellular domain of the influenza A virus M2 protein (figure 3-1). Desirable antibodies should be bispecific in that they bind to $V\beta8^+$ T cells and to the M2 protein expressed on the surface of influenza virus infected cells simultaneously. In this way they trigger the cytotoxic capacity of CTLs only upon encountering virus infected cells culminating in the death of infected cells (figure 3-2).

Screening of 3F12 hybridoma. Hybridomas derived from the fusion of F23.1 cells and M2-specific splenocytes from immunized BALB/c mice were screened for binding to the M2 peptide coated on plastic plates in a radioimmunoassay (RIA) (see table 3-1), MD11 (anti M2 mAb) was used as positive control. A number of hybridomas produced antibodies which bound the peptide. Since many peptide specific antibodies fail to bind to native protein we tested the hybridoma supernatants for binding to influenza virus infected MDCK cells by immunostaining . We tested several influenza virus strains by immunostaining and we found 3F12 to be positive with all of them (PR8, WSN, Japan, Beijing, Udorn), in figure 3-3 results using PR8 and Beijing viruses are shown. Hybridomas producing antibodies binding in both tests were

considered positive for M2. Then the supernatants from M2 positive hybridomas were tested for their ability to redirect $V\beta8^+$ CTLs to kill influenza virus infected target (Bneo) cells to confirm that they are indeed bispecific antibodies (data not shown). 3F12 was best in binding to M2 peptide, purified virus and virus infected cells and redirecting lysis of PR8 infected cells, therefore it was selected and cloned two times by limiting dilution.

Purification of 3F12 BsAb. Supernatants derived from 3F12 hybridoma expressed both, the IgG2a isotype of F23.1 as well as an IgG1, which must be derived from the M2 specific partner. Since all BsAbs represent mixtures of parental antibodies, mismatched pairs and bispecific antibodies (De Lau et al., 1991), we attempted to purify the BsAbs away from the parental antibodies. IgG1 binds poorly to protein A and IgG2a strongly, thus, we used a protein A column for purification and eluted using a pH step gradient (between pH 7 and pH 3.5) of citrate buffer. All IgG1 parental antibodies passed through the column at pH 6 and IgG2a parents eluted at pH 4.5. We looked at fractions between these extremes for a population of antibodies which could redirect CTLs to kill virus infected cells. Figure 3-4 shows that fraction 8, which eluted at pH 4.9 contained most of the antibody capable of redirecting CTL lysis on a 4 hour chromium release assay. This fraction, considered to be the most efficient bispecific one, was used for further experiments.

Binding of 3F12 to different strains of influenza virus. The M2 protein of influenza A virus is highly conserved among different strains of influenza A virus

which have been isolated since 1934 to the present. We wanted to test the ability of our BsAb to recognize the extracellular domain of the M2 of as many strains of influenza virus as possible, thus, we would have an antibody crossreactive for all those strains. The binding of 3F12 to cells infected by different strains of influenza virus was checked by immunostaining of influenza A virus infected MDCK cells (figure 3-3) and by RIA using purified viruses to coat the plates (data not shown). 3F12 bound to cells infected with different strains of influenza A virus such as PR8, WSN, Udorn, Japan and Beijing. Thus, 3F12 is crossreactive for different strains of influenza A viruses. MD11 is a mAb specific for the M2 protein of influenza virus (Rao and Moran unpublished results) and was used as a positive control. The intensity of the staining is weaker for 3F12 than for MD11, which may be due to the fact that 3F12 only has one arm that binds to the M2 protein or to other avidity related differences between 3F12 and MD11.

3F12 does not inhibit influenza virus replication by itself. Since some mAb to M2 have been reported to be neutralizing (Zebedee and Lamb, 1988), we wanted to test if 3F12 had virus neutralization properties. PR8-infected MDCK cells were incubated in the presence of either 3F12, PY206, PY211, HHA6 or PY102 for 48 hours and virus titers in the supernatants were then tested by hemagglutination of red blood cells. Only PY102 could inhibit virus replication by itself in this assay (data not shown).

3F12 can redirect SEB-activated T cells to kill influenza virus-infected cells. Bneo cells were infected for 6 hours with different strains of influenza virus such as PR8, WSN and Udorn and then a cytotoxicity assay was performed as described in general materials and methods. As shown in figure 3-5, 3F12 could redirect SEB T cells to kill cells infected by any of these viruses. On the other hand, HHA6, which is a BsAb specific for the HA protein of PR8, could only redirect the SEB-activated T cells to kill PR8 virus infected cells.

Inhibition of influenza virus multicycle replication in MDCK cells by 3F12. MDCK cells were infected with PR8 virus and incubated with 3F12, SEB T cells, 3F12 + SEB T cells or medium, (see Materials and Methods). Samples were harvested every 12 hours, and, as shown in figure 3-6, 3F12 was able to redirect the SEB T cells to inhibit virus replication. Virus titers in the supernatants were reduced by two $\log_{10}$ units when 3F12 + SEB T cells were present, compared to the titers obtained in the presence of SEB T cells alone. This inhibition of virus replication was maintained throughout the infection. While 3F12 clearly demonstrated the broad specificity predicted for a monoclonal antibody to the M2 protein, the real test of this antibody was whether it could inhibit multicycle replication of influenza virus *in vitro*. To test this we used PR8 virus to infect MDCK monolayers. PR8 virus was chosen because it replicates better than other isolates in MDCK cells.

DISCUSSION.

While cell-mediated immunity has been shown to play a crucial role in recovery from virus infections (Doherty et al., 1974, Doherty et al., 1992 Abbas et al., 1996, Zinkernagel, 1996), antibodies function primarily to prevent reinfection, playing only a minor role in viral clearance in many virus infections (Liang et al., 1993, Palladino et al., 1995). This is clearly documented in influenza virus infection where virus clearance begins at day 3 but neutralizing antibodies do not appear until day 7 (Wells et al., 1981, Bender et al., 1992). Infusions of cytotoxic $CD8^+$ T cells or cytokine releasing $CD4^+$ T cells speed recovery and reduce symptoms associated with influenza virus infection in mice (Doherty et al., 1974, Cambridge et al., 1976, Lukacher et al., 1984, Andrew et al., 1987). While such immunotherapy has been successfully utilized in laboratory experiments for years, very little advantage has been taken of this phenomena in humans. Attempts have been made to use T cell immunotherapy to fight CMV infection in bone marrow transplanted patients (Riddell et al., 1994). There are two primary difficulties which must be overcome to take advantage of T cell immunotherapy. First, the T cells must be MHC restricted and virus specific and second, they should not elicit a host versus graft reaction in the recipient. These drawbacks have severely restricted the usefulness of specific T cell immunotherapy.

Bispecific antibodies have the potential to bind to two different cell types simultaneously. It has been convincingly demonstrated that BsAbs which bind to the T cell receptor of a cytotoxic T lymphocyte with one arm and a tumor target cell with the

other, can trigger the CTL to kill the tumor, a process known as redirected lysis (Perez et al., 1985, Fanger et al., 1991, Gruber et al., 1994, Penna et al., 1994, Kurucz et al., 1995). Binding to the T cell receptor is essential since this is the signal which leads to its degranulation culminating in target cell death. Redirected lysis has two advantages over conventional MHC restricted killing. The killer cell does not have to be MHC restricted to the target cell and it can bind to any antigen expressed on the surface of the infected cell, not only those presented by MHC molecules. We, and others have shown that bispecific antibodies generated by hybridoma fusion (Kohler et al., 1975, Milstein and Cuello, 1983, Milstein and Cuello, 1984) can redirect CTLs to kill virus infected cells, even though those CTLs are not virus specific (Staerz et al., 1986, Paya et al., 1989, Berg et al., 1991, Moran et al 1991, Howell et al., 1994). In addition, we have documented that SEB-activated CTLs and BsAbs can inhibit multicycle virus infection when added to cultures of infected cells (Moran et al 1995). These findings have suggested that BsAbs may be used in an immunotherapeutic protocol against virus infections *in vivo*. The fact that MHC restriction is not necessary is advantageous.

In our previous work we utilized BsAbs with specificity for the hemagglutinin protein of PR8 influenza A virus. The limitation of these antibodies and others directed to strain or subtype specific epitopes is that they will only work against that particular strain or subtype. Many viruses express cell surface proteins on infected cells which are strongly conserved. The fact that they are non-neutralizing epitopes means that they

are not subject to the same selective pressure as subtype specific epitopes. These non variable epitopes are actually better targets for bispecific antibodies not only because they are conserved but because they are often less highly expressed on virions and therefore, most antibodies actually bind to infected cells. The M2 protein of influenza virus is such a determinant. It is an ion channel which is strongly conserved in its extra cytoplasmic domain and is underrepresented on the virion (Lamb et al.1985, Zebedee and Lamb, 1988, Pinto et al., 1992, Wang et al., 1993,). Thus, we produced a bispecific antibody which binds the Vβ8 T cell receptor protein on T cells and the M2 protein of influenza A virus. This antibody behaves as predicted by binding to cells infected with influenza A viruses of all subtypes and isolates tested. The antibody is an IgG1\IgG2a hybrid, which allows relatively easy purification and separation from parental antibodies which are also produced by our hybrid hybridomas. The antibody can redirect superantigen activated T cells to kill virus infected target cells, again demonstrating broad crossreactivity with various virus subtypes. The crucial test was its ability to inhibit multicycle virus replication in virus infected target cells. We demonstrate in this chapter that it efficiently does so.

We used superantigen activated T cells as effector cells in these experiments. Since all our bispecific antibodies have one arm specific for the Vβ8 molecule on the TCR, we have chosen to use staphylococcal enterotoxin B to activate the cells. SEB primarily activates $V\beta8^+$ T cells in the BALB/c mouse (Fleischer and Schrezenmeier, 1988, White et al., 1989, Herman et al., 1991). Thus, in a short three day incubation

we can produce large numbers of activated Vβ8$^+$ T cells which are a combination of CD8$^+$ killer cells and CD4$^+$ cytokine producing cells (primarily IFNγ). The mechanism by which these cells inhibit multicycle virus replication is unclear in the influenza virus model. Our group has demonstrated in a previous publication that both CD4$^+$ and CD8$^+$ T cells are capable of inhibiting influenza virus replication (Moran et al 1995). This parallels the result shown by Doherty and others demonstrating that both subpopulations have the potential to clear virus in an infected animal (Doherty et al 1992).

We envision the use of a patients own T cells which will be collected and activated *ex vivo*, incubated with the antibody and returned to the patient. If such cells can destroy a fraction of the virus infected or tumor cells they may function to help immunosuppressed or immunocompromised patients to better cope with an ongoing or persistent viral infection or tumor. In addition to the ability of these cells to kill virus infected cells, they may also function (particularly the CD4$^+$ population) to release cytokines at the site of virus infection which may aid in recovery. Even in patients infected with HIV it may be possible to infuse antibody bearing superantigen stimulated autologous CD8$^+$ T cells back to decrease the numbers of infected cells. If we can show experimentally that BsAbs can be utilized *in vivo*, we would like to create antibodies such as the one described in this chapter which can redirect non virus specific T cells to inhibit virus replication.

Figure 3-1. Generation of 3F12 hybridoma. F23.1 hybridoma cells, which make antibodies to the Vβ8 molecule of the TCR and were made HAT sensitive, were fused by PEG with splenocytes from mice immunized with the M2 peptide to generate the bispecific hybridomas. The HAT system was used for selection. Bispecific hybridomas will make a mixture of parental and bispecific antibodies.

Figure 3-1

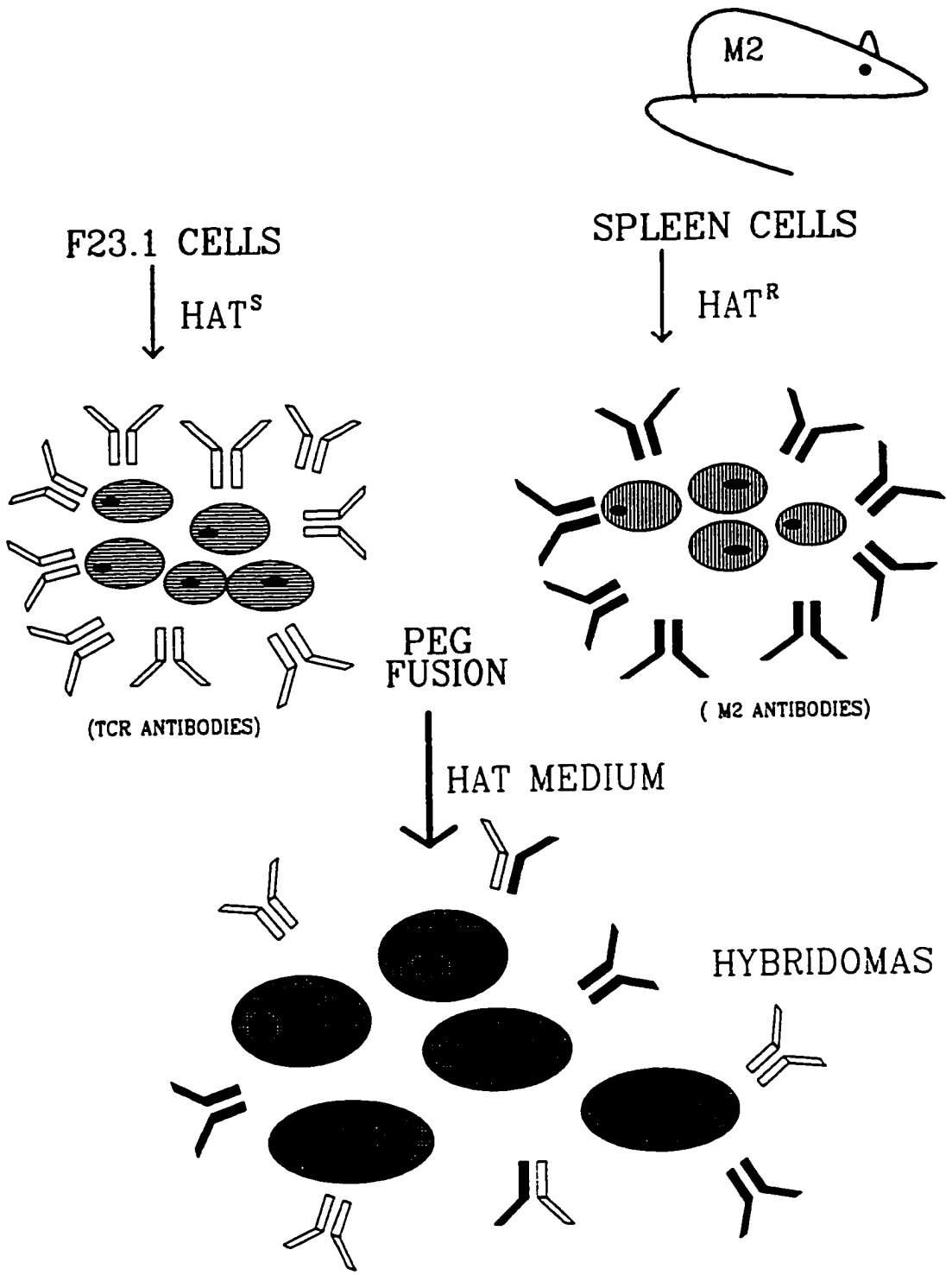


Figure 3-2. Bispecific antibodies can redirect cytotoxic cells to kill virus infected cells. PR8 virus infected Bneo cells were used as target cells (MOI=5) for a 4h Cr^{51} release assay and SEB activated T cells were used as effector cells (effector: target ratio was 20:1) in the presence of supernatant from hybridoma cultures, purified antibodies (MD11 And HHA6) or medium. MD11 (anti-M2 mAb) and HHA6 (anti-TCR + anti-HA BsAb) were used as positive controls at 2μg/ml. Results are shown in % cytotoxicity and are the average of triplicate samples.

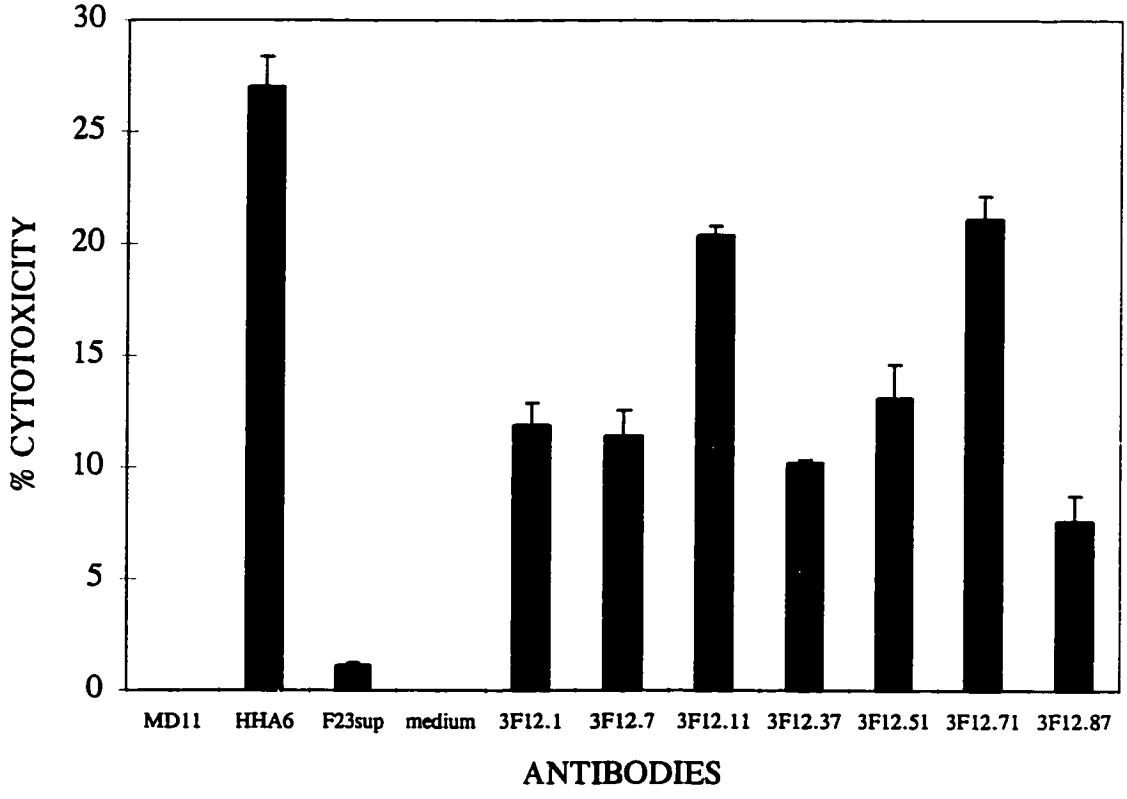


Table 3-1. Screening of hybridoma supernatants for binding to the M2 peptide by RIA. Hybridomas from the fusion of F23.1 cells and M2-specific splenocytes from immunized BALB\c mice were screened by RIA using the M2 peptide SLLTEVETPIRNEWGCRCND (2.5μg/ml) to coat the plates and ^{125}I- labeled goat anti-mouse IgG mAb was used to develop. MD11 (anti-M2 mAb) was used as positive control at 5μg/ml. CPM were counted in a γ-counter.

SUPERNATANTS FROM WELLS	CPM BOUND
MD11	8641
F23 supernatant	144
medium	115
1C3	304
1E9	3162
2A5	269
2D4	1958
3B6	240
3F12	3513
4E6	1703
4H10	288
5C1	643
5E7	254

Figure 3-3. Binding of 3F12 BsAb to cells infected by different strains of influenza virus: Immunostaining of MDCK cells infected overnight at a MOI= 10 with influenza A/PR8/34 virus (**A,B,C**), A/Beijing/93 (**D,E,F**) or uninfected (**G**). In panels **A**, **D** and **G**: MD11 (anti-M2 mAb) was used at 5μg/ml. In **B** and **E** purified 3F12.11 BsAb was used at 5μg/ml. In panels **C** and **F** medium was added. Infected and uninfected monolayers were fixed with 1% paraformaldehyde and incubated with either MD11, 3F12.11 or medium for 1 hour. After washing, POD-linked anti IgG antibodies and AEC substrate were used to detect . Triplicate wells were done for each sample with similar results. This figure was generated by scanning of microscope slides with ADOBE photoshop.

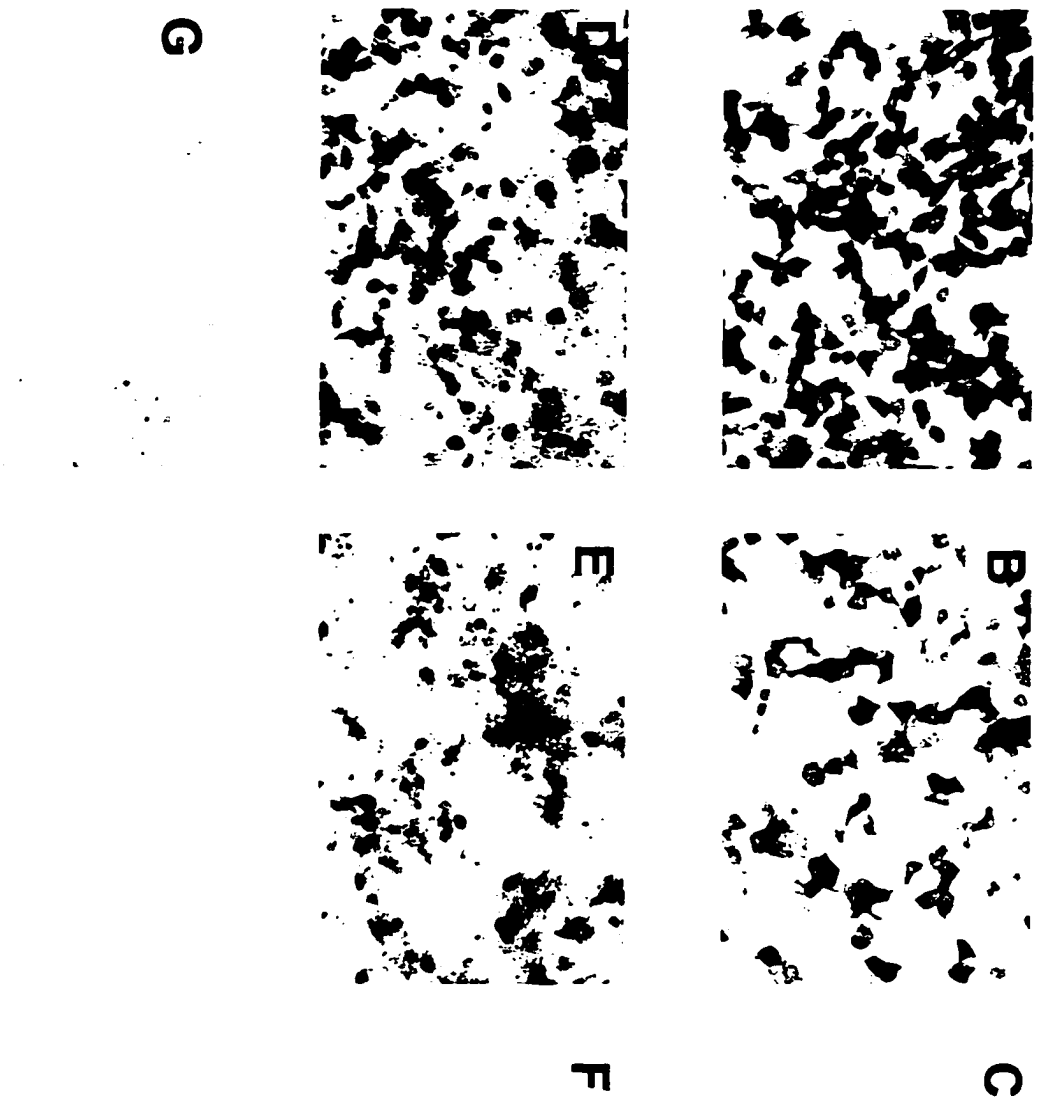


Figure 3-3

Figure 3-4. Killing of influenza virus infected cells by SEB activated T cells redirected by different fractions of pH purified 3F12: Supernatants from 3F12 hybridomas were purified through a protein A column using a pH gradient of citrate buffer (pH 7 to pH 3.5) to elute (see methods). Peak fractions were tested for their ability to redirect SEB activated T cells to kill influenza virus infected cells in a 4 hour ^{51}Cr release assay. Influenza virus PR8 was used to infect Bneo cells at an MOI=5 for 1 hour, all antibody fractions were used at 2μg/ml, effector to target cell (E:T) ratio was 20:1. Samples are the average of triplicates.

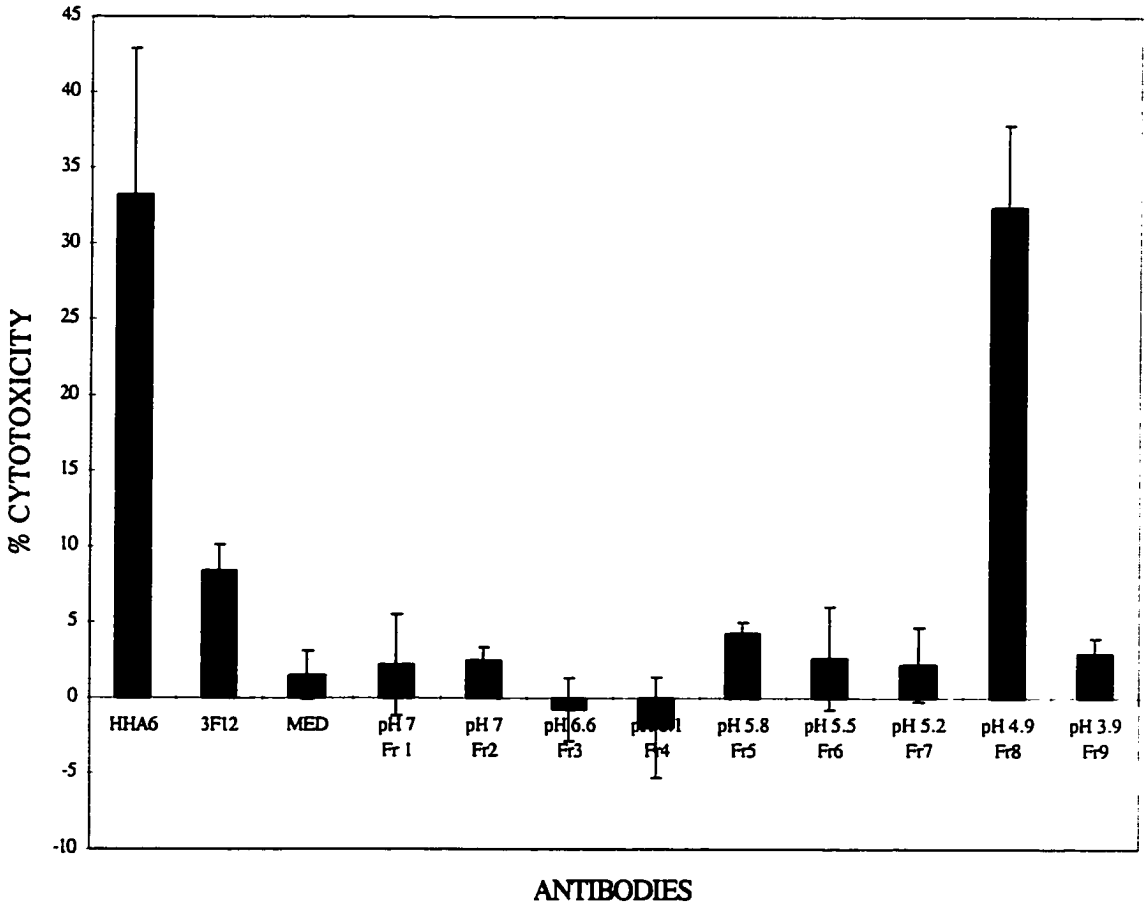


Figure 3-5. 3F12 redirects SEB activated T cells to kill cells infected by different strains of influenza virus: Bneo cells were infected (MOI=5) for 6 hours with PR8, WSN or Udorn. A 4 hour Cr^{51} release assay was performed using SEB activated T cells as effector cells. HHA6 and 3F12 were used to redirect lysis of target cells by the effector cells at a concentration of 2μg/ml and the E:T ratio used was 20:1. Means of triplicates are shown. Standard errors are: 1.32 (mock + HHA6), 1.27 (mock + 3F12), 4.59 (mock), 0.39 (PR8 + HHA6), 1.76 (PR8 + 3F12), 0.63 (PR8 + mock), 5.26 (WSN + HHA6), 7.1 (WSN + 3F12), 2.38 (WSN + mock), 1.33 (Udorn + HHA6), 6.9 (Udorn + 3F12) and 2.32 (Udorn + mock).

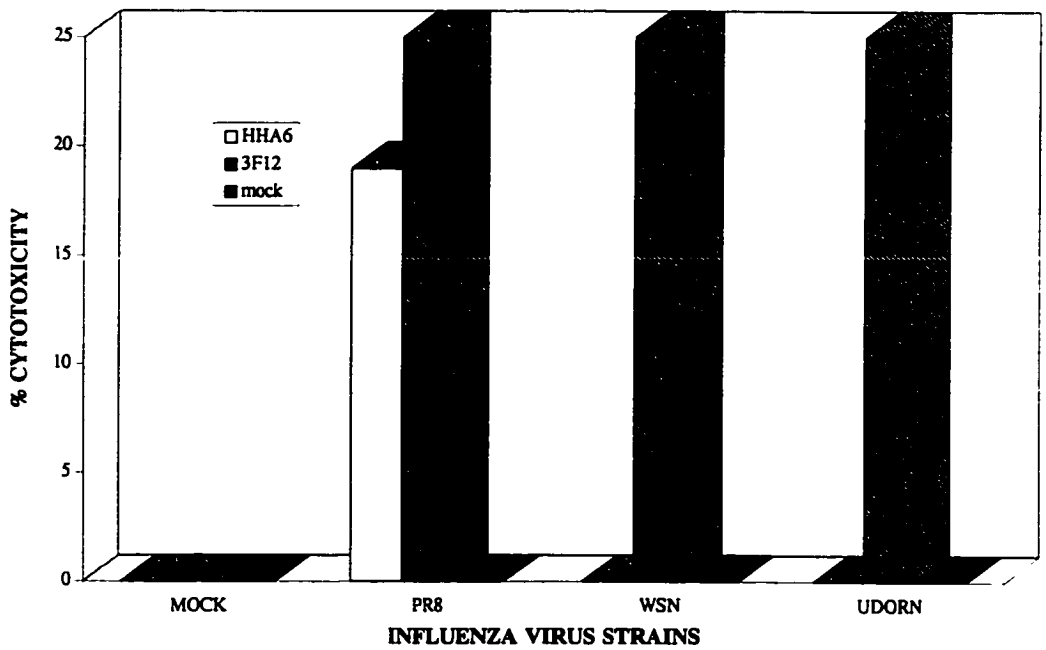


Figure 3-6: 3F12 can redirect SEB activated T cells to inhibit influenza virus replication in tissue culture: MDCK cells were infected with PR8 virus (MOI of 0.1) and incubated for 48 hours in the presence of medium, 3F12 (4μg/ml), SEB T cells (E:T ratio of 20:1), or 3F12 + SEB T cells (4μg/ml + 20:1). Test points were taken every 12 hours and tested for virus titers by hemagglutination of chicken red blood cells. Mean values of triplicates are shown for each sample. The difference in inhibition of virus replication by 3F12 + SEB T cells compared with SEB T cells alone was statistically significant (two-tailed *t* test $p=0.006$ for 12 hours, $p=0.1$ for 36 hours and $p=0.002$ for 48 hours.

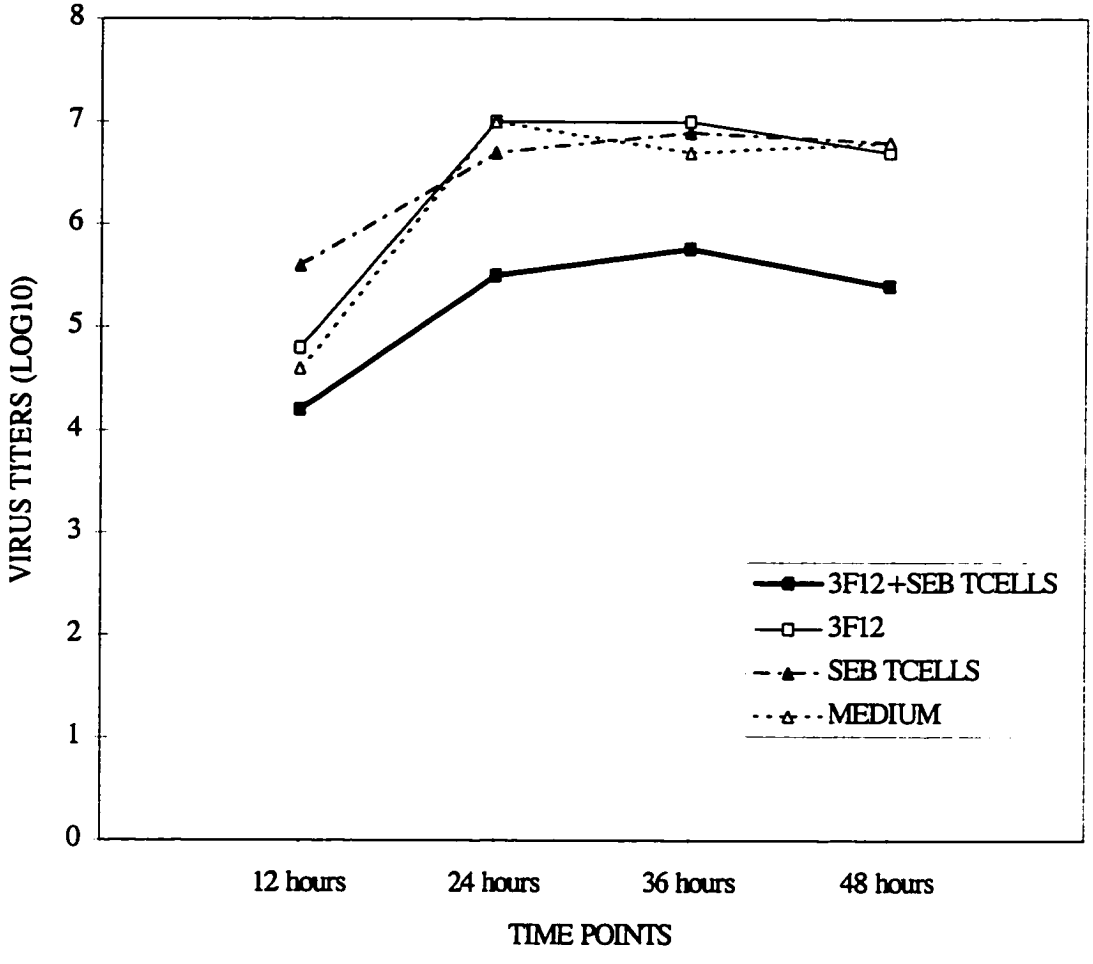


Figure 3-6

Chapter 4

SUPERANTIGEN ACTIVATED T CELLS REDIRECTED BY A BISPECIFIC ANTIBODY INHIBIT VSV REPLICATION *IN VITRO* AND *IN VIVO*.

(Ana Fernandez-Sesma, et al., 1997. in press, J. Immunol.)

ABSTRACT

A bispecific antibody (BsAb) which binds the T cell receptor (TCR) on T cells and the G protein of 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 antibody for the viral protein. IFNγ doesn't play a very important role in this phenomenon, which is mainly mediated by the release of perforin from $CD8^+$ T cells. We have used a Stat1 knockout (KO) mouse model in which VSV infection is lethal. Infusion of SEB T cells and BsAb significantly slowed virus progression and prolonged the survival of VSV infected Stat1 KO mice.

INTRODUCTION

In many virus infections T cells have been shown to play a crucial role in recovery, whereas antibodies predominantly protect against reinfection (Cambridge et al., 1972, Wells et al., 1981, Bender et al., 1992, Hou et al., 1992, Koup, 1994, Zinkernagel, 1996). The demonstrated requirements for costimulatory molecules suggest that activation of virus specific T cells may depend on presentation by professional antigen presenting cells (Bluestone, 1995, Zinkernagel, 1996). Once activated the antigen-specific CTLs proliferate and attack the infected cells preventing the release of infectious progeny. The activation step takes a few days, allowing virus replication and invasion while the CTLs are generated.

Some viruses such as VSV which replicate quickly and have a broad tropism would be lethal if cellular or humoral mechanisms are solely responsible for control. This is confirmed by experiments performed infecting Stat1 KO mice with VSV (Durbin et al., 1996). These mice are unable to respond to IFN and thus, lack proper innate immunity. All Stat1 KO mice died from VSV infection within 48 hours, before any cellular immunity could be generated (Durbin et al., 1996).

Experimentally, the interval between antigen presentation by professional APCs and the generation of CTLs can be overcome by the use of bispecific antibodies (BsAbs) to redirect lysis of virus infected cells by activated T cells in a non MHC restricted fashion (reviewed in Fanger et al., 1992). In previous work our group has demonstrated that T cells activated by the SEB in the presence of a BsAb with virus and T cell receptor

specificity, can kill infected target cells (Moran et al., 1991, Moran et al., 1995, Fernandez-Sesma et al., 1996). Thus, these BsAb-targeted T cells combine the specificity of an antibody with the intracellular attack capability of CTLs.

BsAbs can function without limitation as to the specificity of the T cell. Our group has previously shown that BsAbs with specificity for the Vβ8 molecule on the TCR and for the influenza virus protein (HA) can redirect SEB-activated T cells to kill influenza virus infected cells and to inhibit influenza virus multicycle replication in tissue culture (Moran et al., 1995). Using this approach we have targeted influenza virus infected cells using BsAbs which bind to viral antigens subject to evolutionary drift, such as the hemagglutinin (Moran et al., 1991, Moran et al., 1995) as well as to highly conserved antigens such as the M2, which is found on the surface of virus infected cells (Fernandez-Sesma et al., 1996). Though we were able to demonstrate significant inhibition of virus replication, the influenza virus system was not optimal for the study of BsAbs for two reasons. First, *in vitro* productive infections can only be efficiently generated in xenogeneic cells (MDCK). This would make cytokines and or adhesion molecules with restricted species specificity, ineffective. Secondly, *in vivo*, influenza is believed to bud apically into the lumen of the lungs (Rodriguez-Boulan, 1983) making it difficult to reach with BsAb and T cells infused into the blood stream.

To overcome these limitations we prepared a BsAb with specificity for VSV which replicates productively in many cell types. We chose the mouse mastocytoma, P815 as our *in vitro* culture target cell line. P815 cells are productively infected by VSV,

but the viral titers are lower in this cell line (in our hands, 10^6 VSV infectious particles/25μl in P815 cells compared to $2.5x10^8$ VSV infectious particles/25μl in BHK cells) than in other non-mouse cell lines that are more commonly used to grow VSV. P815 cells grow in suspension culture and therefore are not subject to budding polarity. This gave us a completely syngeneic system in which to study the mechanism by which inhibition of virus replication by non-virus specific T cells takes place. In addition, recent work with Stat1 KO mice has demonstrated that VSV replicates efficiently and is lethal for these mice, thus providing an *in vivo* model to attempt to demonstrate efficacy of BsAb and SEB activated T cells (Durbin et al., 1996).

The VSV specific BsAb, 526 was demonstrated to be specific for VSV (Indiana) and could mediate killing of VSV infected cells in the presence of SEB activated T cells. In addition, it was extremely potent in inhibiting multicycle replication of VSV *in vitro*. The antibody alone was incapable of neutralizing virus in the absence of activated $CD8^+$ T cells. Interestingly, inhibition of virus replication was dependent on perforin mediated lysis and IFNγ did not seem to play an important role.

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 which were given a lethal infection of virus. This is the first evidence that BsAb can be used *in vivo* as immunotherapeutic agents to fight virus infection.

MATERIALS AND METHODS.

Purification of BsAb.

526 VSV BsAb was purified by passing the supernatant of the hybridoma through a protein A-agarose column (Boehringer Mannheim) using PBS at pH 7 as buffer for washing. 0.1M citrate buffer pH 3.5 was used for elution. Fractions were tested for their absorbance at 280 nm and peak fractions were dialyzed against PBS pH 7 for 24 hours.

Inhibition of multicycle virus replication.

$3x10^6$ P815 cells were infected in suspension with VSV at an MOI=0.1 using maintenance medium. One hour later, $3x10^4$ VSV-infected P815 cells with either, 526 (1μg/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 hours in maintenance medium. Supernatants from each well were used to infect BHK cell monolayers plated in 96 well plates. BHK cells were incubated with the supernatants for 24 hours and then stained with crystal violet for titration by 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 ($5x10^6$ cells/ml) and incubated with either anti-CD4 antibody (GK1.5), anti-CD8 antibody (2.43) both at 10 μg/ml or medium alone for 30 minutes at 4^0C. Antibodies then were washed away and cells were incubated with freshly washed anti-IgG linked BioMag magnetic beads (Perseptive Diagnostics, INC.)

(50 beads/cell) for 30 minutes at 4°C. The depletion of antibody-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 then were used for cytotoxicity and inhibition of virus replication.

FACS analysis.

Samples from CD4-depleted, CD8-depleted and unseparated SEB T cells ($3x10^5$ cells/sample) were incubated with 100 ng/ml of anti-CD3-FITC Gibco BRL), anti-CD8-FITC + anti-CD4-PE (Boehringer Mannheim) (100 ng/ml each), anti-IgG2b isotype standard (Pharmingen) (100 ng/ml) or medium alone for 30 minutes at 4°C. Antibodies were washed away with T cell medium and each pelleted sample was incubated for 5 minutes at RT with 50 μl 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). On average 89% of the 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.

Anti-IFNγ ELISA.

Immulon-4 ELISA plates (Dynatech) were coated with the neutralizing anti-IFNγ antibody XMG1.5 at 5μg/ml. Control IFNγ (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 IFNγ to the coating plate at 0.3μg/ml. Based on these

results we used a concentration of XMG1.2 three times higher than needed to block IFNγ in the SEB T cell cultures for inhibition of virus replication (Figure 4-3).

***In vivo* infections and treatment of Stat1 KO mice.**

Stat1 KO mice (generated in David Levy's laboratory) were infected by intraperitoneal (ip) injection with 1-5 LD_{50}/mouse of VSV (for each experiment a single LD_{50} was used) diluted in PBS to a final volume of 1 ml/mouse. One hour after infection, mice were injected ip again with either $95x10^6$ SEB T cells/mouse (prepared from CD1 mice) only, BsAb 526 only (50μg/mouse), SEB T cells ($95x10^6$ cells/mouse)+ BsAb 526 (50μg/mouse) or PBS, all injections in a final volume of 1 ml/mouse. Survival times were recorded in hours post- infection and results are shown in table 4-1.

RESULTS

Production of bispecific antibody, 526. The BsAb 526 was produced by fusion of the HAT sensitive F23.1 (anti-Vβ8) hybridoma with spleen cells from a BALB/c mouse immunized with VSV (Indiana). Hybridomas producing bispecific antibodies were identified by binding of supernatant to purified VSV (Indiana) coated plates in a RIA. All bispecific hybridomas which bound the virus should also produce anti-Vβ8 since F23.1 was the fusion partner. To ensure that we had a bispecific antibody which 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 hour ^{51}Cr release assay (cytotoxicity 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 cytotoxicity 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 cytotoxicity assay. Figure 4-1A 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 bispecific antibody, 3F12,

specific for Vβ8 and influenza A M2 protein (Fernandez-Sesma et al., 1996) failed to lyse VSV infected target cells.

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 2.5μg/ml to 150ng/ml with or without SEB T cells. The results in figure 4-1B demonstrate that in the presence of 526, SEB T cells can be redirected to inhibit virus replication at concentrations of antibodies as low as 600ng/ml. An irrelevant BsAb (3F12), 526 alone or SEB alone have 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 may result from infection of SEB T cells by VSV or cytokine mediated increase of virus replication.

Cytotoxicity and inhibition of virus replication against an irrelevant VSV subtype. To verify the specificity of 526, we tested 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) (Figure 4-2).

Is IFNγ released from SEB T cells responsible for inhibition of multicycle virus replication?. IFNγ has been reported to play an important role in inhibition of virus replication in some studies (Ozmen et al., 1995, Guidotti et al., 1996). Our SEB T cell cultures secrete very high levels of IFNγ (data not shown) thus, we performed an ELISA (see materials and methods) to check how much of the anti-IFNγ antibody XMG1.2 , which has been shown to inhibit IFNγ (Ozmen et al., 1995), we needed to add to our assay to bind all the IFNγ present in the supernatant of our SEB T cell culture. Thus we analyzed what role, if any, IFNγ 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-IFNγ antibody (XMG1.2 at 1mg/ml, 3 three fold excess of the concentration needed to bind all the IFNγ present in our SEB T cell culture). The results shown in figure 4-3 indicate that inhibition of IFNγ 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 bispecific antibody. However, there is evidence that both $CD4^+$ and $CD8^+$ T cells are capable of inhibiting virus replication *in vivo* (Eichelberger et al., 1991, Bender et al., 1992, Hou et al., 1992, Scherle et al., 1992, and Spriggs et al., 1992, Topham et al., 1996). 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

$CD4^+$ cells or $CD8^+$ cells and tested them in assays of both cytotoxicity and inhibition of virus replication. When these different cell populations were mixed with 526 and VSV infected P815 cells and the release of virus progeny measured 48 hours later, mainly unseparated and $CD8^+$ T cells (CD4 depleted) were able to inhibit multicycle virus replication as shown in figure 4-4A. The small inhibition effect observed when only $CD4^+$ T cells together with 526 were used may be attributed to residual $CD8^+$ T cells after depletion or to some soluble factor different from IFNγ present in the cultures. Figure 4-4B shows that significant cytotoxicity is only observed with unseparated cells or $CD8^+$ T cells and 526.

Analysis of the ability of SEB T cells from perforin KO mice to inhibit multicycle replication. Inhibition of virus replication didn't seem to be mediated by IFNγ release and was dependent on the presence of $CD8^+$ T cells. Thus, we set out to determine whether perforin mediated cytotoxicity was essential for inhibition of virus replication. Spleen cells from perforin KO (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 4-5A shows that, as expected, Pfp KO SEB T cells were unable to lyse the target cells in the presence of BsAb in a cytotoxicity assay.

Pfp KO T cells activated by SEB were added to infected P815 along with 526. Under these conditions, no inhibition of virus replication was observed (figure 4-5B) compared to SEB activated T cells from WT C57Bl\6 mice and 526. From these results

we concluded that the BsAb mediated inhibition of virus replication is dependent on cytotoxicity resulting from the release of perforin.

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 (Bi et al., 1995). Recently, it has been demonstrated that Stat1 KO mice, which are unable to react to interferon, are extremely sensitive to VSV. These mice given only a few hundred infectious particles die within 48 hours (Durbin et al., 1996). 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 intraperitoneal injection with VSV. Independent experiments were performed using either 1, 2.5, or 5 LD_{50} 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 4-1 demonstrate that virtually all infected animals given SEB T cells or nothing died within 24-48 hours after infection. In contrast, infected animals receiving SEB T cells and BsAb began to die only after 48 hours. Chi square test showed that the differences in survival rates were statistically significant ($p < 0.005$). Some prolongation was observed with BsAb 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 chapter we describe and analyze the properties of the monoclonal bispecific antibody, 526, which binds the Vβ8 molecule on the TCR of T cells and the G protein of VSV. We have previously generated similar antibodies in our laboratory which differ with respect to the virus protein that 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 (Moran et al., 1991, Moran et al., 1995, Fernandez-Sesma et al., 1996). 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 (replication (Moran et al., 1991, Moran et al., 1995, Fernandez-Sesma et al., 1996). One of the drawbacks of the influenza system was that multicycle virus replication could only be achieved using xenogeneic cells (canine MDCK or bovine MDBK 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 log10 units (3F12) throughout the 48 hour 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* (Eichelberger et al., 1991, Bender et al., 1992, Hou et al., 1992, Scherle et al., 1992, and Spriggs et al., 1992, Topham et al., 1996). 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 (Moran et al., 1995). Thus, we tried to manipulate the system in order to test which T cell population 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 perforin mediated cytotoxicity and, IFNγ 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 hours compared to 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*. We have not tested this hypothesis, since we did not measure virus titers in this mice, but we plan on testing this to identify the mechanism by which Stat1 KO mice infected with VSV and treated with SEB T cells + BsAb 526 show prolonged survival than the rest of the groups of mice. 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 non lethal influenza virus fail to adequately generate a proinflammatory response, and demonstrate impaired virus clearance (Durbin et al., 1996). In these Stat1 KO mice as little as 100 VSV infectious particles can be lethal.

It should be noted that BsAb alone also prolonged the lives of Stat1 KO mice infected with VSV, although to a lower extent that in combination with SEB T cells. As can be clearly seen in figures 4-1B, 4-3, 4-4B and 4-5B, 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 bifunctional antibody's ability to activate the complement cascade and its opsonizing and ADCC activity due to its isotype (IgG2a). Effects of bispecific antibodies alone have been observed by others in an anti-tumor model (Demanet et al., 1991, Demanet et al., 1994). Also, Stat1 KO mice infected with influenza virus had good levels of antibody redirected cytotoxicity in P815 cells with an anti-TCR antibody, 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 and 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 (Renno et al., 1996). 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 pre-existing 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 to be 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 nor perforin nor Fas ligand play a role in recovery from VSV in normal animals (Kägi et al., 1995). The fact that the virus would seem to be controlled by innate immune responses renders such cells unnecessary. Our results however demonstrate that $CD8^+$ T cells using perforin mediated cytotoxicity are very efficient at inhibiting virus replication.

Tools such as BsAbs may prove to be of immunotherapeutic value to fight not only fast spreading viruses, but more importantly, persistent infections (Berg et al., 1991, Chamow et al., 1994, Howell et al., 1994). It is possible to generate BsAbs against the TCR and viral proteins expressed on virus infected cells to be used against viruses which are inefficient inducers of specific CTL. This may result from either a failure to infect cells 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 (Koup et al., 1994, Gilbert et al., 1996).

BsAbs can also be generated to redirect other cells of the immune system to act upon pathogens, i.e. complement binding BsAbs (Kontermann et al., 1997), those which bind Fc receptors on cells of the immune system.(Deo et al., 1997, Holliger et al., 1997 and Kontermann et al., 1997). Also, other groups have successfully used BsAbs to target the immune response against tumors in animal models and humans (Penna et al., 1994, Moreno et al., 1995, Weiner et al., 1995, Robert et al., 1996 and Sahin et al., 1996). The use of BsAbs in virus infections has been more limited, but there have been successful results as well, against HIV (Chamow et al., 1994, Howell et al., 1994, Mabondzo et al., 1994, and Mabondzo et al., 1995), and for targeted delivery of adenoviruses (Wickham et al., 1996). Our *in vivo* results encourage further pursuit of their therapeutic use in experimental models of virus infection.

Figure 4-1. A) Cytotoxicity assay using SEB T cells as effector cells and the BsAbs 526 (anti-VSV and anti-TCR), 3F12 (anti-influenza virus M2 and anti-TCR), or medium alone to redirect lysis of VSV infected EL4 cells. EL4 cells were infected with VSV for 1 hour at an MOI=40, and then chromium labeled for another hour. Then, cells were incubated 4 hours with either BsAb 526 (1 or 2μg/ml), 3F12 (1μg/ml) or medium in the presence or absence of SEB T cells at different E:T ratios or no effectors. % cytotoxicity is shown.

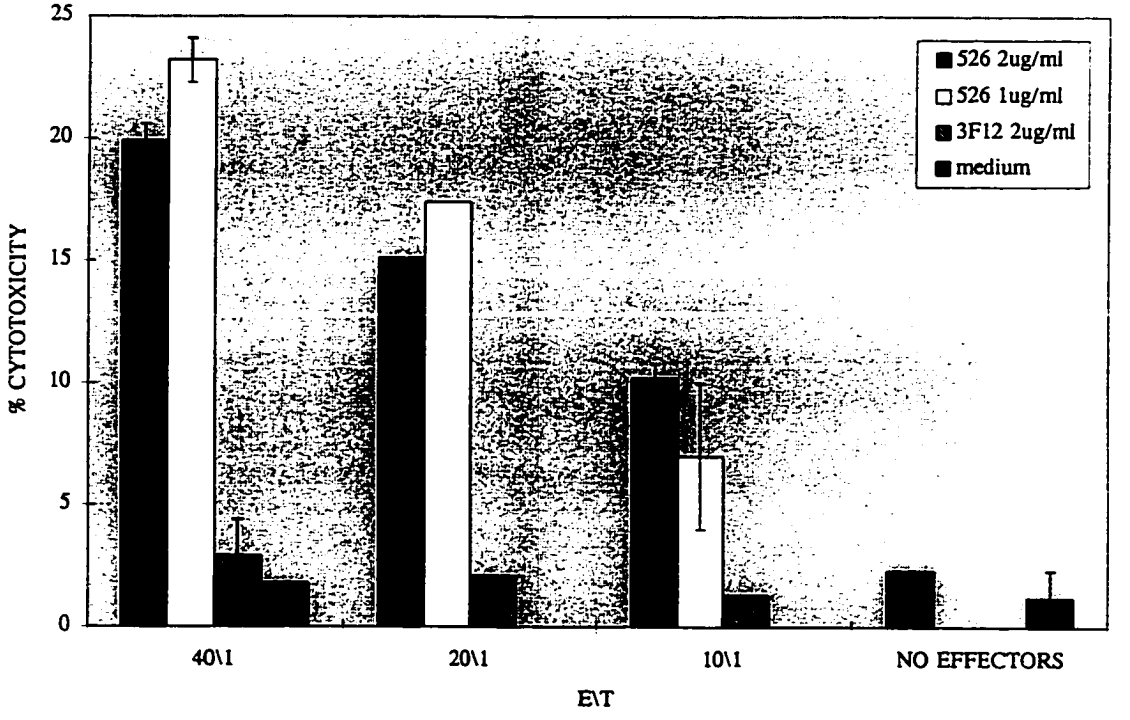


Figure 4-1. B) Inhibition of VSV replication in P815 cells by SEB T cells redirected by 526 at different concentrations, 3F12 (1μg/ml), or medium alone. P815 cells were infected with VSV at an MOI= 0.1 for 48 hours in the presence of SEB T cells at E:T ratio of 20:1 together with BsAbs 526, 3F12 or medium. Supernatants were then collected and the VSV titers determined by inoculation of BHK cell monolayers for 48 hours with log 10 dilutions of the supernatants. CPE was observed following crystal violet staining and end points determined by the method of Reed and Muench (10).

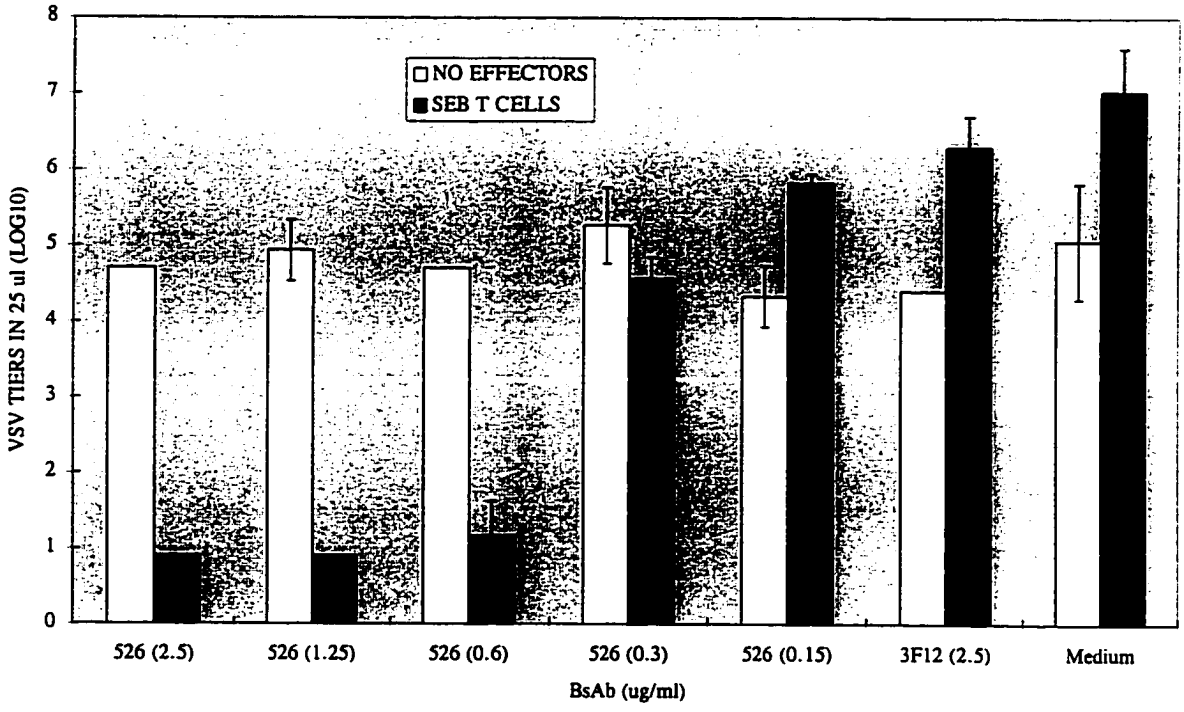


Figure 4-2. Inhibition of VSV replication by SEB T cells (20:1) and 526 (1 μg/ml) using either the Indiana or New Jersey strains of VSV to infect P815 cells. Both VSV strains were used to infect P815 cells at MOI= 0.1 for 48 hours. Virus titers in the supernatants were calculated by performing an infectivity assay on BHK cells for 48 hours and then testing for CPE using crystal violet.

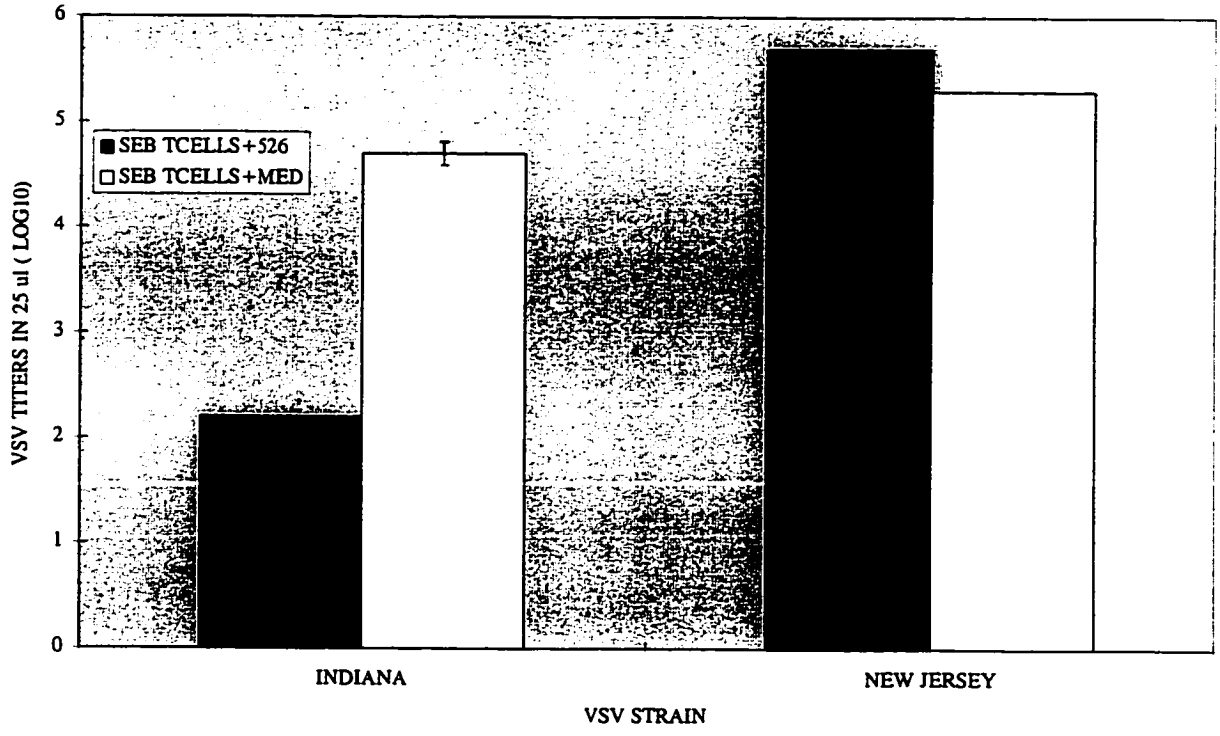


Figure 4-3. Inhibition of VSV replication by SEB T cells and 526 in the absence of IFNγ. SEB T cell cultures (20:1) + 526(1μg/ml) were incubated with either XMG1.2(anti-IFNγ) at 1μg/ml or medium and then the mixtures were added to VSV infected P815 cells (MOI=0.1). After 48 hours, supernatants were used to infect BHK cells for another 48 hours and infectivity titers were calculated by staining BHK monolayers with crystal violet.

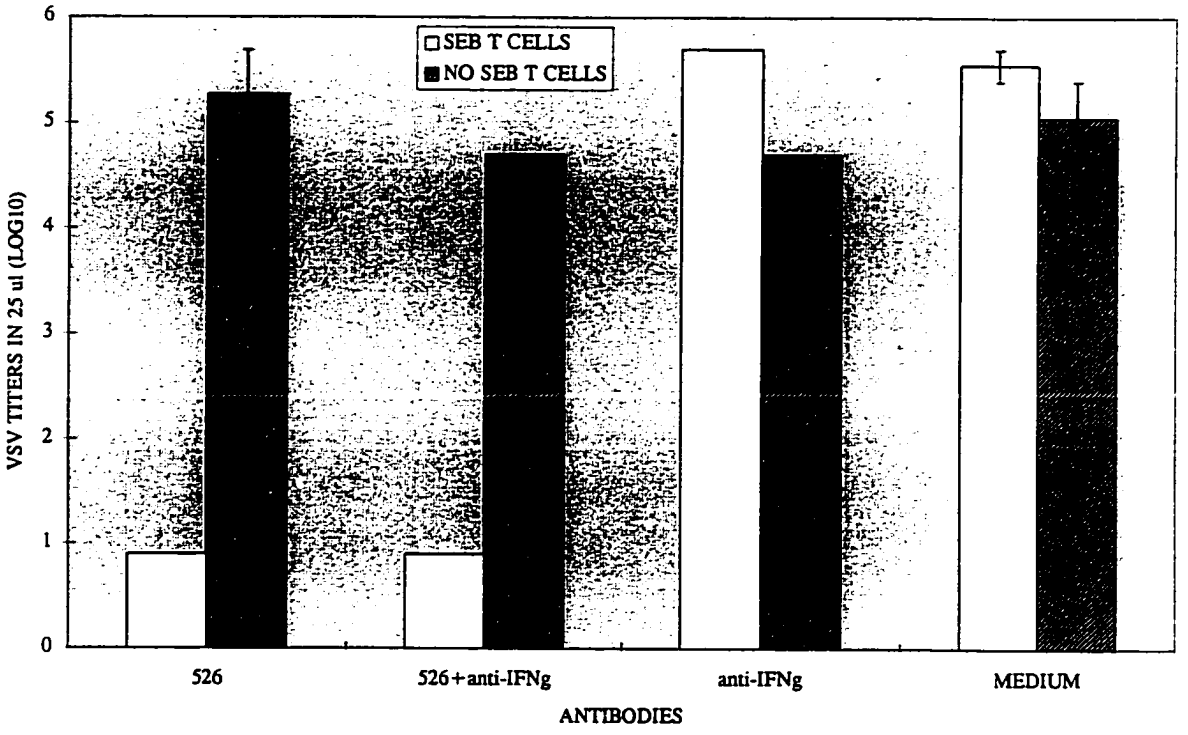


Figure 4-4. A) Inhibition of VSV replication in P815 cells by either CD4$^+$ SEB T cells, CD8$^+$ SEB T cells or unseparated SEB T cells, redirected by 526. P815 cells infected with VSV (MOI=0.1) were incubated 48 hours with either CD4$^+$ SEB T cells, CD8$^+$ SEB T cells or unseparated SEB T cells and 526 (1μg/ml) or medium. Supernatants were then tested on BHK cells for infectivity titers.

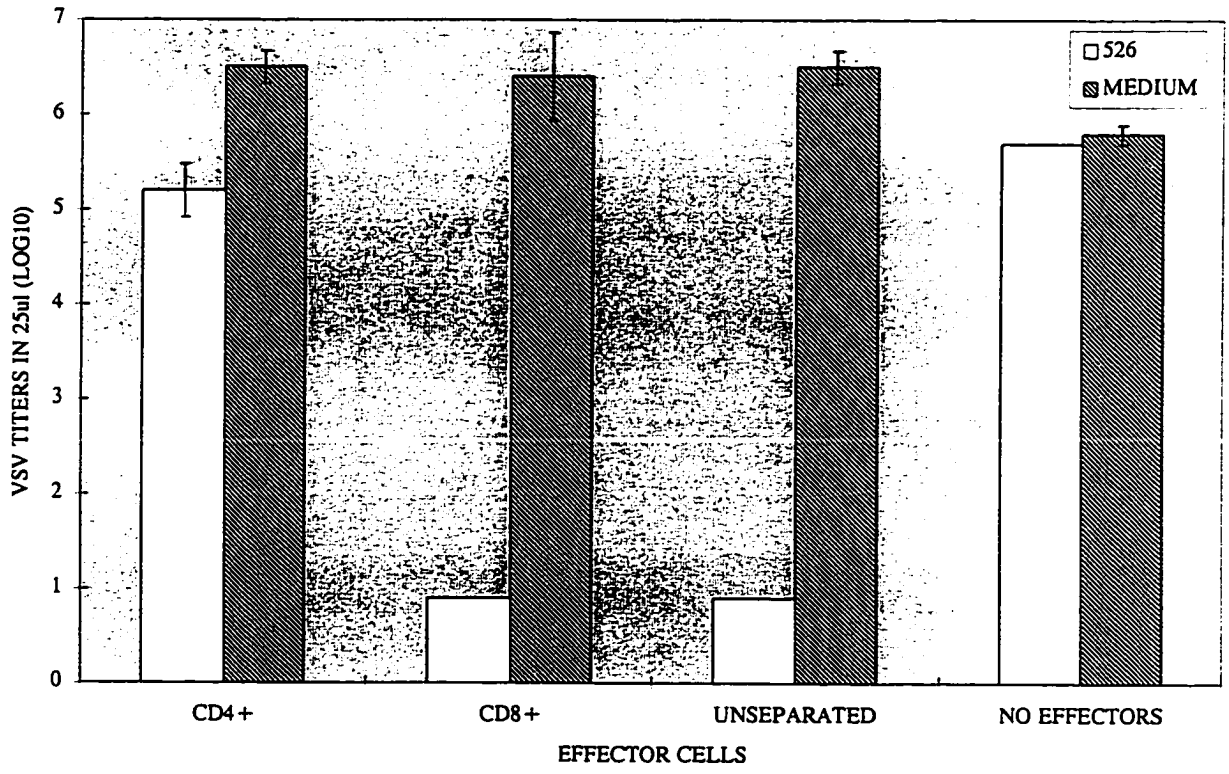


Figure 4-4. B) Cytotoxicity assay using $CD4^+$ SEB T cells (◆), $CD8^+$ SEB T cells (■) or unseparated SEB T cells (▲). EL4 cells were infected with VSV at an MOI=40 for 1 hour and labeled with ^{51}Cr for 1 hour. EL4 cells were then incubated for 4 hours in the presence of either $CD4^+$ SEB T cells, $CD8^+$ SEB T cells or unseparated SEB T cells + 526 (1μg/ml) or medium. Supernatants were then harvested and % cytotoxicity calculated.

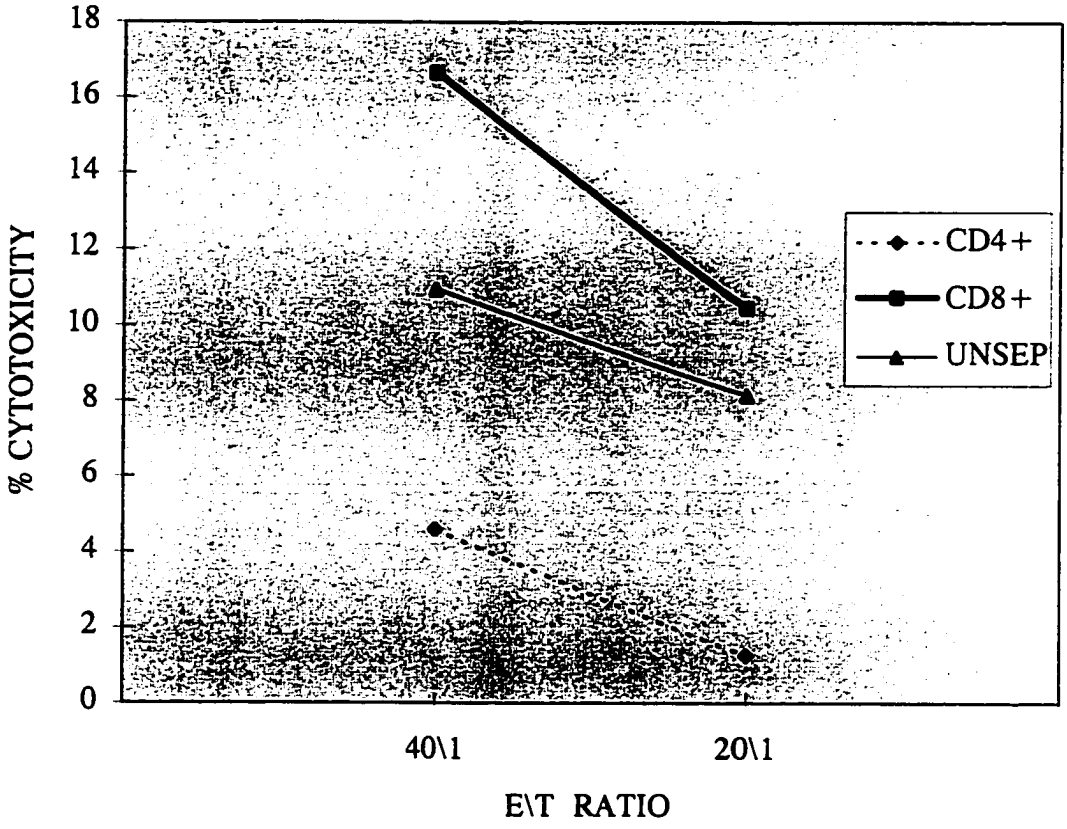


Figure 4-5. A) Cytotoxicity assay by SEB T cells from either C57Bl\6 (WT) mice or perforin deficient mice (Pfp KO) redirected by 526 (1μg/ml). EL4 cells infected with VSV (MOI=40) for 1 hour and chromium labeled for another hour were incubated 4 hours with 526 (1μg/ml) and SEB T cells from either C57Bl\6 (WT) mice or Pfp KO mice. % cytotoxicity was calculated from the supernatants. When medium was used instead of BsAb, %cytotoxicity ranged between 0-6%. Effector cells were used at 20:1 and 40:1 E:T ratios.

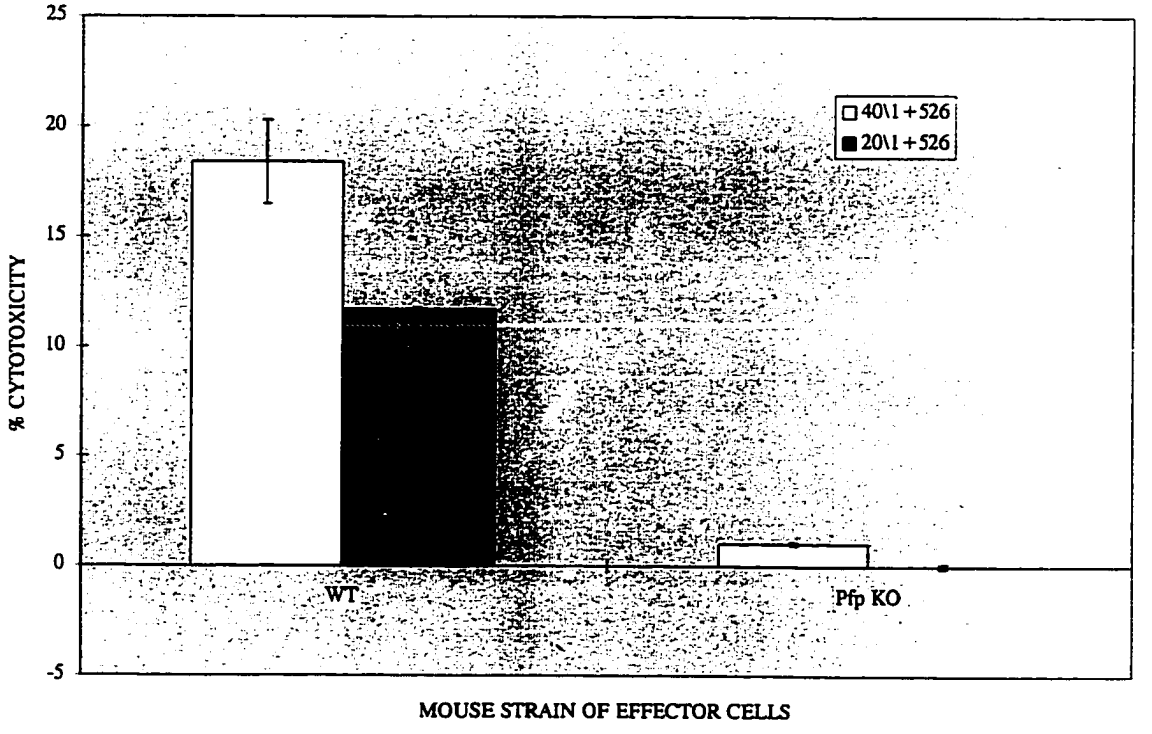


Figure 4-5. B). Inhibition of VSV replication in P815 cells by SEB T cells from WT mice or Pfp KO mice (E:T 20:1) or no SEB T cells redirected by 526 (1 μg/ml). P815 cells infected with VSV (MOI=0.1) were incubated 48 hours with SEB T cells from C57Bl\6 (WT) mice or Pfp KO mice or medium together with 526 (1 μg/ml) or medium. Supernatants were used to infect BHK cells for 48 hours and virus titers were calculated by CPE.

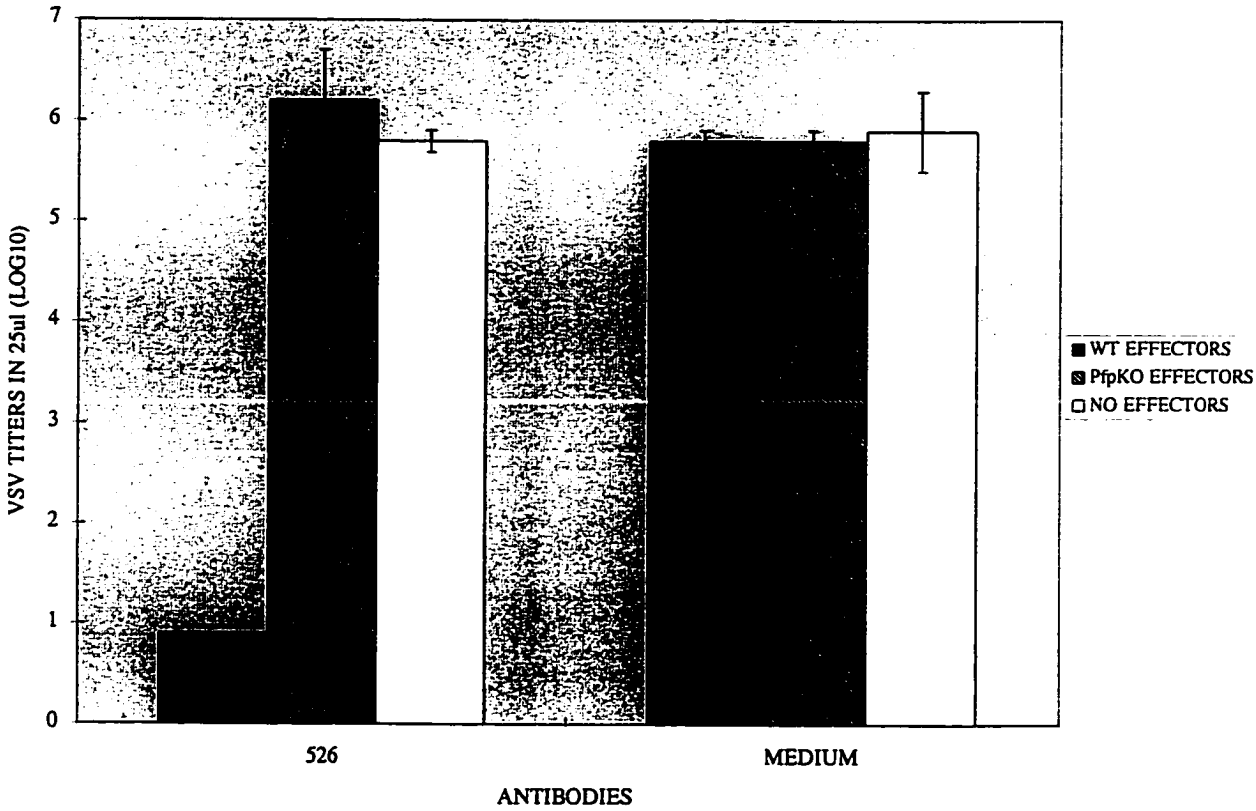


Table 4-1. Survival of VSV-infected Stat1 KO mice after different treatments. Stat1 KO mice were infected ip with 1-5 LD_{50}/mouse of VSV (for each experiment a single LD_{50} was used). One hour later, mice were injected again ip with either $95x10^6$ WT SEB T cells/mouse, BsAb 526 (50μg/mouse), SEB T cells ($95x10^6$ cells/mouse)+ BsAb 526 (50μg/mouse) or PBS. Hours of survival after infection are shown. Values are the sum of several experiments. The differences in the values are statistically significant (Chi Square with $p < 0.005$).

	24h alive/total	**48h alive/total**	**96h alive/total**
MOCK	18/18	5/18	4/18
SEB T CELLS	16/16	6/16	2/16
BSAB	16/16	7/16	7/16
SEB T CELLS + BSAB	20/20	20/20	13/20

Chapter 5

IMPAIRED ANTIVIRAL IMMUNE RESPONSE OF STAT1 KO MICE AFTER INFECTION WITH INFLUENZA VIRUS.

(Part of Durbin et al., 1997, submitted for publication)

ABSTRACT.

Stat1 is a protein involved in signal transduction in response to both type I and type II interferon. Stat1 KO mice were infected with influenza virus and their immune response was studied. Serum from Stat1 KO mice and WT CD1 mice was taken before and after infection and immunoglobulin levels were measured by ELISA. Stat1 KO mice showed higher levels of IgG1 and a lack of IgG2a antibodies specific for influenza virus compared to WT mice. Stat1 KO mice also showed higher levels of total serum IgE antibodies than WT mice even before virus infection. Spleen cells from infected mice were restimulated *in vitro* and cytokine levels were measured in the culture supernatants. Spleen cells from Stat1 KO Mice secreted more IL-4 and IFNγ than spleen cells from WT mice. Surprisingly, splenocytes from both Stat1 KO and WT mice showed comparable levels of cytotoxic activity against influenza virus infected cells. Stat1 KO mice exhibited an antiviral immune response with characteristics of both a Th1 and a Th2 response. Thus, these animals produce IL-4 and IgE but no IgG2a and yet make a good CTL response characteristic of Th1 immunity.

INTRODUCTION.

Stat1 is involved in signal transduction in response to both type I and type II interferons (Müller et al., 1993, O'Shea, 1997). Thus, disruption of the Stat1 gene or malfunction of the Stat1 protein will render cells deficient in their response to interferon (Horvath and Darnell, 1996). After interferons bind to their receptor on the surface of cells, JAK kinase is recruited to the intracellular portion. This process activates the JAK kinase by phosphorylation. Stat1 is then recruited to this activated complex, becomes phosphorylated, and subsequently forms dimers which translocate to the nucleus and activate specific genes by binding to sequences in the DNA (reviewed in Ihle, 1996 and O'Shea, 1997).

Stat1 KO mice are expected to mount an impaired immune response against virus infection, since type I interferon is involved in early innate responses against viruses (Staeheli, 1990, Horvath and Darnell, 1996), and type II interferon is involved in specific immune responses (Graham et al., 1993). Such Stat1 KO mice were generated by Joan Durbin in David Levy's laboratory by disruption in the Stat1 gene in CD1 mice (Durbin et al., 1996). Since they are incapable of responding to interferon, these mice are very susceptible to bacterial and viral infections (Durbin et al., 1996, Meraz et al., 1996). VSV is known to be very sensitive to type I interferon (Staeheli and Pavlovic, 1991). Consistently, Stat1 KO mice were more susceptible to VSV infection than their WT counterparts (Durbin et al., 1996). Influenza virus is not as

sensitive to the action of IFN but Stat1 KO mice infected with influenza virus showed an impaired (100 fold less) capability of clearing infection compared with WT in experiments performed in David Levy's laboratory and related to this work (Durbin et al., 1997, submitted for publication).

For the experiments described in this chapter Stat1 KO mice were infected with either influenza A/WSN/33 virus or an attenuated virus derived from it, NA/B-NS and the course of infection was studied by examining lesions and cellular infiltrates in lungs on different days after infection in Dr. Levy's laboratory. Stat1 KO mice showed more extensive lung lesions than WT mice as well as greater infiltration of eosinophils in the lung compared to WT mice. These results suggested that Stat1 KO mice were generating a different immune response against influenza virus infection than WT mice and that this response could have the characteristics of a Th2 response by the type of cells present at the site of infection. The generation of a Th2 immune response by these mice against influenza virus infection would explain their impaired viral clearance and their susceptibility to this virus at doses not usually lethal for WT mice.

Serum from Stat1 KO and WT mice before and after infection with NA/B-NS and spleens from Stat1 KO and WT mice infected with NA/B-NS were analyzed in our laboratory. The serum from Stat1 KO mice was found to have higher levels of total IgE antibody than WT mice before and after infection. Stat1 KO mice also had serum anti-influenza virus antibodies of the IgG1 isotype while IgG2a antibodies could not be detected in our assay. In contrast, WT mice showed both IgG1 and IgG2a antibodies

against influenza virus in their serum with predominance of the IgG2a isotype. These results alone confirm the results from Dr. Levy's group, but we also studied the cytokines released by spleen cell cultures restimulated *in vitro* from both Stat1 KO and WT mice previously infected with influenza virus. We tested the cytotoxic capacity of those spleen cells against influenza virus infected cells as well. Spleen cells from Stat1 KO mice consistently showed higher levels of both IFNγ and IL-4 in the supernatant than those of WT mice at days 2 and 5 of culture. In contrast, cytotoxicity levels against influenza virus infected cells were comparable in Stat1 KO and WT mice.

Based on the IL-4 levels as well as the antibody isotypes and Dr. Levy's results with the lung infiltrates, the immune response of Stat1 KO mice against influenza virus demonstrate many of the characteristics of a Th2 response. But the IFNγ levels as well as the cytotoxicity data indicate some Th1 component in this immune response. Thus, Stat1 KO mice make a mixed immune response against influenza virus, suggesting that a loss of interferon activity results in an impairment of cytokine regulation in these mice compared to CD1 WT mice.

MATERIALS AND METHODS

Spleen cell cultures.

Spleen cells from influenza virus (NA-B/NS) infected Stat1 KO and WT CD1 mice were taken 3 weeks after infection and treated with 0.17M Tris NH_4Cl to lyse the red blood cells. The splenocytes were then divided in 3 samples. One of the samples was used as the stimulator cells. These cells were irradiated and then infected with influenza virus A/PR8/34 at an MOI=4 for 1 hour at 37^0C and then incubated together with another sample (responders) from the same spleen at a concentration of $4X10^6$ stimulators/$4X10^6$ responders/ml for 5 days at 37^0C, 7% CO_2 in T cell medium. The third sample of splenocytes was stimulated with *S. typhosa* LPS (25μg/ml) (Difco) for 5 days at a concentration of $2X10^6$ cells/ml to be used as target cells in the cytotoxicity assay.

Cytokine detection.

Supernatants from spleen cell cultures were taken at different days of culture to test for the concentration of IFNγ (ELISA) and IL-4 (Bioassay).

Bioassay: IL-4 dependent CT4.S cells ($5x10^3$/well) (kind gift from Dr. W. Paul) were mixed with dilutions of supernatants from the spleen cell cultures, medium containing different concentrations of recombinant mIL-4 (Boehringer Mannheim), or medium alone in flat bottom 96 well plates and incubated at 37^0C for 48h. ^3H-thymidine was added (1 μCi/well) and the plates harvested 18 h later and ^3H incorporation determined by counting in a Packard Matrix counter.

ELISA: ELISA plates were coated overnight at 4^{0}C with anti-IFNγ antibody (Pharmingen) at 4μg/ml. Plates were then blocked with PBS-BSA for 1h at RT and supernatants from spleen cell cultures, recombinant mIFNγ (Boehringer Mannheim) (3-100 ng/ml), or medium alone were added to the plates and incubated for 3h at RT. After washing away the supernatants, 50μl of biotinylated anti-IFNγ mAb (Pharmingen) were added to each well (0.5μg/ml) and incubated for 1h at RT. Streptavidin-POD (horseradish peroxidase) conjugate (Boehringer Mannheim) was added to the plates at (0.05U/ml) and after washing away the unbound antibodies with PBS-BSA plates were incubated 45 min at RT. Plates were washed with PBS-BSA and ABTS substrate (Boehringer Mannheim) was added to wells (1mg/ml) and incubated at RT. Plates were read after 15 minutes in an ELISA reader ELX 800 (Bio-Tek. Inc.) at 405 nm.

Cytotoxicity assays.

1. Virus-specific cytotoxicity

Cells were removed from spleen cell cultures and mixed at 40/1 E/T ratio with autologous ^{51}Cr-labeled LPS blasts (sodium chromate, NEN) either uninfected or infected with PR8 influenza virus (MOI=10) in v-bottom 96 well plates. The plates were incubated at 37^{0}C for 4 hours, spun to pellet the cells and supernatants were harvested. The amount of ^{51}Cr in the supernatants was determined in a γ-counter. Target cells incubated with 0.5% NP-40 (Sigma) were used to determine maximum release, and target cells incubated with medium alone (routinely less than 10% of the maximum release) were used to calculate spontaneous release. The % cytotoxicity was calculated by the

formula [% cytotoxicity=test release-spontaneous release/maximum release-spontaneous release x 100].

2. Antibody-mediated redirected lysis.

This assay was performed exactly as above except that the target cells were ^{51}Cr-labeled P815 cells and anti-CD3 antibody 2C11 (1μg/ml) was added. This antibody bridges activated T cells and Fc receptor-bearing cells leading to target cell lysis.

Immunoglobulin detection.

Detection of anti-influenza virus IgG1 and IgG2a antibodies in serum from naive as well as influenza virus infected Stat1 KO and WT mice was performed by ELISA using purified influenza virus (PR8) at 25μg/ml to coat plates overnight at 4^0C.

Plates were blocked with PBS-BSA and serum dilutions, medium alone or standard IgG antibodies (0.008-1μg/ml) (PY102 anti-PR8 IgG1, ,PY210 anti-PR8 IgG2a and A31G10 as negative IgG) were incubated for 3 hours. POD-linked m Abs anti-IgG1-POD, anti-IgG2a-POD and anti-IgG-POD (Boehringer Mannheim) were used as secondary antibodies. ABTS substrate was used for the reaction as described.

Total serum IgE was determined by ELISA (all reagents kindly provided by Dr. Seiji Haba, Brandeis University). AE19, an A/J anti-mouse IgE was used to coat ELISA plates overnight at 5μg/ml. After blocking the wells, dilutions of serum were added and incubated for 3 hours, plates then were washed. Bound (IgE) was identified by the addition of biotin- labeled rabbit anti-mouse IgE antibody and Streptavidin-POD

for 1h. Lastly, ABTS substrate was added and the reaction allowed to proceed for 15 minutes after which the plate was read on an ELISA reader at 405 nm.

RESULTS.

Stat1 KO mice do not generate antibodies of the IgG2a type after infection with influenza virus. WT CD1 and Stat1 KO mice were infected intranasally with an attenuated strain of influenza virus derived from A/WSN/33 (NA/B-NS) (Muster et al., 1991). Serum from these animals was taken before and after infection and immunoglobulin isotypes were detected by ELISA. As shown in figure 5-1, Stat1 KO mice do not make anti-influenza virus antibodies of the IgG2a isotype after infection with the virus as do WT CD1 mice. This lack of IgG2a antibodies can be taken as an indicator of a Th2 type of immune response after virus infection. Also, Stat1 KO mice seemed to produce higher levels of IgG1 antibodies against influenza virus than WT CD1 mice. The lack of Stat1 protein makes these mice unable to respond to IFNγ, a known switch factor for IgG2a (Coutelier et al., 1987, Bossie and Vitteta, 1991). Thus, B cells of Stat1 KO mice are unable to class switch to the IgG2a isotype after virus infection.

Stat1 KO mice make high levels of IgE antibodies both before and after infection with influenza virus. Figure 5-2 shows levels of total IgE antibodies in serum from mice before (prebleed) and after (infected) infection with influenza virus NA/B-NS (Muster et al., 1991). Stat1 KO mice have higher levels of IgE in serum than WT mice before the infection, but this difference is dramatically accentuated after infection with influenza virus. When anti-influenza virus specific IgE antibodies were

measured (data not shown) an increase was also observed after virus infection (1 fold), but it was less dramatic than the increase in total IgE antibodies (7.5 fold). Since production of IgE is dependent on the Th2 cytokines, IL-4 and IL-5 (Finkelman et al., 1990), this again suggests that these animals are responding to influenza virus infection with a Th2 response.

Stat1 KO mice have similar levels of antiviral cytotoxic T cells to WT mice, but the cytokine patterns in supernatants of spleen cultures from influenza virus infected mice are different. Spleen cells from influenza virus (NA-B/NS) infected Stat1 KO and WT mice were taken 3 weeks after infection and cultured *in vitro* for 5 days. At day 5 splenocytes were tested in either a 4h Cr^{51} release assay (direct cytotoxicity) or antibody mediated cytotoxicity (ADCC) using the anti-CD3 antibody 2C11. In both assays splenocytes from both Stat1 KO mice and WT mice showed similar levels of cytotoxicity against influenza virus infected spleen cells or P815 cells (table 5-1). Samples from the supernatants were taken at days 2 and 5 and tested for the presence of the cytokines, IL-4 (CT4.S cells bioassay) and IFNγ (ELISA). Splenocytes from Stat1 KO mice secreted higher levels of both IL-4 and IFNγ after *in vitro* restimulation with influenza virus (PR8) infected spleen cells as shown in table 5-1. Thus, the mice secrete both Th1 and Th2 cytokines.

Spleen cells from Stat1 KO mice proliferate faster than spleen cells from WT mice. Spleen cells from cultures were checked for their proliferative capacity by taking samples on a daily basis and counting the cells. Consistently there were more

cells in the cultures of Stat1 KO splenocytes compared to WT (data not shown). Also, the morphology of the Stat1 KO cells was different from the WT ones. It has been reported that IFNγ is an important regulator of cell growth. This may explain the rapid growth of cultures from Stat1 KO mice (Novelli et al., 1997).

DISCUSSION.

Stat1 is necessary for the signal transduction of both type I and type II interferons (Müller et al., 1993, reviewed in O'Shea, 1997). IFNα is able to create an antiviral state through the action of Stat1 (Staeheli, 1990, Horvath and Darnell, 1996) which is very potent in preventing the spread of viruses. Mice deficient in the Stat1 protein are unable to respond to viral infection with a proper immune response (Durbin et al., 1996, Meraz et al., 1996) and succumb to infection at extremely low number of virus particles per mouse (Durbin et al., 1996).

The purpose of this work was to study the nature of the immune response of Stat1 KO mice against influenza virus infection. In particular, the antibody isotypes generated by these mice before and after infection and the cytokine profile and cytotoxic functions of their *in vitro* restimulated spleen cultures were analyzed.

Antibody isotypes are associated with different types of immune responses. Immunoglobulins of the IgG1 isotype are generated in Th2 immune responses as are IgE antibodies. (Finkelman et al., 1990, reviewed in Abbas et al., 1996 and Mosmann and Sad, 1996). On the other hand, IgG2a antibodies are the predominant isotype observed in Th1 responses (Coutelier et al., 1987, Bossie and Vitteta, 1991, reviewed in Abbas et al., 1996). IFNγ is produced primarily in Th1 responses and it dictates that B cells switch to the IgG2a isotype. In its absence IgG1 are exclusively made. IgE production is induced only in response to Th2 cytokines, IL-4 and IL-5. Our data suggest that Stat1 KO mice generate antibodies associated with a Th2 immune response

against influenza virus infection. We and others have shown that Th2 immunity leads to an immune response which is very inefficient in clearing virus from infected animals (Moran et al., 1996, Waris et al., 1996).

The cytokine patterns in the supernatant of spleen cells from influenza virus infected Stat1 KO mice was also consistent with a Th2 immune response since high levels of IL-4 secretion was detected. The presence of equally high levels of IFNγ in these supernatants however was in contrast to what is usually observed in Th2 immune responses. These animals seem to be making both Th1 and Th2 cytokines. It should be pointed out however that since the defect in these mice is in the response to interferon, not in its synthesis, the cells of the immune system of these mice are not capable of responding to the effects of their own IFNγ, rendering this cytokine useless.

The most striking result was the presence of similar levels of cytotoxic T cells against influenza virus infected target cells in both Stat1 KO mice and WT mice. This suggests that interferon does not play a major role in the generation of CTL. Nevertheless, these CTLs are not capable of clearing the virus infection *in vitro* (Durbin et al., 1996).

In trying to explain these effects in Stat1 KO mice we propose two not mutually exclusive possible mechanisms. The first mechanism is the loss of negative signaling by interferon-γ. Recent work has demonstrated that Th2 cells have high affinity receptors for interferon-γ while Th1 cells do not (Pernis et al., 1995). Recently, it has been postulated that one way in which Th2 responses are inhibited during a Th1 situation is

by induction of Th2 cells into apoptosis by interferon-γ (Novelli et al., 1997). In Stat1 KO mice this would not occur, thus, explaining why both types of responses are observed simultaneously.

Additionally, the failure of CTL to clear virus may occur because of inefficient trafficking of cells to the infected area. A significant amount of evidence suggests that IFNγ is involved in upregulation of selectins, known to be involved in recruiting cells to sites of inflammation (Austrup et al., 1997). Clearly, this signaling is also lost in these animals. Therefore, CTL may be generated but unable to find the site of infection. It is worth noting that receptors for selectins are found on Th1 cells only. Migration of Th2 cells to inflamed areas either uses other receptors or is independent of selectins.

Based on our results from influenza virus infected Stat1 KO mice, we propose that these mice have both Th1 and Th2 immune responses acting simultaneously, since IFNγ is not capable of downregulating Th2 immune responses. Thus, Stat1 KO mice have an impaired antiviral immunity possibly due to their lack of regulation between Th1 and Th2 responses.

Figure 5-1. IgG isotypes in serum from mice infected intranasally with an attenuated strain of influenza virus. Stat1 KO and WT mice were infected intranasally with NA/B-NS strain and serum was collected and tested for the isotypes of the anti-influenza virus antibodies. ELISA plates were coated with 25μg/ml purified PR8 influenza virus, sera from either influenza virus infected Stat-1 KO or CD1 mice at a 1/1000 dilution were added to triplicate wells. Bound antibodies were identified by the addition of biotin labeled anti-IgG (whole antibody), anti-IgG1 or anti-IgG2a. After addition of streptavidin-POD and substrate (ABTS), plates were read at 405nm. Immunoglobulin levels represent the means of cumulative results of sera from three mice analyzed separately.

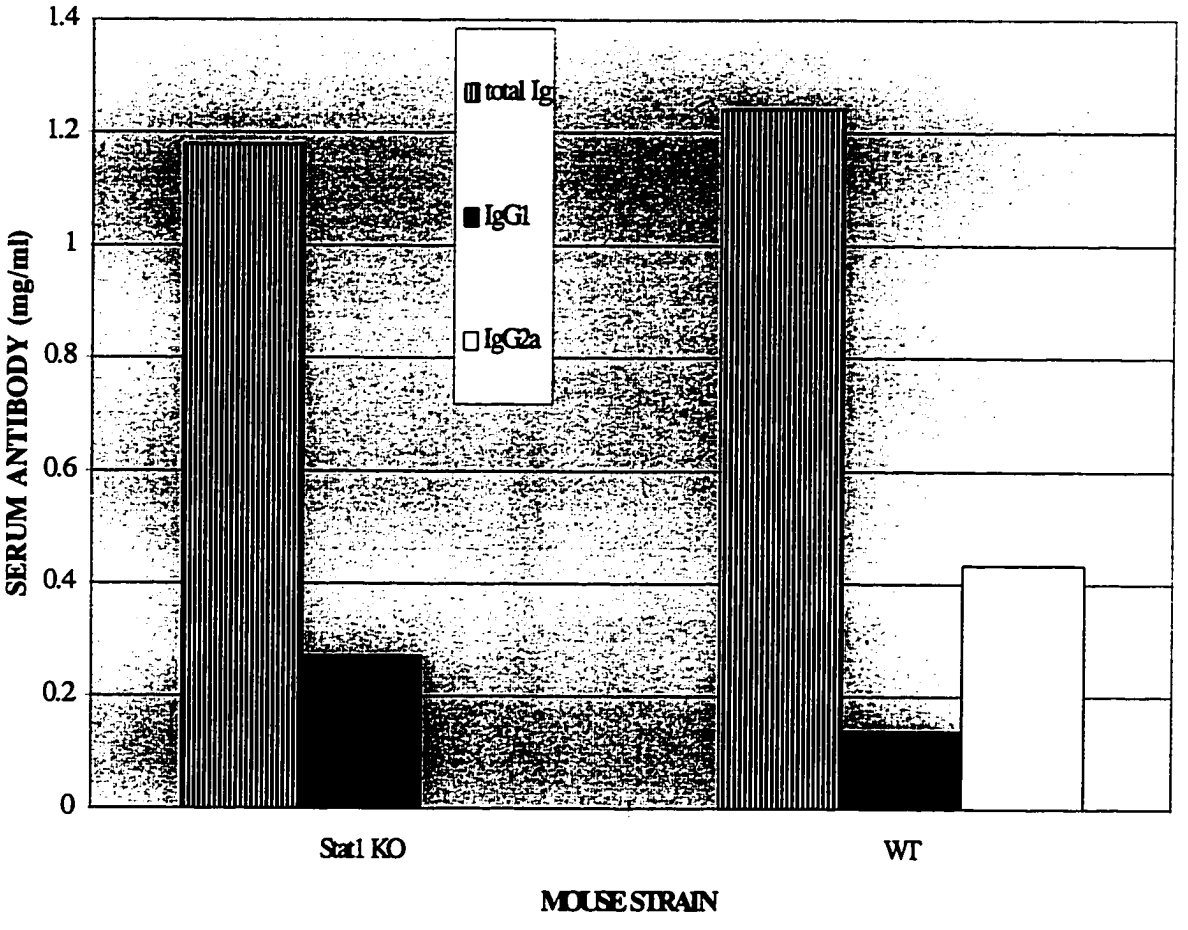


Figure 5-1

Figure 5-2. IgE antibodies in serum from uninfected or influenza virus infected mice. Mice were infected intranasally with the attenuated influenza virus NA/BNS. Serum was taken before and after infection and tested for the IgE antibody concentration by ELISA. Anti-IgE antibody AE19 was used to coat the plates and biotin-labeled anti-IgE antibody was used to detect. Streptavidin-POD and ABTS substrate were added subsequently and plates were read at 405nm. Results are means from 4 mice in each group.

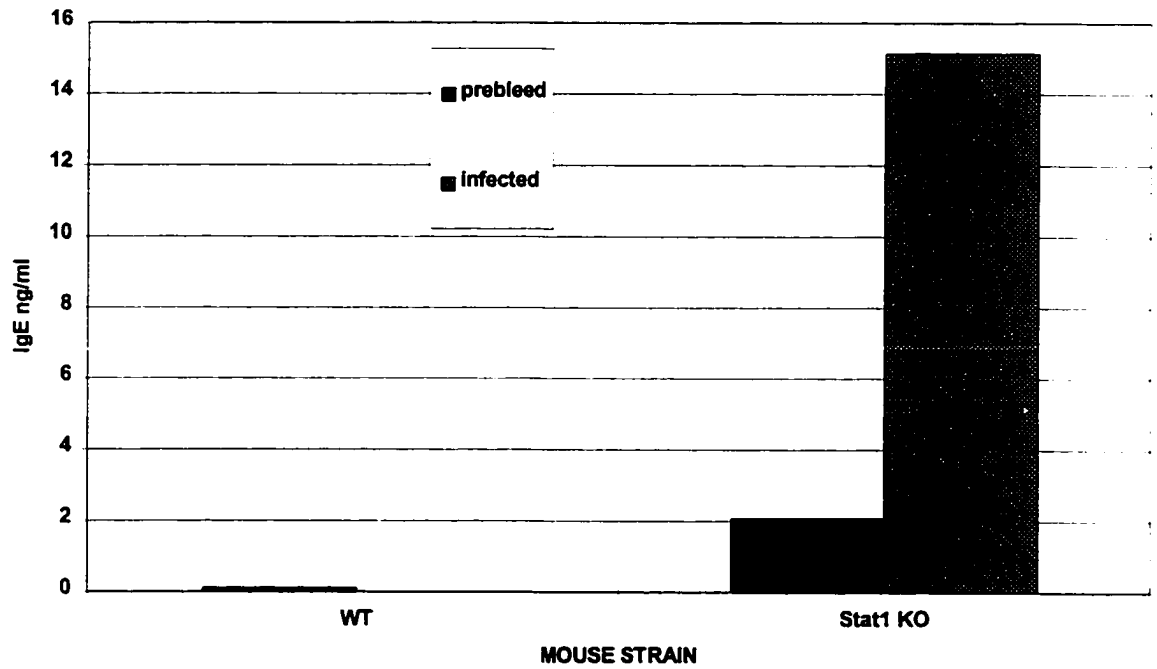


Table 5-1. Cytotoxicity and cytokine profiles of influenza virus stimulated splenocytes from Stat-1 KO and WT mice. The first column shows % cytotoxicity of autologous virus- infected LPS blasts by spleen cells from influenza virus infected mice at an E:T ratio of 40:1. (%CTX). For each group, 2 mice were used. The second column shows % antibody mediated redirected cytotoxicity using 2C11 (anti-CD3) at 1μg/ml and P815 (FcR+) as target cells and spleen cells from influenza virus infected mice as effectors at a 40:1 E:T ratio (%ADCC). Means from 2 mice in each group are shown. Third and fourth columns show levels of IFNγ in supernatants in spleen cultures from influenza virus infected animals restimulated *in vitro* at days 2 and 5 after restimulation respectively. Columns fifth and sixth show levels of IL-4 in supernatants of spleen cell cultures from influenza virus infected mice at days 2 and 5 after restimulation *in vitro*. In both cytokine detections 4 KO mice and 5 WT were used. ND= Not detectable. Experiments were done several times with different animals and comparable results. Results are means ± standard errors.

	% CTX	% ADCC	ng/ml IFNγ (Day 2)	ng/ml IFNγ (Day 5)	ng/ml IL-4 (Day 2)	ng/ml IL-4 (Day 5)
KO	16.65±7.9	39.9 ±13.7	179.7±4.9	74.4 ±2.8	123 ±6.3	6.8 ±5.3
WT	26 ±11.1	59 ±2.12	14.5 ±0.6	30.1 ±1.4	ND	ND

Chapter 6.

EFFECTS OF *IN VIVO* INJECTIONS OF IL-4 ON INFLUENZA VIRUS INFECTED BALB/C MICE.

(Part of Moran et al., 1996).

ABSTRACT.

BALB/c mice infected with influenza virus are able to recover by efficiently clearing the virus from their lungs. When IL-4 was injected at the time of infection and subsequently on a daily basis, this response was significantly modified. The generation of cytotoxic T cells by these mice was inhibited, while mice not treated with IL-4 generated normal levels of cytotoxic T cells. Supernatants of spleen cultures from infected and IL-4 treated mice, restimulated *in vitro* showed high levels of IL-4 compared to mice not treated with IL-4. In contrast, the IFNγ levels in supernatants of spleen cells restimulated *in vitro* were significantly lower in mice treated with IL-4 than in mice not treated. In conclusion, the addition of IL-4 to mice infected with influenza virus switched their antiviral immune response from Th1 to Th2. CTL generation was significantly reduced and the animals were unable to efficiently clear virus from their lungs.

INTRODUCTION.

Influenza virus infection in mice has been used as a model to study antiviral immunity by several groups (Schulman, 1967, Luckacher et al., 1984, Graham et al., 1994, Topham et al., 1996). Mice become infected and are able to generate antibodies against major proteins of the virus, and also make a cellular immune response which clears the virus from the lungs efficiently (Cambridge et al., 1976).

The cellular immune response generated against viruses is composed of both $CD4^+$ T cells (Th) and $CD8^+$ T cells (CTLs) (Cambridge et al., 1976, Wells et al., 1981, Doherty et al., 1992, Hou et al., 1995, reviewed in Zinkernagel, 1996). Since Th1 cells are known to be involved in the generation of CTLs it seemed likely that they would be the subtype most efficient in mediating recovery from virus infection (reviewed in Abbas et al., 1996). Yet, examination of draining lymph nodes from virus infected animal showed evidence of both Th1 and Th2 cytokines (Sarawar et al., 1993, Graham et al., 1994). The importance of T helper cells as well as CTLs for clearing viruses has been shown using models of influenza virus infected mice in which either $CD4^+$ T cells (Allan et al., 1990) or $CD8^+$ T cells (Scherle et al., 1992) were not functional or the infected animals lacked specific MHC elements (Eichelberger et al., 1991, Bender et al., 1992). In both cases, antiviral immunity against influenza virus was diminished but not abolished. Thus, antiviral immunity against influenza viruses was most efficient when both $CD4^+$ and $CD8^+$ T cells acted in synergy.

Th1 responses are mediated by $CD4^+$ T cells that secrete IFNγ and IL-2. These cells develop when they are exposed to antigen in an environment containing IL-12 (Mosmann et al., 1986, Seder and Paul, 1994, reviewed in Abbas et al., 1996, Heufler et al., 1996, reviewed in Zinkernagel, 1996) and $CD8^+$ T cells, which lyse virus infected cells by either release of soluble substances such as perforin or by inducing apoptosis in the infected cells (Kojima et al., 1994, reviewed in Berke, 1995, Nagata and Suda, 1995, Atkinson and Bleackley, 1997, Zajak et al., 1997). The fact that cytokines such as IFNγ and TNFα have been shown to have direct antiviral effects (Chisari and Ferrari, 1995) may provide an explanation for the observation that $CD4^+$ T cells can clear virus infections in the absence of $CD8^+$ T cells (Scherle et al., 1992) or MHC class II (Topham et al., 1996).

Th2 immune responses are generated when the cytokine milieu contains IL-4 at the time of exposure to immunogen. Immune responses of this type are characterized by $CD4^+$ T cells which release IL-4 and IL-5, directing B cells to make antibodies of the IgG1 and IgE isotypes (Finkelman et al., 1990), recruitment of eosinophils, and a failure of CTL to develop.

It has been shown in different experimental models that it is possible to manipulate immune responses to pathogens by using cytokines or anti-cytokine antibodies early in the infection (Haak-Fredescho et al., 1992, Romani et al., 1992, Racke et al., 1994, Sarawar et al., 1994, Leonard et al., 1995, Mocci and Coffman, 1995, Orange et al., 1995). We wanted to determine the effect that the Th2 inducing cytokine IL-4 would

have on immunity to influenza virus. We hypothesized that mice injected with IL-4 would develop a Th2 immune response which might impair their ability to generate CTL and clear virus from their lungs. Therefore, we studied different parameters of antiviral immunity such the generation of cytotoxic T cells and the cytokine profiles of spleen cells from infected mice either untreated or treated with IL-4. This was compared to the level of virus in their lungs as a measure of recovery.

Mice were injected with IL-4 at the time of infection and then daily until day 7. The CTL and cytokines released from spleen cells removed from mice 7 days after infection were restimulated with virus infected stimulator and analyzed. The animals injected with IL-4 made Th2 cytokines while those from the control mice made Th1 cytokines. In addition, CTL generation was vastly reduced in mice treated with IL-4. The same mice sacrificed at different days after infection showed higher virus titers in lungs when IL-4 was injected compared with control mice (virus titrations not performed by Ana Fernandez-Sesma). This demonstrated that injections of IL-4 can switch the immune response of mice infected with influenza virus from a Th1 to a Th2 response. This change reduced the CTL response and significantly inhibited the ability of the mice to clear the virus from their lungs. Moreover, this work emphasizes the importance of the cytokine milieu in the generation of efficient immune responses.

MATERIALS AND METHODS.

Infection Procedure.

Eight to ten week old BALB/c mice were infected by aerosol (Schulman, 1967) as described in general materials and methods. From day 1 to day 7 mice were injected intravenously (iv) with IL-4+anti-IL-4 complexes (Finkelman et al., 1994). Mice were sacrificed at days 5, 7, 8, and 10 and lungs and spleens were taken.

Generation of IL-4 Immune complexes.

IL-4 has been shown to be more stable in complex with anti-IL-4 antibodies (Finkelman et al., 1994). IL-4 immune complexes were prepared by incubating a 1:5 ratio of IL-4 to anti-IL-4(BVD4-1D11.2) in a small tube for 1 minute. The mixture then was diluted with normal saline and 2.5μg:12.5μg (IL-4:anti-IL-4) injected into each animal. The animals were injected iv for the first three days and intraperitoneally (ip) in subsequent injections.

In vivo treatment with IL-4 immune complexes.

Eight week old BALB/c mice were infected with aerosolized PR8 virus (approximately $7.5x10^3$ $TCID_{50}$/mouse) and beginning 24 hours later were injected daily with immune complexed IL-4. Groups of 5 animals were sacrificed on days 5, 7, 8 and 10 and pulmonary virus titers and lung lesions determined. Spleen cells removed from day seven animals were restimulated *in vitro* with PR8 infected irradiated syngeneic spleen cells and cultured for 6 days. The cultured cells then were tested in a cytotoxicity assay and the supernatants collected and tested for cytokine content.

Cytokine determinations.

Microtiter plates were coated with anti-cytokine XMG1.2 (IFNγ) or BVD4-1D11.2(IL-4) antibody at 1μg/ml and incubated overnight at 4^0C. Plates were washed and blocked for 30 minutes with PBS-1% BSA. Plates were washed and then control rIL-4 or rIFNγ (Boehringer Mannheim) were added at different concentrations. Test samples also were added at different dilutions and plates were incubated 90 minutes at room temperature. BVD6-24G2.3 anti-IL-4 and rat anti-mouse IFNγ monoclonal (Upstate Biotechnology) were I^{125} labeled and used for detection at 50,000 CPM in 50μl. Plates were incubated for 90 minutes at room temperature and then washed and counted in a gamma counter. A dose response using purified IL-4 or purified INFγ was performed in each assay and results were used to calculate the absolute concentration of cytokines using samples diluted to fall into the linear portion of the dose response curve.

Cytotoxicity assay.

Spleen cells from influenza virus infected cells were taken at days 4 and 7 after infection and restimulated *in vitro* with syngeneic spleen cells infected with PR8 virus (MOI=5). Cultures were incubated in T cell medium and cells were used as effector cells in cytotoxicity against influenza virus infected P815 cells on day 5 as described in general materials and methods.

Virus titers determinations.

Lungs were taken from infected mice at different days after infection and virus titers were determined by infectivity assay in MDCK cells followed by hemagglutination of chicken red blood cells as described in general materials and methods.

Statistical analysis.

Statistical analysis was performed using a T test for paired means. Differences were considered significant if $P <= 0.05$.

RESULTS.

BALB/c mice were infected with PR8 virus by aerosol as described in general materials and methods ($7.5x10^3$ $TCID_{50}$/mouse). They were also injected iv with IL4+anti-IL-4 complexes , which is more stable than IL-4 alone (Finkelman et al., 1994) or saline from day 1 to 3 and ip from day 4 to 9. At day 7 after infection 5 mice from each group were sacrificed and their spleens were cultured in T cell medium together with freshly PR8 virus infected spleen cells from naïve mice. Spleen cultures were incubated and then tested for their cytokine profile in the supernatant as well as their cytotoxic activity against PR8 virus infected cells.

Cytokine levels in supernatant of spleen cultures. The levels of IL-4 secretion were found to be higher in spleen cultures from mice that were injected with 2.5 μg of IL-4 + 12.5μg of anti-IL-4 per mouse (anti-IL-4 alone didn't have any effect) as compared to control mice (figure 6-1). In the case of IFNγ, supernatants of spleen cultures from control mice showed higher levels than IL-4 injected mice in RIA (figure 6-2). These two cytokine secretion patterns by themselves showed a major difference in the type of CD4 T cells that are activated after infection in a Th2 cytokine milieu.

Cytolytic capacity of spleen cells from influenza virus infected mice. At day 6 of culture, spleen cells from either control or IL-4+anti-IL-4 treated mice were tested in a 4 hour Cr^{51} release assay against PR8 infected P815 cells. As shown in figure 6-3, only spleen cells from control mice were able to lyse PR8 virus infected P815 cells, while splenocytes from or IL-4+anti-IL-4 treated mice could not do so. From these results we

conclude that the injections of IL-4 drastically impaired the ability of infected mice to generate cytotoxic T cells.

Virus titers in lungs. Virus titrations of lungs from either control or IL-4 treated mice were performed by other members of the laboratory showing a correlation with the cytotoxicity and cytokine data. PR8 virus titers in lungs from IL-4 + anti-IL-4 treated mice were consistently higher over a 10 day observation period than in lungs of control mice, which showed decreasing virus levels from day 6 to day 10 (figure 6-4).

DISCUSSION.

The existence of 2 different subtypes of T helper cells, namely Th1 and Th2, has been well documented (Mosmann et al., 1986, reviewed in Abbas et al., 1996, Zinkernagel, 1996). Proinflammatory cytokines like IFNγ and IL-12 in virus infections drive the immune response towards Th1 immunity, which is probably most effective in fighting virus infections (Alwan et al., 1994, Clerici and Shearer, 1993, Graham et al., 1994, Ozmen et al., 1995, Heufler et al., 1996). IL-4 is the key cytokine for generation of Th2 type immunity, which is probably not as effective against virus infections (Clerici and Shearer, 1993, Graham et al., 1994). Not only are these cytokines responsible for generation of different types of response, but they also inhibit the generation or the effector functions of the other type (Swain et al., 1991, Seder and Paul, 1994). IL-12 released by macrophages and dendritic cells is crucial for the development of Th1 cells which lead to the generation of CTL (Heufler et al., 1996). CTLs are believed to be the main effector cells in Th1 responses (Chisari and Ferrari, 1995). IL-4 is not only responsible for differentiation of cells into the Th2 phenotype but it can also inhibit the generation of Th1 immunity, thus it inhibits the generation of inflammatory responses (reviewed in Abbas et al., 1996).

Recently, several cytokines and anti-cytokine antibodies have been used in different experimental models for the modification of immune responses to pathogens (Haak-Fredescho et al., 1992, Finkelman et al., 1994, Orange et al., 1994, Racke et al.,

1994, Leonard et al., 1995, Mocci and Coffman, 1995). Results from these experiments have proven that it is possible to switch the immune response from Th1 to Th2 and vice versa during the onset of infection. In those experimental models it has been shown to be easier to reverse a Th1 response to Th2 than the opposite.

Our results show that it is possible to reverse a Th1 immune response by the addition of IL-4 early following infection with influenza virus. These results clearly document that Th2 responses are not efficient in responding to influenza virus infection. Additionally, these data demonstrate that the cytokine milieu at the time of exposure to infectious agents or vaccination may influenza the type of immune response which will be generated. These findings may have important implication for the design of novel vaccine protocols as well as the study of the response to infectious agents.

Figure 6-1. IL-4 levels in spleen cultures from mice infected with PR8 virus and either treated with daily IL-4 injections or untreated (control). Each mouse received approximately $7.5x10^3$ $TCID_{50}$ of influenza virus strain A/PR/8/34 via aerosol. Mice were untreated or treated with daily injections of IL-4+anti-IL-4 complexes from days1 to 7. Mice were sacrificed at day 7 and spleen cells were cultured together with PR8 virus infected spleen cells from naïve mice for 6 days. Levels of IL-4 in the supernatants were detected by RIA using the antibodies BVD4-1D11 (1μg/ml) to coat and I^{125}-labeled BVD6-24G2.3 (50,000 cpm/50μl) to detect. Values are shown in pg/ml. Each group had 5 mice, with means +/- standard deviations = control 0.04 +/- 0.02 pg/ml and IL-4 treated 24.22 +/- 11 pg/ml (p= 0.05).

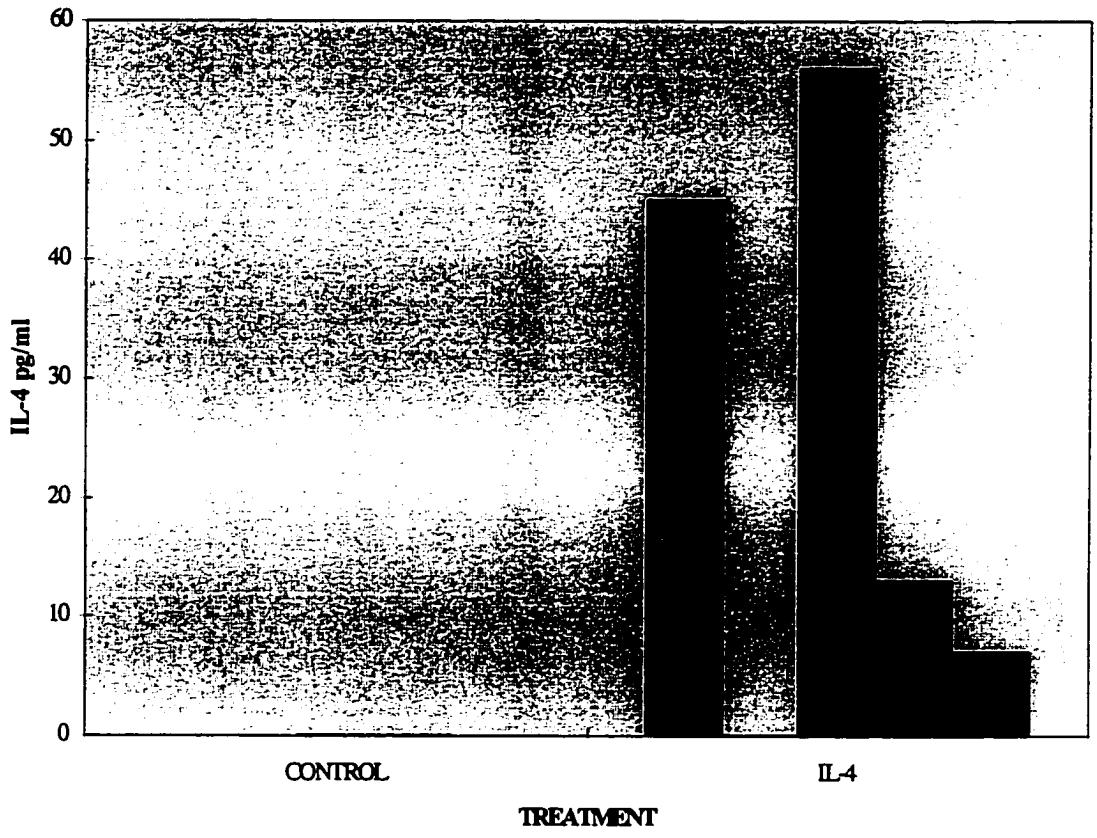


Figure 6-1

Figure 6-2. IFNγ levels in supernatants of spleen cells from mice infected with PR8 virus and untreated or treated with IL-4. Mice infected by aerosol ($7.5x10^3$ $TCID_{50}$ /mouse of PR8 influenza virus) and injected with IL-4 daily from day 1 to 7 were sacrificed at day 7. Spleen cells from these mice were restimulated *in vitro* by splenocytes from naïve mice freshly infected with PR8 virus, and incubated for 6 days. At day 6 supernatants from spleen cultures were tested by RIA with the antibodies XMG1.2 (1μg/ml) to coat and rat-anti mouse IFNγ at 50,000 cpm/50μl (Upstate Biotechnology Inc.)to detect levels of IFNγ. Values are shown in ng/ml. Means and standard deviations of each group of 5 mice are 19.6 +/- 5.8 for the control mice and 3.8 +/- 1.2 ng/ml for IL-4 treated ($p= 0.02$).

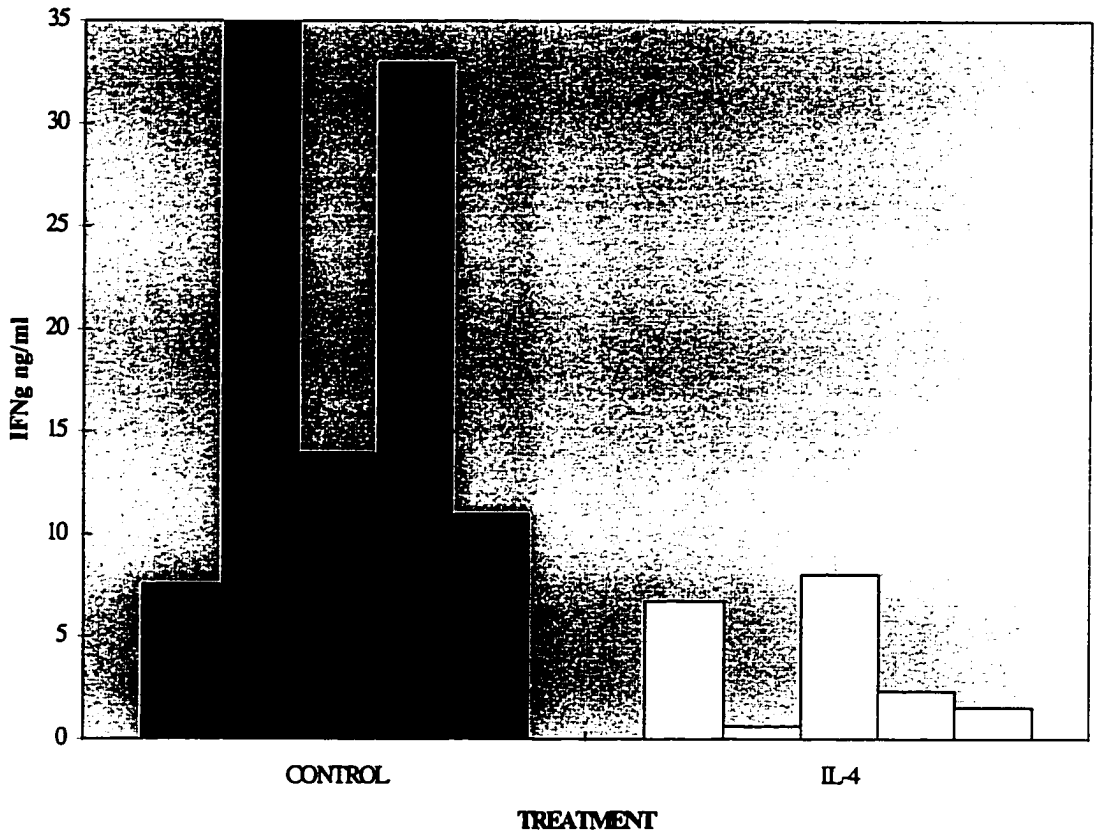


Figure 6-2

Figure 6-3. Lysis of PR8 virus infected cells by spleen cells from mice infected with PR8 virus and either untreated or treated with IL-4. Mice were infected with PR8 influenza virus by aerosol ($7.5x10^3$ $TCID_{50}$ /mouse) and injected on a daily basis with IL-4 from day 1 up to day 7. After sacrifice of the mice at day 7 spleen cells were restimulated *in vitro* with PR8 virus infected splenocytes from naïve mice for 6 days. At day 6 of incubation splenocytes were tested in a 4 hour Cr^{51} release assay against PR8 virus infected P815 cells to test their cytotoxic capacity. Data are shown as % cytotoxicity and means ± standard deviations for each group of 5 mice are 46.2% ± 8% for controls and 14.9% ± 5% for IL-4 treated mice (p= 0.03).

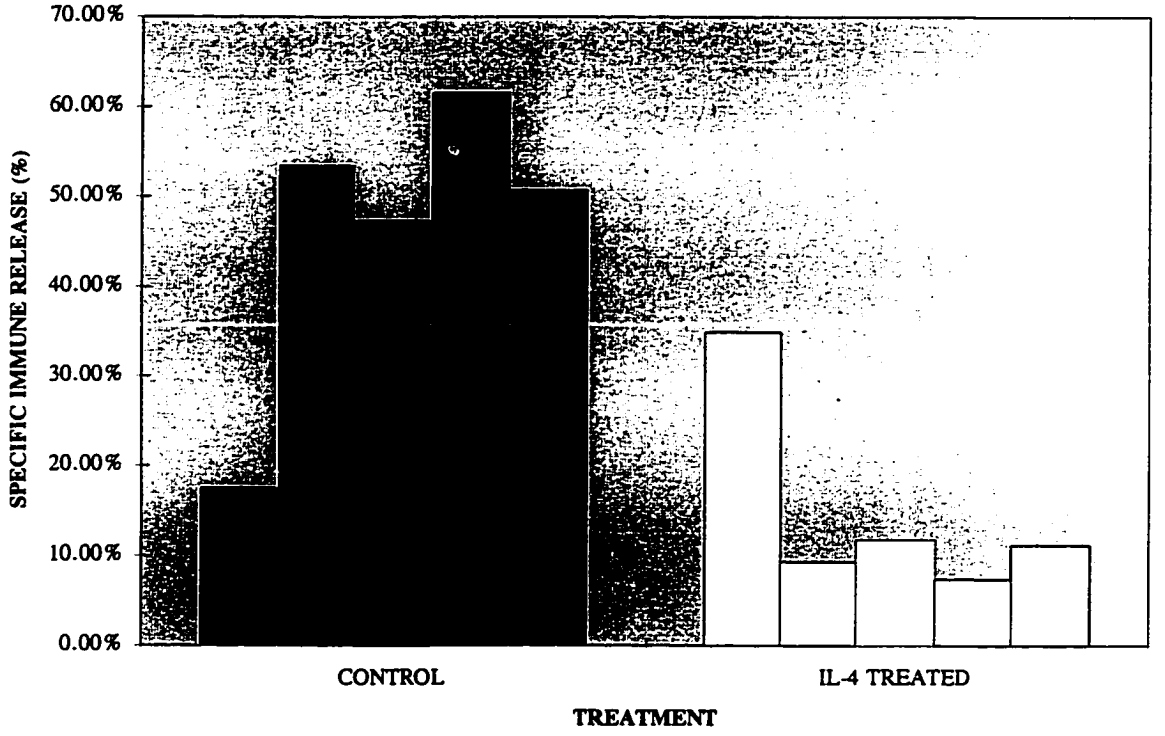
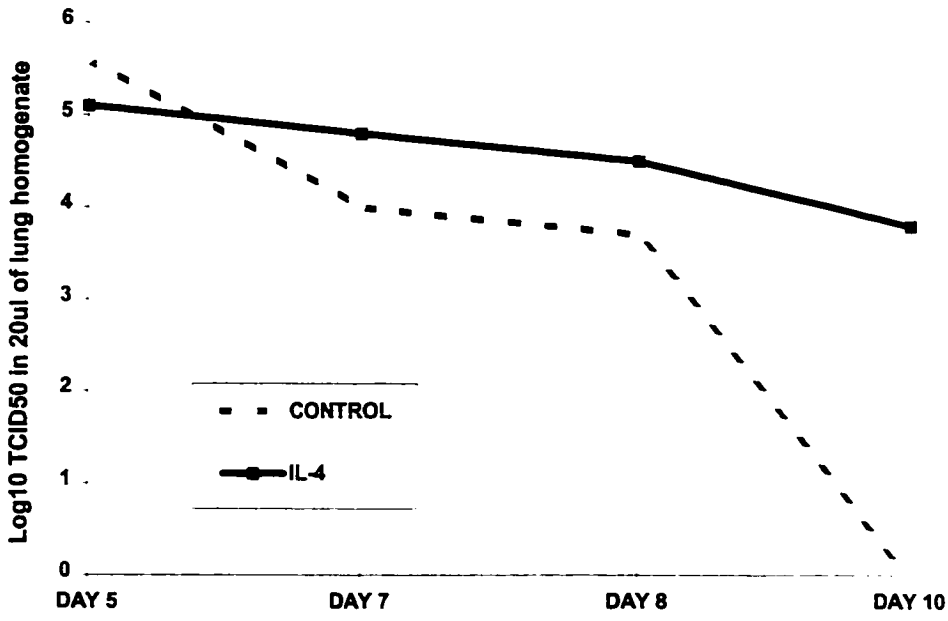


Figure 6-4. Virus titers in lungs of mice infected with PR8 influenza virus. Mice infected with PR8 virus were injected iv with IL-4 + anti-IL-4 from day 0 to day 10 (IL-4), or untreated after infection (control). Virus titers in lung homogenates were determined by infectivity assay on MDCK cells at different days after infection. Samples are means of triplicates with standard errors shown in vertical bars for each time point.



Chapter 7.

VACCINATION OF BALB/C MICE WITH LIVE AND INACTIVATED INFLUENZA VIRUS IN DIFFERENT CYTOKINE ENVIROMENTS LEADS TO DIFFERENT IMMUNE RESPONSES.

(Part of Moran, et al., 1997, manuscript in preparation).

ABSTRACT.

BALB/c mice were immunized with either live or inactivated influenza virus and the nature of the memory immune response was investigated. Vaccination with live virus led to a Th1 response characterized by high levels of anti-influenza virus CTLs, IgG2a antibodies, and secretion of the cytokine, IFNγ. Vaccination with inactivated virus led to the expansion of Th2 cells as shown by the fact that spleen cells from the mice secreted IL-4 but not IFNγ upon restimulation *in vitro*. In addition, mice immunized with inactivated virus were unable to generate CTLs against influenza virus infected cells and the predominant antibody isotype was IgG1.

When immunized animals were infected with a different virus subtype only those given live virus were able to generate heterosubtypic immunity which facilitated the clearance of virus from their lungs. The addition of IL-12 to the immunization protocol with inactivated virus partially switched the immune response towards Th1. These mice demonstrated a small but significant heterosubtypic immune response. In contrast, the addition of IL-4 to the live virus immunization abolished most of the

protective immune response against influenza virus and led to a Th2 response with production of IL-4 and reduced CTL generation.

INTRODUCTION.

Cellular responses to immunogens include the generation of inflammatory cytokines, such as IFNγ and TNF (Th1) as well as other cytokines, like IL-4 and IL-5 (Th2). IFNγ promotes not only the generation of CTLs but also the generation of complement fixing antibodies of the IgG2a in mice (Coutelier et al., 1987, Bossie and Vitteta, 1991, reviewed in Abbas et al., 1996). Th2 responses consist of IgG1 and IgE (mast cell degranulation) antibody generation and expansion and migration of eosinophils. It does not promote generation of CTLs (Finkelman et al., 1990, reviewed in Abbas et al., 1996).

The presentation of immunogens by APCs to T cells in different cytokine contexts has been suggested to be the determining factor for the generation of either Th1 or Th2 responses (Heufler et al., 1996, Ohshima and Delespesse, 1997, Winzler et al., 1997). If cytokine context is high in IL-12 and low in IL-4, then Th1 responses will be generated. On the contrary, if the environment is high in IL-4 and low in IL-12 Th2 responses are made. The origins of IL-4 and IL-12 are the subject of discussion, but recently, evidence has suggested that IL-12 may be released by APCs in response to factors such as inflammatory cytokines, bacterial products and live viruses (Winzler et al., 1997). The origin of IL-4 is not clear.

Our previous results have shown that IL-4 given at the time of infection generated a Th2 response in influenza virus infected BALB/c mice (Moran et al., 1996). These mice did not generate appropriate levels of cytotoxic T cells and were not

able to clear viruses from their lungs. Waris and colleagues (1996) have shown in an RSV mouse model that vaccination with inactivated virus led to the generation of a Th2 memory response that did not protect the mice against reinfection with RSV. When the Th2 memory cells were reactivated upon subsequent infection with RSV, these mice showed more severe disease than mice not vaccinated.

To test whether the induction of Th2 immunity by inactivated virus was restricted to RSV, we decided to vaccinate mice with either inactivated or live influenza viruses and analyze the make up of the immune response generated to each. In this chapter we show results confirming that inactivated virus generated a Th2 immune response. Live viruses used for vaccination in our influenza virus model gave a clear Th1 response, which was protective upon reinfection with a different virus subtype.

We also wanted to test if cytokines used at the time of vaccination would change the type of response and the pattern of protection in mice upon challenge with a different virus subtype. We chose IL-12 to use in combination with inactivated virus vaccine since this cytokine is a potent promoter of Th1 immunity (Ozmen et al., 1995). Based on our results using IL-4 to modify the immune response of mice against influenza virus infection (Moran et al., 1996) we used IL-4 together with live virus to try to change the immune response generated by live virus alone.

In this chapter I present data from experiments in which we used different vaccination protocols using influenza virus X31 in BALB/c mice. When we used live virus for immunization, mice were able to generate a protective Th1 immune response

(confirming results of Waris et al., 1996), that was dramatically switched by the addition of IL-4 in the vaccination protocol to a more Th2 type of response. In contrast, the immune response to vaccinations with inactivated virus could be partially reverted to a Th1 response by the inclusion of IL-12 in the immunizations. Mice showed different levels of protection against challenge with live virus depending on the protocol of vaccination. Vaccination with live virus was the optimal one for protection in this series of experiments.

MATERIALS AND METHODS.

Virus inactivation.

For immunization X31 virus (H3N2) was diluted 1:4 in maintenance medium and half was subjected to 6 minutes of UV irradiation on ice at a distance of 6 inches followed by a 30 minute incubation at 37° C, pH=5.0 after which the pH was adjusted to that of the live virus. Live virus had an infectivity titer in MDCK cells of 10^7 $TCID_{50}$ in 25 μl while inactivated virus demonstrated no infectivity at a 10^{-1} dilution in either MDCK cells or in eggs.

Immunization.

Immunization was performed by injecting 200µl of live ($8x10^7$ $TCID_{50}$ /mouse) or inactivated X-31 virus ($8x10^{-1}$ $TCID_{50}$ /mouse) ip. In some experiments, animals were reimmunized two weeks later in an identical fashion. For the cytokine experiments, animals were immunized with virus plus IL-4-11B11 (anti-IL-4) immune complex as described (Finkelman et al., 1994) (2.5µg IL-4-12.5µg 11B11/mouse) or IL-12 (10ng/mouse) in 0.1% BALB/c serum.

Infection procedure and titrations.

Two weeks after the last immunization mice were infected with PR8 virus by aerosol ($7.5x10^3$ $TCID_{50}$/mouse) as described in general materials and methods (Schulman, 1967) and then sacrificed at days 4 and 7 after infection. Lungs were taken and virus titers were determined as described in chapter 1. Spleen cells were

restimulated *in vitro*, cytokines in supernatants were measured and at day 5 of culture spleen cells were tested in cytotoxicity assay.

Statistical analysis.

Degrees of significance between groups of animal were determined by using the T test: paired two sample for means.

RESULTS.

The experiments shown in this chapter were performed by all members of the group in a cooperative way due to the volume of samples involved. None of the experiments were performed by Ana Fernandez-Sesma alone but she participated in all of them.

Virus titers in lungs from mice immunized by different protocols and challenged with live virus. Table 7-1 shows influenza virus titers in lungs from mice immunized with either: live virus, inactivated virus, live virus+IL-4 or inactivated virus+IL-12. All mice subsequently were infected by aerosol with live virus and lungs were taken at days 4 and 7 after infection. Infectivity titers were calculated by infecting either MDCK cell monolayers or 9 day embryonated eggs. Mice immunized with live virus+IL-4 and mice immunized with inactivated virus+IL-12 show similar levels of protection against challenge. In both cases the level of protection was higher than the one for mice immunized with inactivated virus alone. All groups of immunized mice with the exception of inactivated virus immunization seemed to generate heterotypic immunity.

Cytotoxic characteristics of spleen cells from mice immunized twice with influenza virus under different protocols. Mice were immunized twice in a two week interval with inactivated virus, live virus, inactivated virus+IL-12 or live virus+IL-4. Two weeks after the last immunization spleens were taken and restimulated *in vitro* with syngeneic spleen cells infected with PR8 virus. Five days later spleen cells were

tested in a 4 hour ^{51}Cr release assay against PR8 virus infected P815 cells. As shown in figure 7-1, mice immunized with live virus showed the highest levels of lysis of infected cells, this activity was drastically reduced by the addition of IL-4 to the immunization protocol. Mice immunized with inactivated virus under different protocols didn't show significant levels of cytotoxicity. Only mice immunized with live virus under different protocols were able to generate cytotoxic T cells against influenza virus infected targets.

Isotypes of anti-influenza virus antibodies in serum from mice previously vaccinated under different protocols. Figure 7-2 shows the isotypes of antibodies against influenza virus in serum taken from mice two weeks after the second immunization with live influenza virus, inactivated influenza virus, live virus+IL-4 or inactivated virus+IL-12.

Levels of IgG2a were comparable in serum from mice immunized with live virus and with those of mice given inactivated virus+IL-12. The inclusion of IL-4 with live virus in the immunization protocol resulted in high levels of IgG1 antibodies against influenza virus in serum from those mice. Mice immunized with inactivated virus alone showed high levels of IgG1 antibodies in serum as well. The addition of IL-4 to live virus immunization switched the antibody isotypes towards more Th2 type.

DISCUSSION.

It is now generally accepted that immune responses can proceed by either of two possible pathways, namely Th1 or Th2. Both helper immune responses are capable of negatively regulating each other (reviewed in Abbas et al., 1996, Mosmann and Sad, 1996). But that regulation has to occur early in the response, because once one type of response is initiated the other one is inhibited (Swain et al., 1991, reviewed in Abbas et al., 1996). The early events during the onset of an infection are crucial for the generation of an appropriate immune response. Thus, for vaccination protocols it is important to create the right environment to obtain the desired immune response.

One very interesting aspect of our results is the fact that live virus immunization generated a different immune response from that elicited by inactivated virus. This means that the right environment is created by the way the immunogen first interacts with the immune system. Live pathogens have been suggested to induce the release of IL-12 by APCs (Winzler et al., 1997) which initiates a Th1 immune response against them (Heufler et al., 1996). Inactivated viruses may not be able to induce the release of IL-12 by the APC and thus, when used in vaccinations, they generate a more Th2 type immune response.

Our results using different cytokines to change the immune response after immunization with either live virus or inactivated virus clearly show the efficacy of this approach. Looking at cytokine profiles and cytotoxic activity of spleen cells, the immunization with live virus generated a clear Th1 type immune response. The

addition of IL-4 in the immunization protocol changed the antiviral immune response in these mice to a Th2 type, since these mice showed less protection upon reinfection with a different subtype of influenza virus and less cytotoxic T cells generated.. The Th2 immune response generated after inactivated virus immunization could be partially switched to a Th1 response by the addition of IL-12 at the time of the immunization. In terms of heterotypic immunity, mice immunized with live virus+IL-4 and mice immunized with inactivated virus+IL-12 showed some levels of protection against challenge, which is a parameter for Th1 immunity. Some CTL-independent mechanism must be responsible for reducing virus titers in lungs from mice immunized with inactivated virus+IL-12, since they could not generate influenza virus specific CTLs. IL-12 could be having a direct effect against viruses that slows down their replication in the lungs of these mice.

The different types of immune responses are determined by the nature of the immunogen as well as the cytokine environment. These results can be very useful in designing vaccination protocols for different pathogens. For some viruses, such as HIV it is complicated to develop vaccines using live virus, due to the high rate of mutation, which will make this approach risky for human use (Coffin, 1995). One way to generate a Th1 immune response would be to immunize with IL-12 and inactivated virus, thus avoiding the risk of real infection with the virus. At the same time, the use of IL-12 overcomes the need for the right signal from the APC, since it can initiate a Th1 response (Ohshima and Delespesse, 1997)

Another application of our results concerns the use of IL-4 to "trick" the immune system about the existence of a replicating virus, like in gene therapy using adenovirus vectors to deliver the gene. In this case, the fact that a non-replicating virus delivers the gene alerts the immune system to generate a Th1 immune response and particularly CTLs that can clear the virus and block the expression of the gene as well (Yang et al., 1995). Results in this report as well as our previous demonstration suggest that IL-4 used in combination with an adenovirus vector gene therapy could start a Th2 immune response that does not eliminate the virus and the transgene. Theoretically, a Th2 response of this type could permanently prevent rejection of transgene by continuing to produce IL-4 in response to the gene product.

The results from this chapter give us a broad spectrum of possibilities in terms of vaccination experiment as well as deviation of the immune response experiments which we plan on performing in the near future.

Table 7-1. Influenza virus titers in lungs from mice immunized by indicated protocols following aerosol infection with PR8 influenza virus. Animals were immunized twice in a two week interval with mock, live influenza virus X31(H3N2), UV-inactivated X31 virus, live X31 virus+IL-4 or inactivated X31 virus+IL-12. Two weeks after the last immunization mice were challenged by aerosol infection with PR8 influenza virus (7500 $TCID_{50}$/mouse). Animals were sacrificed at days 4 and 7 after infection and virus titers in lungs were calculated by infectivity of dilutions of lung homogenates in MDCK cells or 9 day embryonated eggs followed by hemagglutination of chicken red blood cells. Virus titers shown in Log_{10} $TCID_{50}$ in 25μl of lung homogenate. Titers of live virus, live virus+IL-4 and inactivated virus+IL-12 immunized mice were significantly different from titers of naïve mice ($p<0.05$).

Days after infection	naive	Live virus	Inact. virus	Live virus +IL-4	Inact. Virus +IL-12
4	6.4	4.5	6.0	5.4	5.5
7	6.0	3.0	5.8	4.8	5.0

Figure 7-1. Only live virus immunization leads to the generation of cytotoxic T cells. Five days after restimulation *in vitro* with PR8 virus infected syngeneic cells, spleen cells from immunized mice were tested in 4 hour ^{51}Cr release assay for lysis of PR8 virus infected P815 cells at the indicated effector to target ratios. Results are indicated in % lysis in which cytotoxicity versus uninfected P815 has been subtracted. For each group the values represent means of five individual animals.

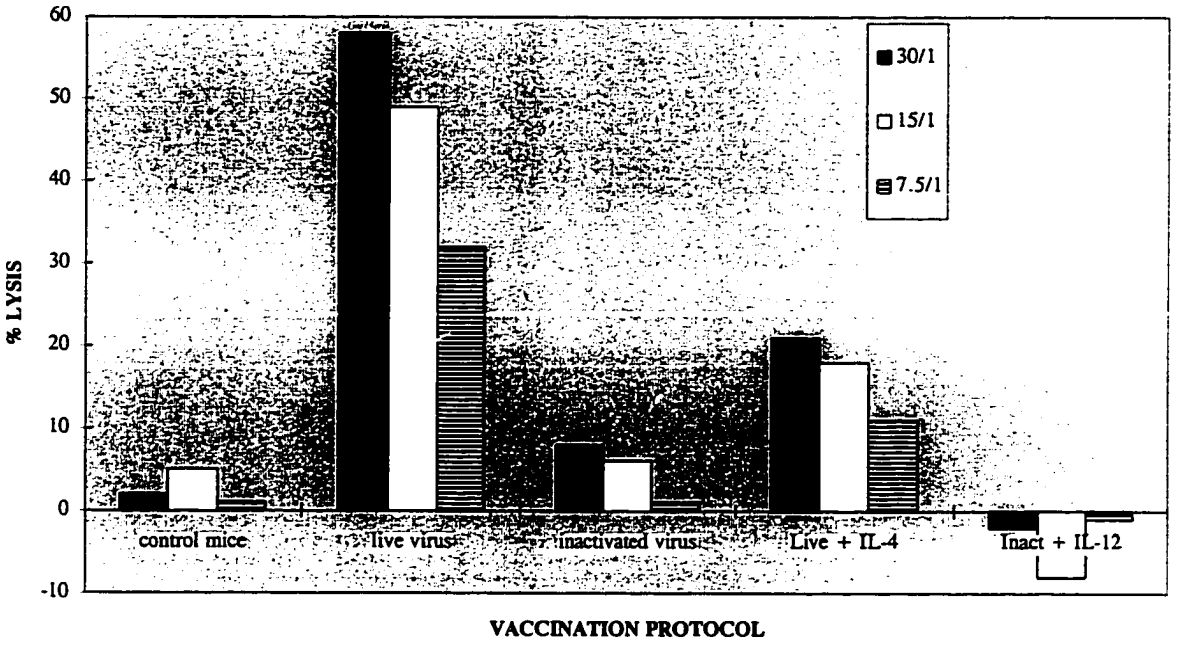


Figure 7-2. Ratio of influenza virus specific IgG1 to IgG2a antibodies differs following various immunization protocols. Animals immunized by the indicated protocols were bled two weeks after the second immunization and the relative ratio of IgG1 to IgG2a in serum was determined by ELISA. Plates were coated with 25ug/ml of sucrose purified virus and serum from individual mice added at 10^{-2}, 10^{-3}, and 10^{-4} dilutions. Bound antibodies were identified by the addition of either biotin-labeled goat anti-mouse IgG, IgG1 or IgG2a, followed by streptavidin-HRP and substrate. Plates were read in ELISA reader at 405nm. The results were interpolated using standard curves generated with anti-influenza virus HA monoclonal antibodies of the correct isotype. Values (ng/ml) for each group are (IgG1, IgG2a)= Live virus (227 ± 51, 119 ± 30), Inactivated virus (176 ± 63, 49 ± 18), Live+IL-4 (249 ± 64, 46 ± 27) and Inactivated+IL12 (143 ± 45, 65 ± 14).

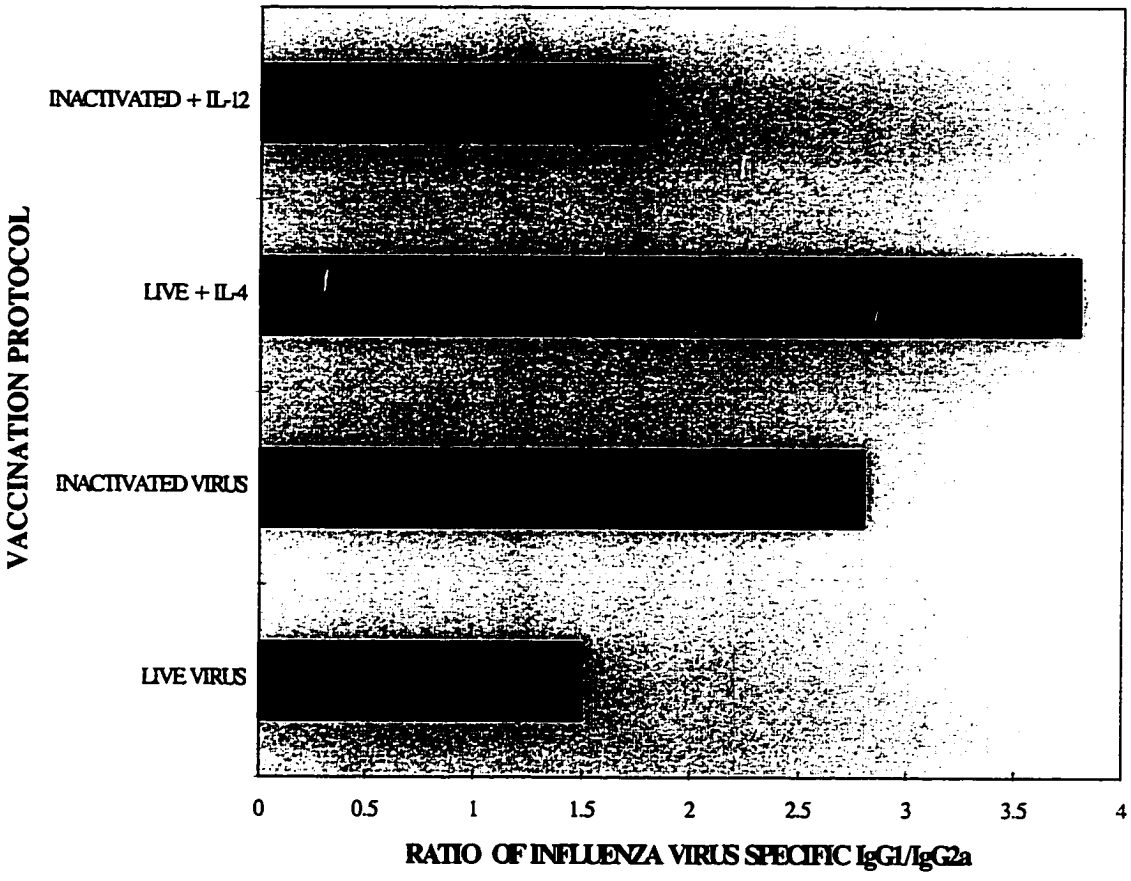


Figure 7-2

Chapter 8.

GENERAL DISCUSSION.

Summary

The results shown in this thesis were generated using different tools to study the immune system, such as bispecific antibodies and cytokines in the context of either vaccination or virus infections. The goal of my work was to study and analyze different aspects of the immune responses generated against viruses as well as to develop and use appropriate strategies to improve and better understand these antiviral responses.

The use of BsAbs was very useful for me in order to study the mechanisms by which virus replication is inhibited by non virus specific T cells redirected by BsAb (chapters 3 and 4). This thesis shows that this inhibition of virus replication was mainly due to by perforin released by $CD8^+$ T cells which did not require IFNγ. I should emphasize however, that this may not be true for all viruses, as previous results from our laboratory using a similar system, demonstrated that both $CD4^+$ and $CD8^+$ T cells could inhibit influenza virus replication. The most important finding in these studies with BsAbs was our ability to demonstrate applicability *in vivo*, since we could delay the lethal effects of VSV in Stat1 KO mice. The concept of using BsAbs for immunotherapy is very attractive. If one envisions their use as a treatment for persistent virus infections or against virus infections which are resistant to host immune responses, they could be valuable immunotherapeutic tools. The fact hat the BsAbs need not to be directed to a neutralizing epitope makes it possible to design a single antibody which can bind to most

virus subtypes. The use of superantigen stimulated T cells makes it conceivable that patients own cells could be expanded *ex vivo* and returned along with the BsAb.

It was also very interesting to study the immune response of Stat1 KO mice against influenza virus, since it showed how important innate immune responses are in fighting virus infections (chapter 5). These mice showed cytokine disregulation. Possibly due to the incapability of IFNγ to negatively regulate the initiation of Th2 immune responses. The fact that Stat1 KO mice were able to generate CTLs but failed o clear virus infection may be related to defects in the ability of these CTLs to migrate to the site of infection due to lack of expression of the proper adhesion molecules.

The use of cytokines such as IL-4 at the time of virus infection showed us how potent the effect of cytokines can be at the time of infection, since we could revert the antiviral immune response from an efficient Th1 type to an inefficient Th2 type (chapter 6). These results proved to us the importance of timing when trying to modify immune responses and they opened the door to other experiments using cytokines in vaccination. They provided verification of the role of the cytokine milieu in dictating the nature of the immune response which is generated. They forced us to speculate on the mechanism by which the early cytokine environment is created . This led us to a new type of investigation such as the vaccination studies, studies of APCs and the substances which may be involved in the generation of this cytokine milieu.

Results from cytokines administered at the time of vaccinations show how crucial it is to generate the right immune response, Th1, against viruses to get protection

(chapter 7). Our results prove that inactivated viruses don't provide the right protection, since they didn't promote the generation of CTLs or heterosubtypic immunity, but they can be improved as vaccines by using IL-12 in the vaccination protocols (such as in the case of designing of HIV vaccines).

In summary, the results emphasize the importance of the primary events after virus infection. In particular, the way antigens are presented to the immune system. The conclusions from my work could be summarized as follows:

1) It is possible to use tools such as BsAbs that recognize proteins on the TCR of T cells and viral proteins to fight virus infections, since they combine the specificity of the antibody response and the effectiveness of the cellular immune response. BsAbs can fill in for the time lapse needed for antigen presentation by APCs and may also be useful in situations where the immune response is of the wrong type or is not generated at all.

2) Mice with defects in the signal transduction responses to interferons cannot generate an appropriate immune response to virus, thus, not only antibodies and CTLs are crucial in a successful response against viruses, but innate immunity is also an important part against influenza virus and VSV.

3) It is also possible to modify antiviral immune responses from Th1 to Th2 by the addition of cytokines like IL-4 at the time of infection. This modification can occur if the cytokines are added at the time of the infection. This may be a useful technique to inhibit inappropriate immune responses.

4) Live and inactivated virus used for vaccines give different immune responses. The Th1 immune response generated after live virus vaccination, which is protective upon challenge, is reversed to Th2 by the addition of IL-4 in the vaccine. The addition of IL-12 to the vaccination protocol with inactivated virus improved the non protective Th2 immune response to a more Th1, which was protective upon challenge. This opens up the possibility of using inactivated virus vaccines to generate cellular immunity in situations where live or attenuated virus is not feasible.

Future perspectives.

Regarding the use of BsAbs, we plan on trying different *in vivo* models to test their efficacy as immunotherapeutic tools. Such models could be IFNγ KO mice or other models with deficiencies in the generation of Th1 immune responses.

The use of IL-4 at the time of virus infection is been currently performed in our laboratory in the context of adenovirus vector-delivery of genes into livers of mice. After obtaining prolongation of transgene expression, our laboratory is trying to improve the protocols using various approaches. We are trying to develop the optimal protocol to deliver IL-4 together with the transgene.

The use of IL-12 at the time of vaccination can be very useful for development of vaccines against viruses such as HIV. We could use this approach in order to generate the appropriate immune response against this virus after vaccination with inactivated virus. An interesting experiment might be to combine vaccination with inactivated virus and IL-12 with BsAb- targeted CTLs. Since the addition of IL-12 activates Th1 cells but is still

unable to provoke the generation of CTLs, this combination might fully restore cellular immunity.

REFERENCES.

Abbas, A. K., Murphy, K. M., and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature,* 383:787-793.

Alber, G., Hammer, D. K. and B. Fleischer. 1990. Relationship between enterotoxic and lymphocyte stimulating activity of staphylococcal enterotoxin B. *J. Immunol.*, 144(12):4501-4506.

Allan, W., Tabi, Z., Cleary, A. and P. C. Doherty. 1990. Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting $CD4^+$ T cells. *J. Immunol.*, 144:3980-3986.

Alwan, W. H., Kazlowska, W. J. and P. J. M. Openshaw. 1994. Distinct types of lung disease caused by functional subsets of antiviral T cells. *J. Exp. Med.*, 179:81-89.

Andrew, M. E., Coupar, B. E. H., Boyle, D. B. and G. L. Ada. 1987. The roles of influenza virus haemagglutinin and nucleoprotein in protection: Analysis using vaccinia virus recombinants. *Scand. J. Immunol.*, 25:21-28.

Atkinson, E. A., and R. C. Bleackley. 1997. Mechanisms of lysis by cytotoxicity. *Crit. Rev. Immunol.*, 15:359-384.

Austrup, F. Vestweber, D., Borges, E., Lohning, M., Brauer, R., Herz, U., Renz, H., Hallmann, R., Scheffold, A., Radbruch, A. and A. Hamann. 1997. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflammed tissues. *Nature,* 385:81-83.

Baeuerle P. A. and T. Henkel. 1994. Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol.*, 12:141-179.

Bender, B. S., Croghan, T., Zhang, L . and P. A. Small, Jr. 1992. Transgenic mice lacking class I major histocompatibility complex - restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. *J. Exp. Med.*, 173:1143-1145.

Berg, J., Lotscher, E., Steimer, K. S., Capon, D. J., Baenziger, J., Jack, H-M. and M. Wabl. 1991. Bispecific antibodies that mediate killing of cells infected with human immunodeficiency virus of any strain. *Proc. Natl. Acad. Sci. USA*, 88:4723-4727.

Berke, G.. 1995. The CTL's kiss of death. *Cell,* 81(1):9-12.

Bi, Z., Quandt, P., Komatsu, T., Barna, M., and C. S. Reiss. 1995. IL-12 promotes enhanced recovery from vesicular stomatitis virus infection of the central nervous system. *J. Immunol.*, 155(12):5684-5689.

Bluestone, J. A.. 1995. New perspectives of CD28-B7-mediated T cell costimulation. *Immunity,* 2:555-559.

Bossie, A. and E. S. Vitteta. 1991. Interferon gamma enhances secretion of IgG2a committed LPS-stimulated murine B cells: implication for the role of interferon gamma in class switching. *Cell. Immunol.*, 135:95-104.

Brutkiewicz, R. R. and R. M. Welsh. 1995. Major histocompatibility complex class I antigens and the control of viral infections by natural killer cells. *J. Virol..*, 69(7):3967-3971.

Cambrige, G., Mackensie, J. S. and D. Keast. 1976. Cell mediated immune response to influenza virus infections in mice. *Infect. Immunol.*,13:36-43.

Chamow, S. M., Zhang, D. Z., Tan, X. Y., Mhatre, S. M., Marsters, S. A., Peers, D. H., Byrn, R. A., Ashkenazi, A., and R. P. Junghans. 1994. A humanized bispecific immunoadhesin-antibody that retargets CD3+ effectors to kill HIV-1-infected cells. *J. Immunol.* 153(9):4268-4280.

Chisari, F. V. and C. Ferrari. 1995. Hepatitis B virus immunopathogenesis. *Ann. Rev. Immunol.*, 13:29-60.

Clerici, M. and G. M. Shearer. 1993. A Th1/Th2 switch is a critical step in the etiology of HIV infection. *Immunol. Today,* 14:107-111.

Coffin J. M.. 1995. HIV population dynamics *in vivo*: implications for genetic variation, pathogenesis, and therapy. *Science,* 267:483-489.

Coutelier, J. P., Van Der Logt, T. M., Heesen, F. W., Warnier, G. and J. V. Snick. 1987. IgG2a restriction of murine antibodies elicited by viral infections. *J. Exp. Med.*, 165:64-69.

De Lau, W. B., Heije, K., Neefjes, J. J., Oosterwegel, M., Rozemuller, E. and B. J. Bast. 1991. Absence of preferential homologous H/L chain association in hybrid hybridomas. *J. Immunol.*, 146(3):906-914.

Demanet, C., Brissnick, J., Van Mechelen, M., Leo, O., and K. Thielemans. 1991. Treatment of murine Bcell lymphoma with bispecific monoclonal antibodies (anti-idiotype x anti-CD3). *J. Immunol.* 147(3):1091-1097.

Demanet, C., Brissnick, J., Leo, O., Moser, M., and K. Thielemans. 1994. Role of T- cell subsets in the bispecific antibody (anti-idiotype x anti-CD3) treatment of the BCL1 lymphoma. *Cancer Res.* 54(11):2973-2978.

Deo, Y. M., Graziano, R. F., Repp, R, and J. G. Van de Winkel. 1997. Clinical significance of IgG Fc receptors and Fc gamma R-directed immunotherapies. *Immunol. Today.* 18(3):127-135.

Doherty, P.C., and R.M. Zinkernagel. 1974. T cell mediated immunopathology in viral infections. *Transpl. Rev.*, 19:89-120.

Doherty, P.C., Allan, W. and M. Eichelberger. 1992. Roles of αβ and γδ T cell subsets in viral immunity. *Annu. Rev. Immunol.* 10:123-151.

Durbin, J. E., Hackenmiller, R., Simon, M. C., and D. E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell.* 84(3):443.

Durbin, J. E., Fernandez-Sesma, A., Garcia-Sastre, A., Rao, T. D., Frey, A. B., Moran, T. M. and D. E. Levy. 1997. Essential role of Stat1 in repression of Th2-mediated response to influenza virus infection.. (Submitted for publication).

Eichelberger, M., Allan, W., Zijlstra, M., Jaenisch, R. and P.C. Doherty. 1991. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-resricted $CD8^+$ T cells. *J. Exp. Med.* 174:875-880.

Fanger, M. W., Morganelli, P. M. and P. M. Guyre. 1992. Bispecific antibodies. *Crit. Rev. Immunol.*, 12(3-4):101-124.

Fanger, M. W., Segal, D. M. and J.L. Romet-Lemonne. 1991. Bispecific antibodies and targeted cellular cytotoxicity. *Immunology Today.*, 12(2):51-54.

Fernandez-Sesma, A., Schulman, J. L., and T. M. Moran. 1996. A bispecific antibody recognizing influenza A virus M2 protein redirects effector cells to inhibit virus replication *in vitro*. *J. Virol.* 70(7): 4800-4804.

Fernandez-Sesma, A., Peluso, R. W., Bai, X., Schulman, J. L., Levy, D. E. and T. M. Moran. 1997. Superantigen activated T cells redirected by a bispecific antibody inhibit VSV replication *in vitro* and *in vivo*. In press (*J. Immunol.*).

Fleischer, B., and H. Schrezenmeier. 1988. T cell stimulation by staphylococcal enterotoxins. Clonally variable response and requirement for major histocompatibility complex class II molecules on accessory or target cells. *J. Exp. Med.*, 167:1697-1707.

Finkelman, F. D., Holmes, J., Katon, J., Urban, J., Beckman, Park, L., Schooley, K., Coffman, R., Mosmann, T. and W. Paul. 1990. Lymphokine control of *in vivo* immunoglobulin isotype selection. *Annu. Rev. Immunol.*, 8:303-334.

Finkelman, F. D., Madden, K., Cheever, A., Katona, I., Morris, S., Gately, M., Hubbard, B., Gause, W. and J. Urban, Jr. 1994. Effect of interleukin 12 on immune responses and host protection in mice infected with intestinal nematode parasites *J. Exp. Med.* 179:1563-1572.

Gilbert, M. J., Riddell, S. R., Plachter, B., and P. D. Greenberg. 1996. Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. *Nature.* 383:720-722.

Graham, M. B. , Braciale, V.L. and T.J. Braciale. 1994. Influenza virus-specific $CD4^+$ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection. *J. Exp. Med.* 180:1273-1282.

Graham M. B., Dalton D. Y., Giltinan D., Braciale V. L., Stewart T. A. and T. J. Braciale. 1993. Response to influenza infection in mice with a targeted disruption in the interferon gamma gene. *J. Exp. Med*, 178:1725–1732.

Gruber, M., Schodin, B . A., Wilson, E. R. and D. M. Kranz. 1994. Efficient tumor cell lysis mediated by a bispecific single-chain antibody expressed in Escherichia coli. *J. Immunol.* 152:5368-5374.

Guidotti, L. G., Ishikawa, T., Hobbs, M. V., Matzke, B., Schreiber, R., and F. V. Chisari. (1996). Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity.* 4:25-36.

Haak-Fredrescho, M., Brown, J.F., Itzawa, Y., Wagner, R.D. and C.J. Czuprynski. 1992. Administration of anti-IL-4 monoclonal antibody 11B11 increases the resistance of mice to *Listeria monocytogenes* infection. *J. Immunol.*, 148:3978-3985.

Herman, A., Kappler, J. W., Marrack, P. and A. M. Pullen. 1991. Superantigens: mechanism of T cell stimulation and role in immune responses. *Annu. Rev. Immunol.*, 9:745-772.

Heufler, C., Koch, F., Stanzl, U., Topar, F., Wysocka, M., Trinchieri, G., Enk, A., Steinman, R. M., Romani, N. and G. Schuler. 1996. Interleukin 12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-γ production by T helper 1 cells. *Eur.. J. Immunol.*, 26:659-668.

Holliger, P., Wing, M., Pound, J. D., Bohlen, H., and G. Winter. 1997. Retargeting serum immunoglobulin with bispecific diabodies. *Nature Biotechnology,* 15:632-636.

Holsinger, L. J., Nichani, D., Pinto, L. H. and R. A. Lamb. 1994. Influenza A virus M_2 ion chanel protein: a structure-function analysis. *J. Virol.*, 68(3):1551-1563.

Horvath, C. M. and J. E. Darnell Jr. 1996. The antiviral state induced by alpha interferon and gamma interferon requires transcriptionally active Stat1 protein. *J. Virol.*, 70(1):647-650.

Hou, S., Doherty, P. C., Zijlstra, M., Jaenisch, R. and J. M. Katz. 1992. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. *J. Immunol.* 149:1319-1325.

Howell, A. L., Guyre, P. M., You, K-S and M. W. Fanger. 1994. Targeting HIV-1 to FcγR on human phagocytes via bispecific antibodies reduces infectivity of HIV-1 to T cells. *J. Leukoc. Biol.*, 55:385-391.

Ihle, J. N. 1996. STATs: signal transducers and activators of transcription. *Cell,* 84:331-334.

Kägi, D., Seiler, P., Pavlovic, J., Ledermann, B., Bürki, K., Zinkernagel, R.M., And H. Hengartner. 1995. The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. *Eur. J. Immunol.*, 25:3256-3262.

Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256:495.

Kojima, H., Shinoara, N., Hanaoka, S., Someya-Shirota, Y., Takagaki, Y., Ohno, H., Saito, T., Katayama, T., Yagita, H., Okumura, K., et al. 1994. Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. *Immunity*, 1:357-364.

Kontermann, R. E., Wing, M. G., and G. Winter. 1997. Complement recruitment using bispecific antibodies. *Nature Biotechnology,* 15:629-631.

Koup, R. A.. 1994. Virus escape from CTL recognition. *J. Exp. Med.*, 180:779.

Kurucz, I., Titus, J. A. Jost, C. R. Jacobus, C. M. and D. M. Segal. 1995. Retargeting of CTL by an efficiently refolded bispecific single-chain Fv dimer produced in bacteria. *J. Immunol.*, 154:4576-4582.

Lamb, R. A., Zebedee, S. L. and C. D. Richardson. 1985. Influenza virus M2 protein is an integral membrane protein expressed on the infected cell surface. *Cell*, 40:627-633.

Lanzavecchia, A., and D. Scheidegger. 1987. The use of hybrid hybridomas to target human cytotoxic T lymphocytes. *Eur. J. Immunol.*, 17:105-111.

Leonard, J. P., Waldburger, K. E. and S. L. Goldman. 1995. Prevention of experimental autoimmune encephalitis by antibodies against interleukin 12. *J. Exp. Med.*, 181:381-386.

Lefrancois, L. 1984. Protection against lethal viral infection by neutralizing and nonneutralizing monoclonal antibodies: distinct mechanisms of action *in vivo*. *J Virol.*, 51(1):208-214.

Liang, S., Mozdzanowska, K. Palladino, G. and W. Gerhard. 1993. Heterosubtypic Immunity to influenza type A virus in mice. *J. Immunol.*, 152:1653-1661.

Long, E. O. and S. Jacobson. 1989. Pathways of viral antigen processing and presentation to CTL: defined by the mode of virus entry? *Immunol. Today*, 10(2):45-48.

Lukacher, A. E., Braciale V.L. and T.J. Braciale. 1984. *In vivo* effector functions of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med.*, 160:814-826.

Mabondzo, A., Boussin, F., Raoul, H., Le Naour, R., Gras, G., Vaslin, B., Bartholeyns, J., Romet-Lemonne, J. L., and D. Dormont. 1994. Antibody-dependent cellular cytotoxicity and neutralization of human immunodeficiency virus type 1 by high affinity cross-linking of gp41 to human macrophage Fc IgG receptor using bispecific antibody. *J. Gen. Virol.*, 75(Pt 6):1451-1456.

Mabondzo, A., Le Naour, R., Le Grand, R., Vaslin, B., Benveniste, O., Cheret, A., Raoul, H., Romet-Lemonne, J. L., and D. Dormont. 1995. Functional consequences of macrophage infection by human immunodeficiency virus: bispecific antibody- targeting of HIV-1-infected cells to Fc gamma RI expressing effector cells. *J. Hematother.*, 4(6):579-585.

Meraz, M. A., White, J. M., Sheehan, K. C., Bach, E. A., Rodig, S. J., Dighe, A. S., Riley, J. K., Greenlund, A. C., Campbell, D., Carver-Moore, K., DuBois, R. N., Clark, R., Aguet, M., and R. D. Schreiber. 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in JAK-STAT signalling pathway. *Cell,* 84:431-442.

Milstein, C., and A. C. Cuello. 1983. Hybrid hybridomas and their use in immunochemistry. *Nature*, 305:537-541.

Milstein, C., and A. C. Cuello. 1984. Hybrid hybridomas and the production of bi-specific monoclonal antibodies. *Immunology Today*, 5(10):299.

Mocci, S., and R. L. Coffman. 1995. Induction of a Th2 population from a polarized Leishmania-specific Th1 population by *in vitro* culture with IL-4. *J. Immunol.*, 154:3779-3787.

Moran, T. M., O. Usuba, E. Shapiro, L. J. Rubinstein, M. Ito, and C. A. Bona. 1990. A novel technique for the production of hybrid antibodies. *J. Immunol. Methods,* 129:199-205.

Moran, T. M., O. Usuba, H. Kuzu, Y. Kuzu, J. Schulman, and C. A. Bona. 1991. Inhibition if multicycle influenza virus replication by hybrid antibody-directed cytotoxic T lymphocyte lysis. *J. Immunol.*, 146:321-326.

Moran, T. M., Toy, E. Kuzu, Y. Kuzu, H. Isobe, H. and J.L. Schulman. 1995. Staphylococcal enterotoxin B-activated T cells can be redirected to inhibit multicyle virus replication. *J. Immunol.,* 155:759-765.

Moran, T. M., Isobe, H., Fernandez-Sesma, A. and J. L.Schulman. '1996. Interleukin-4 causes delayed virus clearance in influenza virus-infected mice. *J. Virol.*, 70)8):5230-5235.

Moran, T. M., Park, H., Fernandez-Sesma, A. and J. L.Schulman. 1997. Inactivated influenza virus elicits a Th2 immune response. (Manuscript in preparation).

Moreno, M. B., Titus, J. A., Cole, M. S., Tso, J. Y., Le, N., Paik, C. H., Bakacs, T., Zacharchuk, C. M., Segal, D. M., and J. R. Wunderlich. 1995. Bispecific antibodies retarget murine T cell cytotoxicity against syngeneic breast cancer *in vitro* and *in vivo*. *Cancer Immunol. Immunother.*, 40(3):182-190.

Morrison L. A., Lukacher A. E., Braciale V. L., Fan D. P. and T. J. Braciale. 1986. Differences in antigen presentation to MHC class I-and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J. Exp. Med.,* 163(4):903-921.

Mosmann, T. R., Cherwinski, H. Bond, M. W. Gredlin, M. A. and R. L. Coffman. 1986. Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.,* 136:2348-2357.

Mosmann, T. and S. Sad. 1996. The expanding universe of T cell subsets: Th1, Th2 and more. *Immunology Today,* 17:138-146.

Müller, M., Laxton, C., Briscoe, J., Schindler, C., Improta, T., Darnell, J. E. Jr, Stark, G. R., and Kerr, I. M. 1993. Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-alpha and -gamma signal transduction pathways. *EMBO. J.,* 12(11):4221-4228.

Murphy, T. L., Cleveland, M. G., Kulesza, P., Magram, J. and K. Murphy. 1995. Regulation of interleukin 12 p40 expression through an NF-kB half-site. *Mol. Cell. Biol.,* 15(10):5258-5267.

Muster, T., Subbarao, E. K., Enami, M., Murphy, B. R. and P. Palese. 1991. An influenza A virus containing influenza B virus 5' and 3' noncoding regions on the neuraminidase gene is attenuated in mice. *Proc. Natl. Acad. Sci. USA,* 88:5177-5181.

Nagata, S. and T. Suda. 1995. Fas and Fas ligand: lpr and gld mutations. *Immunol. Today,* 16(1):39-43.

Novelli, F., Delios, M. M., Bernabei, P., Ozmen, L., Rigamonti, L., Almerigogna, F., Forni, F. and G. Del Prete. 1997. Expression and role in apoptosis of the alpha and beta chains of the IFN-gamma receptor on human Th1 and Th2 clones. *J. Immunol.*, 159:206-213.

Ohshima, Y. and G. Delespesse. 1997. T cell-derived IL-4 and dendritic cell-derived IL-12 regulate the lymphokine-producing phenotype of alloantigen-primed naïve human CD4 T cells. *J. Immunol.*, 158:629-636.

Orange, J. S., Wolf, S. F. and C. A. Biron. 1994. Effects of IL-12 on the response and susceptibility to experimental viral infections. *J.Immunol.*, 152: 1253-1264.

O'Shea, J. J. 1997. Jaks, STATs, cytokine signal transduction, and immunoregulation: Are we there yet? *Immunity*, 7:1-11.

Ozmen, L., Aguet, M., Trinchieri, G., and G. Garotta. 1995. The *in vivo* antiviral activity of interleukin-12 is mediated by gamma interferon. *J. Virol.*, 69(12): 8147-8150.

Pahl H. L. and P. A. Baeuerle. 1995. Expression of influenza virus hemagglutinin activates transcription factor NFkB. *J. Virol.*, 69:1480-1484.

Palladino, G., Mozdzanowska, K. Washko, G. and W. Gerhard. 1995. Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. *J. Virol.*, 69(4):2075-2081.

Paul, W. E., and R. A. Seder. 1994. Lymphocyte responses and cytokines. *Cell*, 76:241-251.

Paya, C., McKean, D. J. Segal, D. M. Schoon, R. A.. Showalter, S. D and P. J. Leibson. 1989. Heteroconjugate antibodies enhance cell mediated anti-herpes simplex virus immunity. *J. Immunol.*, 142:666-671.

Penna, C., Dean, P. A. and H. Nelson. 1994. Antitumor x anti- CD3 bifunctional antibodies redirect T cells activated *in vivo* with staphylococcal enterotoxin B to neutralize pulmonary metastases. *Cancer Research,* 54:2738-2743.

Pennisi, E. 1996. Teetering on the brink of danger. *Science,* 271:1665-1667.

Perez, P., Hoffman, R. W. Shaw, S. Bluestone, J. A. and D. M. Segal. 1985. Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody. *Nature,* 316:354-356.

Pernis, A., Gupta, S., Gollob, K. J., Coffman, R. L., Schindler, C. and P. Rothman. 1995. Lack of interferon gamma receptor beta chain and the prevention of interferon gamma signaling in Th1 cells. *Science,* 269:245-247.

Pinto, L. H., Holsinger, L. J. and R. A. Lamb. 1992. Influenza virus M2 protein has ion channel activity. *Cell,* 69:517-528.

Racke, M. K., Bonomo, A., Scott, D. E. Cannella, B., Levine, A., Raines, C.S., Shevach, E. M., and M. Rocken. 1994. Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J. Exp. Med.*, 180:1961-1966.

Reed L. J. and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hygiene,* 27:493-497.

Renno, T., Hahne, M., Tschopp, J., and H. R. MacDonald. 1996. Peripheral T cells undergoing superantigen-induced apoptosis *in vivo* express B220 and upregulate Fas and Fas ligand. *J. Exp. Med.*, 183(2):431.

Riddell, S. R., Walter, B. A., Gilbert, M. J. and P. D. Greeenberg. 1994. Selective reconstitution of $CD8^+$ cytotoxic T lymphocyte responses in immunodeficient bone marrow transplant recipients by the adoptive transfer of T cell clones. *Bone. Marrow. Transplant.*, 14(suppl. 4):S78-84.

Ridge, J. P., Fuchs, E. J. and P. Matzinger. 1996. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science*, 271:1723-1726.

Robert, B., Mach, J. P., Mani, J. C., Ychou, M., Folli, S., Artus, J. C., and A. Pelegrin. 1996. Cytokine targeting in tumors using a bispecific antibody directed against carcinoembryonic antigen and tumor necrosis factor alpha. *Cancer Res.*, 56(20):47584765.

Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D. and A. L. Goldberg. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generatioin of peptides presented on MHC class I molecules. *Cell*, 78:761-771.

Rodriguez-Boulan, E.. 1983. Polarized assembly of enveloped viruses from cultured epithelial cells. *Methods. Enzymol.*, 98:486-501.

Romani, L., Menacci, A., Grohmann, V., Mocci, S., Mosci, P., Pucetti, P., and F. Bistoni. 1992. Neutralizing antibody to interleukin-4 induces systemic protection and T-helper type 1-associated immunity in murine candidiasis. *J. Exp. Med.*, 176:19-25.

Sahin, U., Kraft-Bauer, S., Ohnesorge, S., Pfreundschuh, M., and C. Renner. 1996. Interleukin-12 increases bispecific antibody-mediated natural killer cell cytotoxicity against human tumors. *Cancer Immunol. Immunother.*, 42(1):9-14.

Salgame, P., Abrams, J. S., Clayberger, C., Goldstein, H., Convit, J., Modlin, R. L. and B. R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science* (Wash. DC), 254:279-281.

Sarawar, S. R., Carding, S. R., Allan, W., McMickle, A., Gujihashi, D., Kiyono, H., McGhee, J. R. and P. C. Doherty. 1993. Cytokine profiles in bronchioalveolar lavage cells from mice with influenza pneumonia: consequences of $CD4^+$ and $CD8^+$ T cell depletion. *Reg. Immunol.*, 5:142-150.

Sarawar, S. R., Sangster, M., Coffman, R. L. and P. C. Doherty. 1994. Administration of anti-IFNγ antibody to B2-microglobulin-deficient mice delays influenza virus clearance but does not switch the response to a T helper 2 phenotype. *J. Immunol.*, 153:1246-1253.

Scherle, P. A., Palladino, G. and W. Gerhard. 1992. Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. *J. Immunol.*, 148:212-217.

Schulman, J. L. and E. D. Kilbourne. 1965. Induction of partial specific hetrotypic immunity in mice by a single infection with influenza A virus. *J. Bacteriol.*, 89:170-174.

Schulman, J. L. 1967. Use of an animal model to study transmission of influenza virus infection. *Am. J .Pub. Health,* 58:2092-2096.

Schwartz, R. H.. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell*, 71(7):1065-8.

Seder, R. A., and W.E. Paul. 1994. Acquisition of lymphokine producing phenotype by $CD4^+$ T cells. *Ann. Rev. Immunol.*, 12:635-637.

Spriggs, M. K., Koller, B. H., Sato, T., Morrissey, P.J., Fanslow, W. C., Smithies, O., Voice, M R., Widmer, F. B. and C.R. Maliszewski. 1992. β-Microglobulin, CD8+ T cell deficient mice survive inoculation with high doses of vaccinia virus and exhibit altered IgG responses. *Proc. Natl. Acad. Sci USA,* 89:6070-6074.

Staeheli P.. 1990. Interferon-induced proteins and the antiviral state. *Adv. Virus., Res.*, 38:147-200.

Staeheli, P. and J. Pavlovic. 1991. Inhibition of vesicular stomatitis mRNA synthesis by human MxA protein. *J. Virol.*, 65(8):4498-4501.

Staerz, U. D., and M. J. Bevan. 1986. Hybrid hybridoma producing a bispecific monoclonal antibody that can focus effector T cell activity. *Proc. Natl. Acad. Sci. USA,* 83:1453-1457.

Swain, S. L., Bradley, L. M., Croft, M., Tonkonagy, S., Atkins, G., Weinberg, A. D., Duncan, D. D., Hedrick, S. M., Dutton, R. W. and G. Huston. 1991. Helper T

cell subsets: phenotype, function and the role of lymphokines in regulating their development. *Immunol. Rev.*, 123:115-144.

Taylor, C. E.. 1995. Cytokines as adjuvants for vaccines: antigen-specific responses differ from polyclonal responses. *Infect. Immunity,* 63:3241-3244.

Topham, D. J., Tripp, R. A., Sarawar, S. R., Sangster, M. Y. and P.C. Doherty. 1996. Immune $CD4^+$ T Cells Promote the Clearance of Influenza Virus from Major Histocompatibility Complex Class II -/- Respiratory Epithelium. *J. Virol.,* 70:1288-1291

Townsend, A. R. M., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D. and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T cells can be defined with short synthetic peptides. *Cell,* 44:959-968.

Wang, C., K. Takeuchi, L. H. Pinto, and R. A. Lamb. 1993. Ion channel activity of influenza virus M2 protein: characterization of the amantadine block. *J. Virol.*, 67(9):5585-5594.

Waris, M. E., Tsou, C. Erdman, D.D. Zaki, S. R. and L. Anderson. 1996. Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominantly Th2 like cytokine pattern. *J. Virol.,* 70:2852-2860.

Weiner, L. M., Clark, J. I., Davey, M., Li, W. S., Garcia-de Palazzo, I., Ring, D. B., and R. K. Alpaugh. 1995. Phase I Trial of 2B1, a bispecific monoclonal antibody targeting c-ERB-2 and Fc gamma RIII. *Cancer Res.*, 55(20): 4586-4593.

Welsh, R. M., Selin, L. K. and E. S. Razvi. 1997. Role of apoptosis in the regulation of virus-induced T cell responses, immune suppression and memory. *J. Cell. Biochem.*, 59:135-142.

Wells, M. A., Albrecht, P. and F. A. Ennis. 1981. Recovery from a viral respiratory infection. I. Influenza pneumonia in normal and T-deficient mice. *J. Immunol.*, 126:1036-1041.

White, J., Herman, A., Pullen, A. M., Kubo, R., Kappler, J. W. and P. Marrack. 1989. The Vβ-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell,* 56:27-35.

Wickham, T. J., Segal, D. M., Roelvink, P. W., Carrion, M. E., Lizonova, A., Lee, G. M., and I. Kovesdi. 1996. Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J. Virol..*, 70(10):6831-6838.

Wilson, I. A., and N. J. Cox. 1990. Structural basis of immune recognition of influenza virus hemagglutinin. *Annu. Rev. Immunol.*, 8:737-771.

Winzler, C., Rovere, P., Rescigno, M., Granucci, F., Penna, G., Adornini, L., Zimmermann, V. S., Davoust, J. and P. Ricciardi-Castagnoli. 1997. Maturation stages of mouse dendritic cells in growth factor-dependent long term cultures. *J. Exp. Med.*, 185(2):317-328.

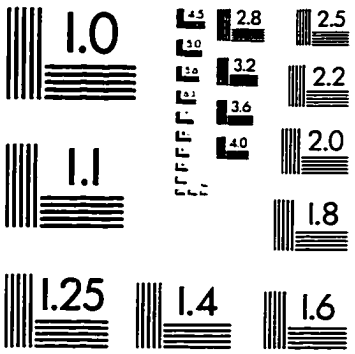
Yang, Y., Li, Q., Ertl, H. C. J., and J. M. Wilson. 1995. Cellular and humoral immune responses to viral antigens create barriers to lung- directed gene therapy with recombinant adenoviruses. *J. Virol.*, 69(4):2004-2015.

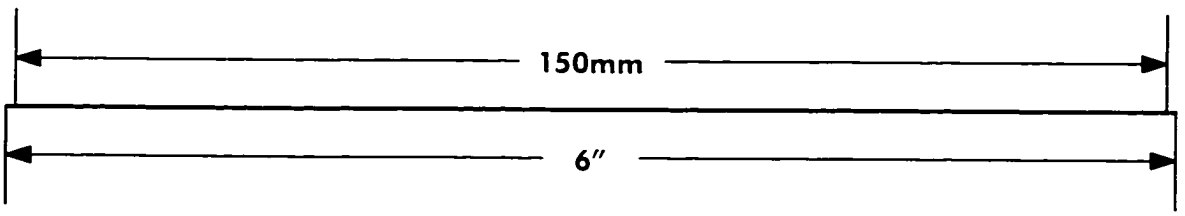
Zajac, A. J., Quinn, D. G., Cohen, P. L. and J. A. Frelinger. 1997. Fas-dependent $CD4^+$ cytotoxic T cell-mediated pathogenesis during virus infection. *Proc. Natl. Acad. Sci. USA,* 93:14730-14735.

Zebedee, S. L., and R. A. Lamb. 1988. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *J. Virol.*, 62(8):2762-2772.

Zinkernagel, R. M. 1996. Immunology taught by viruses. *Science,* 271:173-178.

IMAGE EVALUATION TEST TARGET (QA-3)





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