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VSV infection of resting and activated T lymphocytes

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
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Department of Biochemistry, Microbiology & Immunology
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ABSTRACT

Resting T lymphocytes are uniquely resistant to VSV even at high multiplicities of infection but they can be rendered fully permissive for VSV replication following *in-vitro* activation with monoclonal anti-CD3 and PMA with ionomycin. The block to VSV replication is at the level of viral RNA production and is independent of transcription following infection. T lymphocytes must be activated before infection for at least 24 hours to be rendered susceptible to VSV and transcription is an absolute requirement during this process. Fusion of resting and activated T cells results in a resistant cell indicating that the resistant state is dominant. Resting T cells do not produce interferon (IFN) α or β in response to VSV infection whereas activated T cells produce both but have a down modulated response to the antiviral activity of type I IFNs. Gene expression array analysis demonstrates that the interferon response factors, IRF-4 and IRF-8, are up regulated during activation and a number of interferon stimulating genes (ISG), including ISG20, MxA and GBP-1 are down regulated during activation. IRF-4 and IRF-8 have both been described to bind to and inhibit transactivation from the interferon stimulated response elements (ISRE) in the promoters of a number of ISGs and this could explain the down modulated antiviral response to type I IFNs in activated T cells. The IFN-independent constitutive expression of these ISGs in resting T cells is a possible mechanism for their unique resistance to VSV replication.

"Sometimes wrong, never in doubt"

-A surgeon's motto-

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ABREVIATIONS

6-16	alpha inducible protein 6-16
APC	antigen presenting cells
AV1	interferon inducing mutant VSV
CD	clusters of differentiation
CKII	casein kinase II
DAG	diacylglycerol
DC	dendritic cell
DiD	dialkylcarbocyanines – blue lipophillic dye
DiI	dialkylcarbocyanines – pink lipophillic dye
dsRNA	double stranded RNA
eIF	eukaryotic initiation factor
ELISA	enzyme-linked immunosorbent assay
GAF	Gamma activated Factor
GAS	Gamma associated sequence
GBP	guanylate binding protein
GFP	Green Fluorescent Protein
ICSBP	interferon consensus sequence binding protein
ICSAT	interferon consensus sequence binding protein for activated T cells
IFI27	alpha inducible protein 27
IFIT	Interferon-induced protein with tetratricopeptides repeats
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNGR	Interferon gamma receptor
Ig	immunoglobulin
IL	Interleukin
Io	Ionomycin
IP_3	inositol 1,4,5-triphosphate
IRF	interferon regulatory factor
ISG	Interferon stimulated gene

ISGF3	Interferon stimulated transcription factor 3 (complex with p48)
ISRE	Interferon stimulated response element
HR	heat resistant wild type VSV
ITAM	immunoreceptor tyrosine-based activation motifs
Jak	Janus-activated kinase
JNK	c-Jun NH2-terminal kinase
LD50	lethal dose for 50%
LPS	Lipopolysaccharide
mAb	monoclonal antibody
MAP kinase	mitogen activated protein kinase
MEF	murine embryonic fibroblast
MHC	major histocompatibility complex
moi	multiplicity of infection
Mx1	myxoma (influenza) resistance 1
MxA	murine myxoma (influenza) resistance A
NFAT	nuclear factor of activated T cells
OAS	oligoadenylate synthetase
OKT3	murine monoclonal anti-CD3 antibody
OVCAR	ovarian carcinoma cell line
PBMC	Peripheral blood mononuclear cells
PE	phycoerythrin
PI	propidium-iodide
PKC	protein kinase C
PKR	double-stranded RNA protein kinase
PLC	phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PFU	Plaque forming units
RNP	ribonucleoprotein
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
T_C	cytotoxic T cell

TCR	T cell receptor
TNF	Tumour necrosis factor
T_H	T helper cell
Ub	ubiquitin
VAK	Virus-Activated Kinase
VSV	Vesicular stomatitis virus

1. INTRODUCTION

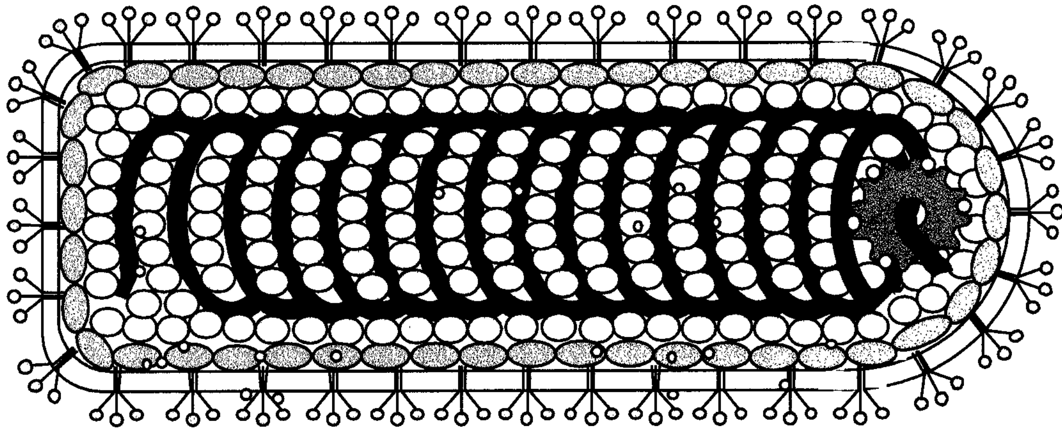
Vesicularstomatitis virus (VSV) is currently being developed as a replicating cancer therapeutic. VSV is highly susceptible to the the antiviral effects of interferons, the key cytokines in the innate immune response, and its oncolytic activity is dependent upon the widespread defects demonstrated in the interferon pathway of many tumours. The ability of the acquired immune system to clear VSV infection from the host is both essential for the safety but detrimental to the efficacy of VSV as a cancer therapeutic. In this thesis I demonstrate that resting T lymphocytes are uniquely resistant to VSV infection but can be rendered susceptible through the process of immunological activation, and I explore the molecular regulation responsible for this phenomenon. An understanding of the interaction of VSV and the innate and acquired immune systems will provide insight into the mechanisms that govern viral susceptibility at the cellular and organismal level.

Vesicular Stomatits Virus as an Oncolytic Agent

1.1 The biology and epidemiology of VSV

As a member of the genus *Vesiculovirus* of the family Rhabdoviridae, VSV is among the simplest of the RNA viruses in terms of its structure, genetics and physiology and for this reason it is exploited as a model system for the study of viral replication and cytopathology. VSV is an enveloped virus with a nonsegmented RNA genome of 11kb encoding 5 mRNAs and 9 proteins. Structurally it is divided into the ribonucleoprotein core and the viral membrane as illustrated in Figure 1. The single strand negative sense RNA, the N (nucleocapsid) protein collectively with the P (phosphoprotein) and L (large) protein form the ribonucleoprotein core; a transcription unit which will synthesize, cap and polyadenylate mRNAs *in vitro*. The L protein serves as the RNA-dependent RNA

a.



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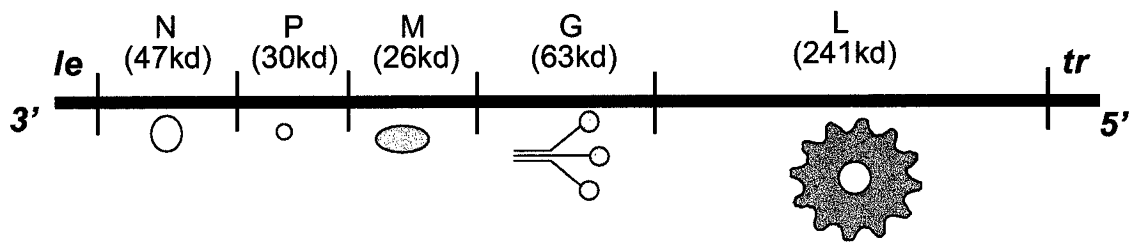


Figure 1. VSV virion and genome structure

Diagram of Vesicular Stomatitis Virus (VSV), illustrating virion sturcture (***a***) and genome structure (***b***). VSV forms a bullet shaped particle, enveloped in a host derived plasma membrane, containing all five viral proteins and the single stranded negative sense RNA genome. The L (Large) protein forms the major component of the RNA dependent RNA polymerase, along with P (Phosphoprotein) and N (Nucelocapsid) protein, which also has a role packaging the genome. The G (Glycoprotein) forms a glycosylated trimer that binds the host receptor, phosphatidylserine and mediates pH dependent membrane fusion. The M (Matrix) protein has a role in virus assembly and budding, cytopathic effects, and shutoff of host cell defences. The virion polymerase transcribes the five mRNAs in sequential order from the 3' end, as shown in (***b***). The polymerase pauses at the end of each transcript, and reinitiates with imperfect efficiency, such that genes at the 3' end (N) are transcribed more than genes at the 5'end (L) providing a mechanism of regulation of gene expression. The leader (*le*) and trailer (*tr*) regions are transcribed but not translated. Diagram courtesy of Jenn Paterson.

polymerase but phosphorylated P is an absolute requirement for transcription[1]. The P protein is sequentially phosphorylated at two separate domains by two distinct kinases. Casein kinase II, a ubiquitous cellular kinase phosphorylates the N-terminal domain followed by phosphorylation at the C-terminus by a kinase associated with the L protein[2]. The genome RNA when tightly complexed with the N protein is ribonuclease resistant and serves as the template for transcription[3]. The viral membrane is composed of the membrane spanning G (glycoprotein) and the M (matrix) protein along with lipids and cholesterol derived from the host cell. The G protein mediates binding of VSV to the cell surface and membrane fusion. Not surprisingly, this viral surface protein is also the major antigenic determinant of VSV and it stimulates a very strong antibody response[4]. The M protein is multifunctional in that it plays a crucial role in directing viral assembly and budding and it has a number of cytopathogenic functions ascribed to it. These include inhibition of host transcription[5], disorganization of the cytoskeleton resulting in cell rounding and detachment[6], induction of apoptosis[7] and the inhibition of nucleocytoplasmic transport of mRNAs and proteins[8]. Both the P and the M mRNAs encode additional proteins whose functions have not been fully elucidated[9, 10].

The life cycle of VSV (Figure 2) begins with viral adsorption to the cell membrane by G protein binding to the ubiquitous phosphatidylserine and possibly other receptors. Penetration follows by receptor-mediated endocytosis and subsequent pH-dependent membrane fusion releases RNPs into the cytoplasm. The negative strand RNA is transcribed sequentially into the five mRNAs: N, P M, and L are translated by free cytoplasmic ribosomes whereas G is translated on ribosomes bound to the endoplasmic reticulum, where the protein product becomes glycosylated and shuttled to the cell

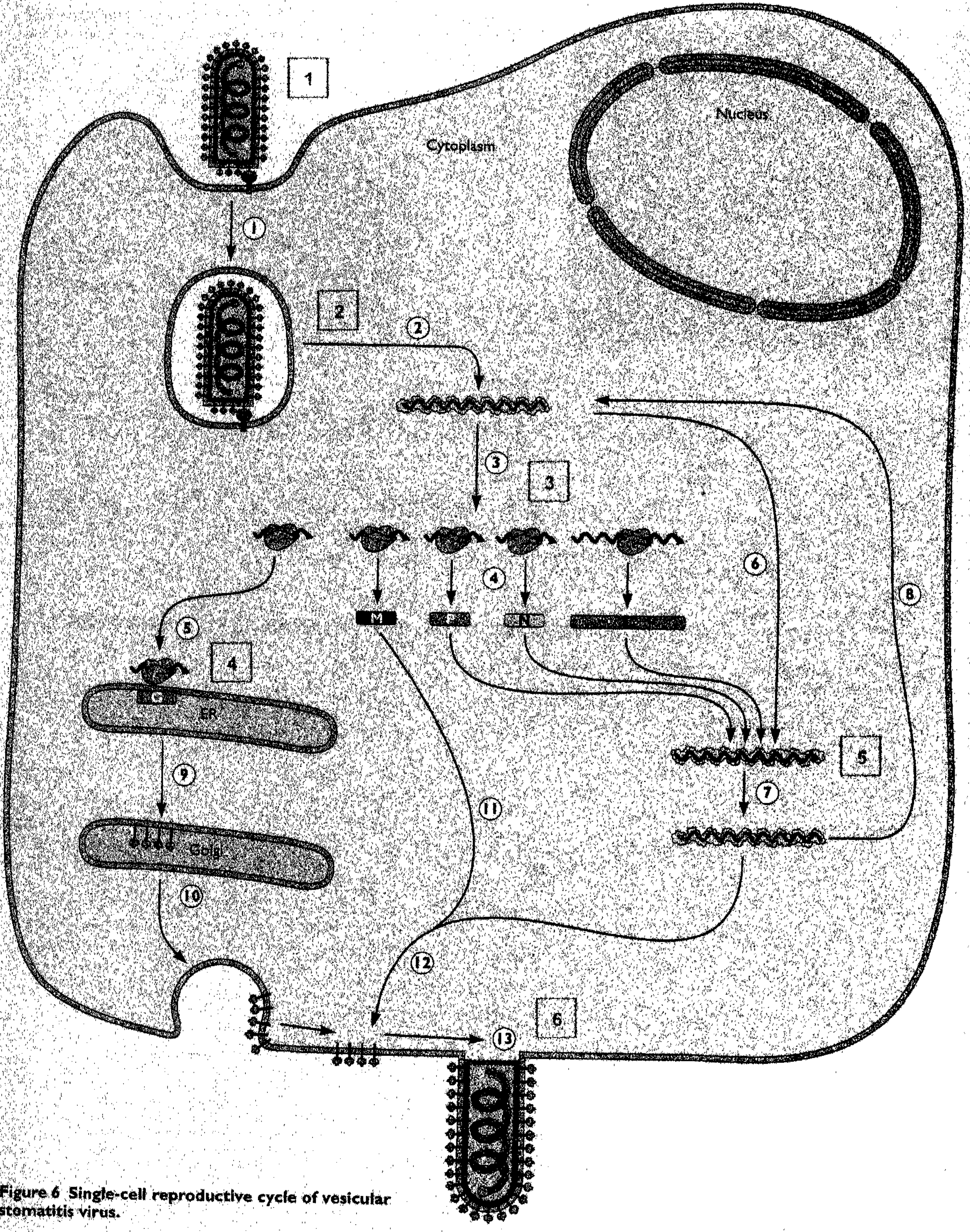


Figure 6 Single-cell reproductive cycle of vesicular stomatitis virus.

Figure 2. The life cycle of VSV

The life cycle of VSV begins with virion adsorption to the cell membrane by G protein binding to the ubiquitous phosphatidylserine receptor (*box1*). Penetration follows by receptor-mediated endocytosis and subsequent pH-dependent membrane fusion releases RNPs into the cytoplasm (*box2*). The negative strand RNA is transcribed sequentially into the five mRNAs: N, P M, and L are translated by free cytoplasmic ribosomes (*box3*) whereas G is translated on ribosomes bound to the endoplasmic reticulum, where the protein product becomes glycosylated and shuttled to the cell membrane (*box4*). Viral RNA replication begins with synthesis of a full-length positive strand copy of RNA which serves as a template for the progeny negative strand genomic RNA (*box5*). These newly synthesized RNPs and the M protein are transported to the G protein containing plasma membrane where viral assembly and budding is initiated (*box6*). Reproduced from Principles of Virology, Flint et al. 2000.

membrane. Viral RNA replication begins with synthesis of a full-length positive strand copy of RNA which serves as a template for the progeny negative strand genomic RNA. These newly synthesized RNPs and the M protein are transported to the G protein containing plasma membrane where viral assembly and budding is initiated[11]. A complete understanding of the viral components of VSV and their interaction in the host cell will be invaluable for elucidating the mechanisms responsible for viral resistance and sensitivity to VSV. Moreover it will provide a rational framework to guide the manipulation of VSV to enhance its oncolytic properties.

The epidemiological host for VSV is livestock. In cattle and swine, the two main serotypes, New Jersey (NJ) and Indiana (IN), both cause a self-limiting disease clinically undistinguishable from foot and mouth disease. VSV is endemic from northern South America to southern Mexico, where seasonal outbreaks infect roughly 10% of cattle every year, while sporadic outbreaks have been described in southwestern USA[4]. Although the natural lifecycle of VSV is unknown, evidence suggests that insects, notably black flies and sand flies, carry the virus in endemic areas and are capable of infecting animals and presumably also humans[4]. However, animal hosts with sustained viremia have never been found strongly suggesting that they are dead-end hosts from which the virus does not return to its cycle in nature[12]. Under laboratory conditions VSV cultures can be subjected to strong selective forces[3], however, extensive population studies among livestock have shown no evidence of immunological selection - VSV is genetically stable despite high levels of neutralizing antibodies among cattle populations[12]. In non-endemic areas, VSV infection in humans is rare and results in an influenza-like illness that is uniformly non-fatal[4].

This epidemiological data has important implications for the development of VSV as a therapeutic agent. First, it alleviates concern regarding the spread of VSV from the index treatment case to the population by establishing animals as dead-end hosts. Second, because VSV is a genetically stable virus *in vivo*, it is unlikely that VSV will mutate to a more virulent form following infection. Third, naturally occurring human VSV infection results in tolerable flu-like symptoms, and the virus is quickly and completely cleared with no evidence of persistent infection. Moreover, because most humans are seronegative, the virus has an opportunity to replicate before neutralizing antibodies develop.

1.2 VSV is exquisitely sensitive to the antiviral effects of interferons

VSV is so well known for its IFN sensitivity that an International Unit of interferon activity is defined by the ability to inhibit VSV replication[13]. IFN α and β are cytokines that belong to the family of Type I IFNs responsible for conferring an antiviral state in cells by the expression of IFN stimulated genes (ISG). The interferon response results in an antiviral state consisting of cell cycle arrest, inhibition of host translation, mRNA degradation and apoptosis (Figure 3). Certainly, an appreciation of the molecular pathways involved in IFN signaling is critical when attempting to delineate the mechanisms responsible for restricted viral replication in resistant cells, such as resting T cells, and permissive viral replication in sensitive cells, such as activated T cells and malignant cells.

IFN α and β signal through Type I IFN receptors (IFNAR) which are associated with two 'Janus' tyrosine kinases (Jak) and two 'signal transducer and activator of transcription' molecules (STAT). The binding of Type I IFNs to their receptor results in

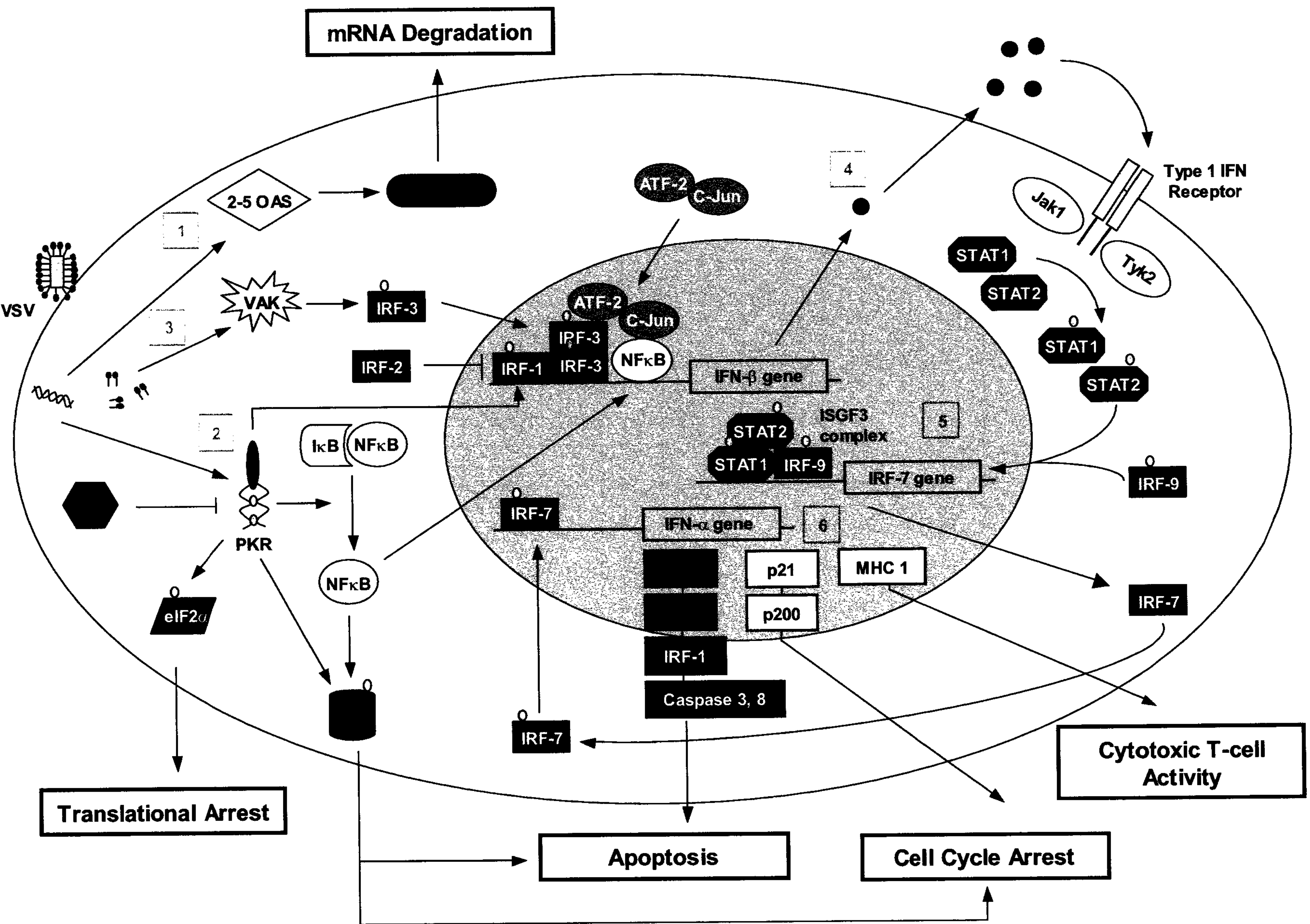

mRNA Degradation
Type 1 IFN Receptor
Jak1
Tyk2
STAT1
STAT2
ATF-2
C-Jun
4
1
2-5 OAS
VSV
3
VAK
IRF-3
IPF-3
NFκB
IRF-2
IRF-1
IFN-β gene
ISGF3 complex
5
2
IκB
IRF-9
IRF-7 gene
PKR
IRF-7
IFN-α gene
6
p21
MHC 1
eIF2α
p200
Caspase 3, 8
Cytotoxic T-cell Activity
Translational Arrest
Apoptosis
Cell Cycle Arrest

Figure 3. The interferon signalling pathway

Infection with VSV initiates the interferon signalling cascade that results a number of antiviral processes including mRNA degradation, translational arrest, cytotoxic T-cell activity, cell cycle arrest and apoptosis. **1.** Double-stranded RNA activates 2-5 oligoadenylate synthetase leading to the production of RNAse L and viral mRNA degradation. **2.** PKR is activated by autophosphorylation in response to dsRNA and it in turn effects several pathways including phosphorylation of eIF2α and subsequent translational arrest, activation of NFκB and IRF-1, components of the 'enhancesome' complex formed on the IFNβ gene promoter, and the transcriptional up-regulation and activation of p53 whose downstream effects include cell cycle arrest and apoptosis. **3.** Viral proteins result in the activation of the Virus Activated Kinase (VAK) leading to the phosphorylation of IRF-3 and its translocation to the nucleus where, along with NFκB and several other transcription factors, it forms the 'enhancesome' and transcriptional up-regulation of IFNβ. IFNβ is synthesized and secreted from the cell where it binds to Type 1 IFN receptors on the cellular surface (itself and neighbouring cells). This interaction results in the phosphorylation of STAT 1 and 2 and, along with phosphorylated IRF-9 leads to the formation of the ISGF-3 complex on the IRF-7 promoter. **6.** Once synthesized, IRF-7 is phosphorylated by the VAK, resulting in its translocation to the nucleus and transcriptional up-regulation of IFNα and a number of other ISGs responsible for the variety of downstream effects of IFNs. Within the pathway blue indicates factors where defects are known to occur some cancer cells, yellow indicates factors where defects are known to confer sensitivity to VSV, and green indicates where defects both occur in cancer cells and are known to confer sensitivity to VSV. (*orange dot indicates phosphorylation*)

transphosphorylation of Jaks and subsequent phosphorylation of STAT 1 and 2. The phosphorylated STATs form a heterodimer that translocates to the nucleus where it associates with the DNA binding protein interferon regulatory factor 9 (IRF-9, previously termed p48). This heterotrimeric complex, known as ISGF3, binds to an interferon stimulated response element (ISRE) to induce the transcription of more than 300 different ISGs[14].

The induction of the IFN α and β genes themselves are mediated by two interferon regulatory factors, IRF-3 and IRF-7 which define the first and the second wave of interferon production[15]. In the first wave following viral infection IRF-3, which is constitutively expressed in all cell types, is activated via phosphorylation by Virus-Activated Kinases (VAK), recently reported to be members of the IKK family of kinases[16]. IRF-3 translocates to the nucleus as a homodimer and binds to the IFN-β promoter as part of a multiprotein transcription-promoting complex called the 'enhancesome' that also contains NFκB, IRF-1 and ATF-2/c-Jun[17]. Once synthesized and secreted, IFN-β signals via the Type I IFN receptor to induce the synthesis of IRF-7, resulting in the second wave of the cascade. Unlike IRF-3, IRF-7 is only constitutively expressed in cells of lymphoid lineage, including T and B lymphocytes. IRF-7 is also a transcriptional activator that turns on the synthesis of IFN-α and many other ISGs. IFN-α also signals via the Type 1 IFN receptor resulting in a feedback loop that is essential for IFN-priming, a phenomenon whereby pre-treatment of cells with a low dose of interferon enhances the interferon production during viral infection[15]. It is also essential for protective interferon signaling from infected cells to neighboring uninfected cells. In

addition, the constitutive expression of an ISG in a cell may confer an antiviral state to that cell in the absence of IFN.

Of the many ISGs, the best understood IFN regulated pathways include the Mx family of proteins and the two double-stranded RNA (dsRNA) dependent enzymes - the 2-5 oligoadenylate synthetase/RNase L system (2-5 OAS), and the protein kinase PKR. The Mx proteins belong to the dynamin family of GTPases and the human cytoplasmic MxA protein has been shown to inhibit viral replication at the level of transcription, as is the case with VSV[18], or at the posttranscriptional and translational levels, as seen with influenza[19] and measles[20]. The 2-5 oligoadenylate synthetases are transcriptionally induced by IFN but require double stranded RNA (dsRNA) as a cofactor for activation. These enzymes polymerize ATP to produce 2'-5' linked oligoadenylates whose only known function is to activate the latent ribonuclease RNase L. Activated RNase L mediates the antiviral activity through cleavage of single-stranded RNA as demonstrated by the correlation between RNase L activation and IFN-induced inhibition of encephalomyocarditis virus[21], vaccinia virus[22] and reovirus infection[23].

The most extensively studied IFN-induced protein is PKR. Similar to 2-5 OAS, the transcription of PKR is upregulated by IFNs but the PKR protein needs to be activated by dsRNA. PKR is a serine-threonine kinase that undergoes autophosphorylation upon activation, which enables it to phosphorylate substrate targets. The translation initiation factor eIF2α is one such target, resulting in the effective shutdown of protein synthesis[24]. PKR upregulates the activity of several transcription factors including NFκB, IRF-1[25], both components of the 'enhancesome' complex critical for the induction of IFN-β. PKR is also found in a complex with STAT1, which dissociates upon

PKR activation, allowing STAT1 to participate in the signaling cascade via the Type 1 IFN receptor[26]. In addition, PKR has been shown to regulate the activity of the tumour suppressor p53 and members of the stress activated protein kinases, p38 MAP kinase and c-Jun NH2-terminal kinase, JNK[27]. The modulation of these varied targets by PKR confers a regulatory ability on diverse cellular processes such as cell growth and differentiation, apoptotic, antitumour and, of course, antiviral activities. The biological importance of PKR in regulating virus infection is underscored by the existence of a multitude of viral inhibitors to PKR action including adenovirus (inhibition of PKR activation), reovirus (sequestration of dsRNA), HCV (inhibition of PKR dimerization), Herpesvirus (synthesis of PKR pseudosubtrates) and poliovirus (PKR degradation)[24]. VSV does not possess such an inhibitor to PKR.

Interferons also signal through a number of alternative pathways that mediate cellular resistance to viral replication, including VSV. These pathways include upregulation of caspases involved in the apoptotic cascade, cyclin-dependent kinase inhibitors, such as members of the p200 family and c-myc, exerting negative regulation of the cell cycle, and of MHC class I proteins for immune stimulation via cytotoxic T-cells[17]. One recently identified alternative pathway is defined by ISG20, a novel IFN-induced RNAse specific for single stranded RNA[28]. ISG20 is strongly expressed in peripheral blood leukocytes and in lymphoid tissue in the absence of IFN but its expression can be induced in most tissues by both IFNα/β and IFNγ[29]. This induction is strictly dependent upon IRF-1 binding to an ISRE within the ISG20 promoter[30]. Espert et al.[28]demonstrated that overexpression of ISG20 in Hela cells conferred resistance to VSV, even in the absence of IFN, by inhibiting VSV mRNA and protein

accumulation, whereas the expression of a dominant negative mutant of ISG20 significantly reduced the ability of IFN to block VSV replication. Taken together these data suggest that ISG20 mediates a novel IFN-induced antiviral pathway and moreover, its constitutive expression, such as that seen in lymphoid tissue[29], may confer resistance to VSV in the absence of IFN. Another alternative pathway is mediated by the IFN-induced guanylate binding protein, GBP-1. Anderson et al.[31] demonstrated that constitutive expression of GBP-1 in the absence of IFN treatment resulted in an inhibition of viral replication and conversely cells constitutively producing antisense RNA to GBP-1 produced a weaker antiviral effect against VSV in IFNγ but not IFNα treated cells. Because IFNα treatment results in the preferential induction of MxA and IFNγ treatment results in the preferential induction of GBP-1, the authors suggest that MxA and GBP-1 may serve as nonredundant effectors of IFN-mediated antiviral activities.

Murine *in-vivo* studies underscore the importance of the IFN pathway during VSV infection. In mouse experiments of VSV infection, treatment with Type I IFNs, even several days after inoculation, increases survival whereas blocking the interferon response, either with antibodies or by using IFN α/β receptor knockout mice, decreases survival[32]. Similarly, mice lacking STAT1[33] or p48/IRF-9[34] genes are also remarkably susceptible to lethal infection with VSV. A clear role for PKR in IFN-mediated resistance to VSV has also been established by Stojdl et al[35]. In this study, fibroblasts derived from PKR-/- mice were shown to be more permissive to VSV infection than wild-type fibroblasts and to be deficient in IFN-mediated protection. In addition, PKR-/- mice were acutely susceptible to intranasal VSV infection and succumbed to an overwhelming respiratory infection with an LD50 of <15 PFU per

mouse. By comparison, wild-type mice had an LD50 of 10^6 and manifested only central nervous system pathology, most notably hind limb paralysis. Moreover, pretreatment of wild-type mice with IFN-α/β completely alleviated symptoms of VSV infection whereas pretreatment of PKR-/- mice resulted in no increase in survival or alleviation of symptoms. In an earlier study by Zhou et al.[36] VSV infection of fibroblasts from mice deficient in either PKR or triply deficient in PKR, Mx1 and RNAse L was 50-100 fold less inhibited by IFN than in RNAseL-/- or control mice. This suggests that PKR is largely responsible for the difference in the antiviral effect of IFN observed between the control and triply deficient fibroblasts following VSV infection. This difference notwithstanding, at the highest dose of IFN more than 10^3 units of inhibition were observed in the PKR-/- and triply deficient mice providing clear evidence for the existence of alternative antiviral pathways. Indeed ISG20 might represent one such alternative pathway since the level of IFN-mediated antiviral activity in triply deficient MEFs correlated with the induction of ISG20 expression in the study by Espert et al.[28].

IFN-γ is a Type II IFN that signals through the IFN-γ receptors, IFNGR1 and IFNGR2. Binding of IFN-γ to these receptors triggers dimerization and transphosphorylation by Jak1 and Jak2 respectively and the binding and phosphorylation of STAT1. Activated STAT1 forms a homodimer, known as γ-activated factor (GAF), which translocates to the nucleus where it binds to specific GAS elements and stimulates transcription[17]. IFNγ plays an important role in the differentiation of activated helper and cytotoxic T cells, which is discussed in detail later. The relative antiviral activities of IFNα/β versus IFNγ are dependent on the cell type being studied[37]. While both types of IFN are able to confer antiviral resistance to VSV in many cell lines studied, including

the fibroblast cell lines L-929 and TS/A, primary T cells and a number of T cell lines, including ST4 and MOLT4 are not sensitive to the antiviral effects of IFNγ[38]. In fact IFNγ may increase VSV yields in primary T cells[39].

1.3 VSV selectively replicates in cancer cells with defects in the interferon pathway

While tumour cells have gained a growth and survival advantage by acquiring defects in the IFN signalling pathway, they have simultaneously compromised their ability to defend themselves against certain pathogens. Since IFN is clearly essential for the defence against VSV it was hypothesized that VSV might replicate and spread more efficiently in cancer cells than in normal cells. Indeed, as shown by Stojdl et al.[35] human tumour cell lines infected with VSV demonstrated earlier and more extensive cytopathic effects and yielded 10^1-10^4 more viral PFU/ml than normal human primary cell cultures. Similar to the results comparing wild type and PKR-/- mice, IFN pre-treatment completely protected normal primary cells and reduced viral production to less than 1,000 PFU, whereas tumour cell lines remained susceptible to VSV and continued to produce high viral titres. Moreover in a mixed culture of leukemic cells from an AML cell line and normal bone marrow cells, VSV demonstrated selective destruction of leukemic cells, even in the absence of IFN, illustrating the potential to use VSV in *ex vivo* bone marrow purging. In a similar study, Balachandran et al.[40] also established the utility of VSV as an oncolytic agent by demonstrating efficient replication and cytolysis in a number of different human cancer cell lines with incomplete protection following IFN-β pretreatment. Interestingly, a second study by the same group[41] also demonstrated normal PKR activation and eIF2α phosphorylation in response to VSV infection in rat C6 glioblastoma cells, implying that other components of the IFN

pathway and not PKR itself are responsible for the oncolytic activity of VSV in this cell line. This study also established the induction of VSV induced apoptosis as the key mechanism responsible for oncolysis.

Both Stojdl et al. and Balachandran et al. used athymic mouse models to investigate the oncolytic efficacy of VSV toward the human melanoma or rat glioblastoma xenografts respectively. These studies demonstrated significant tumour regression following intratumoral injection of VSV with residual virus in the tumour tissue but virtually no virus detectable in the lung, brain, kidney, spleen or liver 21 days after infection. Similar results were seen in athymic mice implanted with *c-myc* or *K-ras* transformed BALB/3T3 cells, suggesting the potential of VSV against malignancies exhibiting a wide range of genetic lesions[41]. In terms of viral toxicity, whereas Balanchandran et al. used a fairly low dose of VSV ($2.5X10^7$ PFU/ml) and observed no overt sickness apparent in the treated animals during the study, Stojdl et al. used a higher dose (10^8 PFU/ml) and found these immune compromised mice began to die by day 10 of infection. In the latter study however, mice that received IFN treatment during the infection were protected and survived symptom free for more than 45 days. Finally VSV is capable of reaching and replicating distal to the site of administration and oncolytic viral replication has been demonstrated in tumours following intravenous injection and contralateral intratumoral injection[41]. Similarly, results from our lab have established the *in-vivo* efficacy of intravenously administered VSV in a metastatic mouse model of colon cancer disseminated to the lung.

Several IFN inducing VSV mutants have been characterized in the literature [42, 43] as having substitutions in the M protein that result in decreased ability of the virus to

inhibit host gene expression, including IFN induction. Our lab has shown that the VSV mutant AV1 induces 20-50 times more IFN than the parental wild type virus on epithelial cell lines. Furthermore, these viruses retain their tumour killing ability against 75-80% of cell lines in the NCI 60 panel of human cancer cell lines. As expected, most of the cell lines in this panel have defects in their response to IFN.

There exist several theories for the mechanism by which some mutants induce more IFN than the wild type VSV. The wild type M protein has been demonstrated to inhibit host transcription and, in particular reduce the expression of genes under the control of the human IFNβ promoter [44] whereas other studies have provided evidence that the wild type M protein actually inhibits nucleocytoplasmic trafficking, including mRNA export [8] and that VSV M protein inhibits STAT activation [45]. Our lab has performed quantitative RT-PCR to show that although AV1 and wild type VSV Indiana induce equivalent levels of IFNβ mRNA in the nucleus, cytoplasmic IFNβ mRNA was reduced by almost 100 fold in wild type virus infected cells, providing further support for the nucleocytoplasmic transport hypothesis. Differential susceptibility to wild type and AVI VSV allows for the identification of cells that depend on IFNβ signalling for their antiviral response.

1.4 VSV infects a particularly broad range of mammalian cells in tissue culture but not resting T lymphocytes

Most normal mammalian cells have an intact IFN pathway and are therefore resistant to VSV when infected at a low moi (0.1 PFU/cell). At this moi less than 1 in 10 cells are infected with input virus and these cells produce IFN thereby protecting neighbouring cells from subsequent infection. When infected at a high moi (10 PFU/cell)

however, the protective effects of IFN are not seen because every cell is productively infected with input virus. With only a few notable exceptions, mammalian cells succumb to VSV infection at a high moi. These exceptions include a rabbit cornea cell line[46, 47], lymphoblastoid cell lines derived from Burkitt's lymphoma[48, 49] (e.g. Raji and Daudi) and primary lymphocytes[50-54]. These cell types represent a unique response to VSV infection that is unlike most mammalian cells studied to date.

T lymphocytes Response to Viral Infection

1.5 T lymphocyte response to viral infection is unique

It has been known for more than 40 years that resting T lymphocytes fail to support the replication of a number of viruses including herpes simplex[55], New Castle Disease virus, yellow fever[50], polio[56] and VSV[56]. It was also well described that activation of small lymphocytes with mitogens - such as phytohemagglutinin (PHA), rabbit anti-human lymphocyte serum, and streptolysin - or specific antigens rendered T cells susceptible to viral replication[56]. In fact the ability of VSV to selectively replicate in activated T cells was exploited in an assay for quantifying T suppressor cell activity[57]. Over the years only a few studies have attempted to address the mechanism underlying this phenomenon.

The earliest studies were able to correlate the morphological features of T cell transformation, such as increase in cytoplasm and endoplasmic reticulum volume and cellular division, to VSV susceptibility[51]. This led to the hypothesis that resting T cells have insufficient metabolic capacity to support viral replication, while the process of activation increases the overall biosynthetic capability of T cells, supporting the synthesis of viral proteins and nucleic acids[53]. VSV does not depend on the cells transcriptional

machinery to synthesize viral mRNA because it encodes its own RNA polymerase, the L and P proteins. VSV does, however, exploit the cellular translational machinery to synthesize viral proteins. A block to viral protein translation would prohibit the synthesis of new L and P proteins and thwart the subsequent exponential increase in RNA and protein synthesis that is characteristic of productive VSV infections.

Restricted replication at the level of protein synthesis has been demonstrated in the non-permissive rabbit cornea cell line[47]. In this study, the VSV mRNA recovered from the polysome region directed the synthesis of VSV protein in an *in-vitro* rabbit reticulocyte translational system, suggesting that protein synthesis was blocked at a step beyond initiation. Schmidt et al.[53] studied the progression of VSV infection in resting and stimulated murine B lymphocytes and also concluded that the process of elongation during viral translation is restricted - initiation did not appear restricted because viral mRNA was detected in the polysome containing fractions and there was no evidence of rapid viral protein degradation using proteasome inhibitors and S^{35} methionine labelling. Interestingly, this group determined that VSV infection was in fact restricted at multiple levels that were associated with different stages of B lymphocyte activation[54]. Whereas cytokines such as IL-2 and IL-4 (but not IFNγ) increased the steady state level of viral mRNA they did not increase viral protein synthesis. Activation with anti-Ig resulted in increased viral protein production but not a correspondingly high level of viral particles while activation with PMA and ionomycin or lipopolysacharide increased both viral protein and PFU production.

Although VSV encodes its own polymerase, its transcriptional activity is dependent on phosphorylation of the P protein at two sites – one of which is catalyzed by the

cellular casein kinase II[2]. This reliance on a host protein kinase for transcriptional activity is a potential step at which viral replication might be restricted. Sleat et al.[58] postulated that restricted replication of VSV in T lymphocytes was due to a deficiency in casein kinase II (CKII). In this study the activity of casein kinase on the P protein of VSV increased by approximately 2-fold and the protein level of the α subunit of CKII increased by 3-fold in T lymphocytes following activation with concanavalin A. By contrast, the CKII activity in other non-permissive cells lines, including Raji and a rabbit cornea cell line, was similar to the activity in permissive cell lines, such as BHK-21 cells. Moreover IFNα treatment did not alter CKII activity in a permissive cell line, despite almost complete inhibition of viral replication. Although this study demonstrates a relative deficiency in CKII activity in resting T lymphocytes they provide no evidence to support a conclusion that restricted replication of VSV in resting T cells is due to this deficiency. Given that T cells are resistant to a variety of other viruses, none of which depend on CKII activity, restricted replication is likely secondary to a more generalizable mechanism.

In general, establishing the level in the viral life cycle at which replication is inhibited is an essential first step in the rational approach to evaluating cellular mechanisms of viral resistance.

1.6 Resting T lymphocytes are activated to become effector cells by infection *in-vivo*

Resting T lymphocytes are either naïve or memory cells that live for many years without dividing. These small resting cells have condensed chromatin, a scanty cytoplasm, and synthesize little RNA or protein. On activation, these cells re-enter the

cell cycle and divide rapidly to produce the large numbers of progeny that will become armed effector T cells, all bearing the same receptor for antigen. Effector cells increase in size with an extended cytoplasm and begin to synthesize cytokines and receptors responsible for the differentiation of these cells into various activated T lymphocyte subsets[59].

Mature T lymphocytes that have not yet encountered their antigens are known as naïve T cells and they can be identified by the cell surface expression of the CD45Ra isoform of CD45[60]. Activation of a naïve T cell requires an encounter with a specific antigen in the presence of the required co-stimulatory signals, such as the B7 molecules which bind to the CD28 receptor on T cells or CD40 which binds to the CD40 ligand. This process is facilitated by professional antigen-presenting cells (APC), including dendritic cells, macrophages and B lymphocytes, which process antigens and present them to T lymphocytes on MHC class II, and in some cases MHC class I molecules (Figure 4). Dendritic cells reside in the tissue and are stimulated by local infection and inflammation to migrate to lymph nodes and express the co-stimulatory molecules that are required for the activation of naive T cells. Dendritic cells can take up, process, and present a wide variety of pathogens and antigens and appear to be the most important activators of naive T cells, whereas macrophages and B cells specialize in processing and presenting antigens from ingested pathogens and soluble antigens, respectively[59]. The generation of effector cells from a naive T cell takes several days. In contrast, memory T cells are more sensitive to stimulation by antigen and display a more rapid and vigorous response to such stimulation. These cells can be differentiated from naïve T cells because they express the CD45Ro isoform, as do effector T cells[61]. At the end of the activation

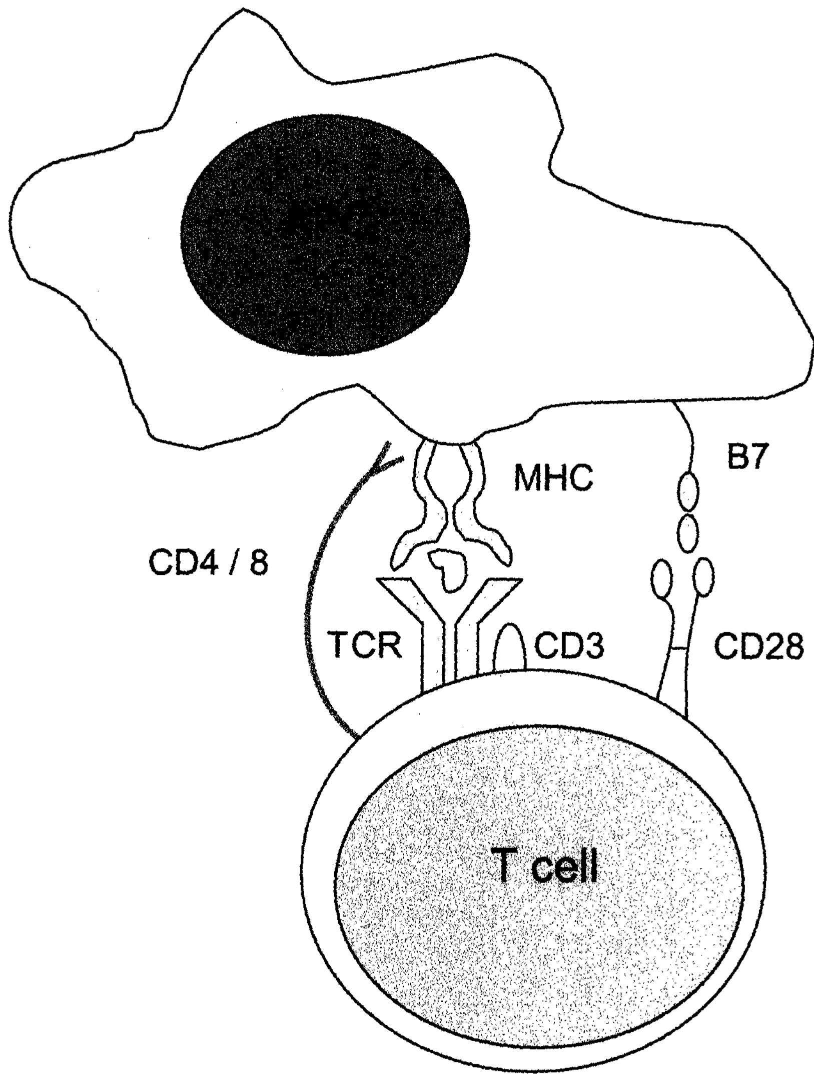

MHC
B7
CD4 / 8
TCR
CD3
CD28
T cell

Figure 4. The activation of resting T cells requires antigen presentation

Activation of a naïve T cell requires an encounter with a specific antigen in the presence of the required co-stimulatory signals, such as the B7 molecule that bind to the CD28 receptor on T cells or CD40, a receptor which binds to the CD40 ligand (not shown). This process is facilitated by professional antigen presenting cells (APC), including dendritic cells, macrophages and B lymphocytes, which process antigens and present them to T lymphocytes on MHC class II, and in some cases MHC class I molecules.

period, armed effector T cells leave the lymphoid organs and re-enter the bloodstream to migrate to site of infection. The following sections will focus in the biological process of T cell activation, both *in-vivo* and *in-vitro*, and the cellular changes that are hypothesized to contribute to the transformation from a VSV-resistant resting T cell to a VSV-permissive activated T cell.

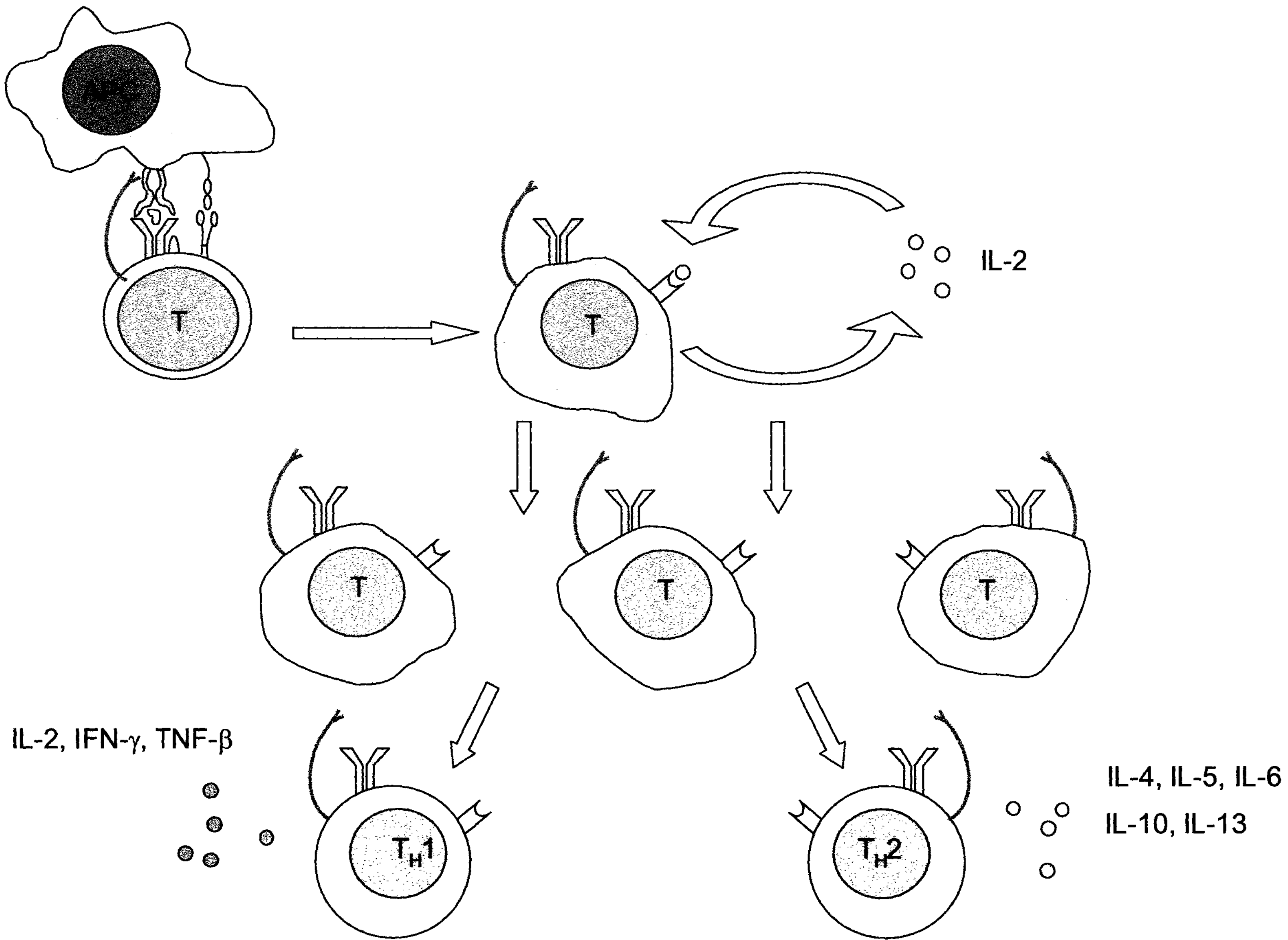
1.7 Activation stimulates T lymphocytes to produce cytokines that promote proliferation and differentiation into effector cell subsets

The process of activation induces T lymphocytes to synthesize and secrete IL-2 which interacts with the IL-2 receptor to promote antigen induced T cell survival and proliferation in an autocrine and paracrine manner. Specifically IL-2, through activation of STAT5, triggers progression through the cell cycle from the G1 to M phases and upregulates the transcription of the IL-2 gene along with the α chain of the IL-2 receptor, CD25[59]. The IL-2 receptor has three chains: α, β, and γ and resting T cells express a form of this receptor composed of β and γ chains which bind IL-2 with intermediate affinity, allowing them to respond only to very high concentrations of IL-2. Association of the α chain with the β and γ chains during activation creates a receptor with a much higher affinity for IL-2, allowing the cell to respond to very low concentrations of IL-2. Although IL-2 is not the only cytokine that can induce T cell proliferation, this feed-forward loop plays the central role in most immune responses[61].

T lymphocytes can be divided into two distinct and non-overlapping populations: a subset which expresses CD4 and mainly induces or 'helps' immune responses in B cells, T cells and macrophages (Th), and a subset which expresses CD8 and is predominantly cytotoxic (Tc). Peptides from intracellular pathogens that replicate in the cytoplasm are

processed by dendritic cells onto MHC class I molecules and presented to CD8 cytotoxic T cells that in turn destroy infected target cells. Peptide antigens from pathogens that replicate in intracellular vesicles or are derived from extracellular bacteria or toxins, are processed on MHC class II molecules and presented to CD4 helper T cells[59]. The helper T cells can differentiate into two types of effector T cell, called T_H1 and T_H2 which differ in the cytokines they produce and thus in their function. The decision on which fate the progeny of a CD4 T cell will follow is made during clonal expansion and is dependant on the type of antigen. Pathogens that accumulate in large numbers inside macrophage and dendritic cell vesicles tend to stimulate the differentiation of T_H1 cells, whereas extracellular antigens tend to stimulate the production of T_H2 cells[59]. T_H1 cells and T_H2 cells release different, but overlapping, sets of cytokines, which define their distinct actions in immunity (Figure 5). T_H1 cells secrete IL-2, IFNγ and TNFβ and mediate a cellular immune response through enhanced phagocytosis by macrophages, induction of B cell synthesis of IgG antibodies that effectively opsonize extracellular pathogens, and activation of cytotoxic T cells. T_H2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 and initiate the humoral immune response by inducing B cells to produce IgM antibodies, essential for the primary antibody response and complement activation[59].

The same cytokines are also regulators for the differentiation of T_H1 and T_H2 phenotypes. IFNγ induces macrophages to synthesize and secrete IL-12 and promotes T cell expression of the IL-12β1 receptor[62]. IL-12 directs the T_H1 response via phosphorylation of STAT4, which is required for IFNγ production, and via the upregulation of several transcription factors including IRF-1[63, 64]. IL-12 has two effects on T_H1 cells - the production of IFNγ, and the internalization of the IFN-γ receptor


APC
T
T
IL-2
T
T
T
IL-2, IFN-γ, TNF-β
TH1
TH2
IL-4, IL-5, IL-6
IL-10, IL-13

β-chain which results in impaired IFNγ induced signalling in T_H1 cells[65, 66]. The net effect is that T_H1 cells secrete abundant IFNγ but they cannot respond to it. IL-4, on the other hand, is the primary cytokine promoting the differentiation of naive T cells into T_H2 cells. The molecular mechanisms by which IL-4 induces expression of the IL-4 gene are not known, although the transcription factors nuclear factor of activated T cells (NFAT) and STAT6 are required for this effect[67]. IL-6 and IL-11 also induce the T_H2 phenotype indirectly through upregulation of IL-4[62]. Importantly, the two T_H subsets can regulate each other and once one subset becomes dominant it is difficult to shift the response to the other subset. This occurs because cytokines from one type of T_H cell inhibit the activation of the other. Thus, IFNγ induces IRF-1 and IRF-2 which bind to the IL-4 promoter and function as transcriptional repressors[68] whereas IL-10 down regulates the T_H1 subset indirectly by suppressing IL-12 production by macrophages[62]. The overall effect is that certain responses are dominated by either humoral (T_H2) or cell-mediated (T_H1) immunity.

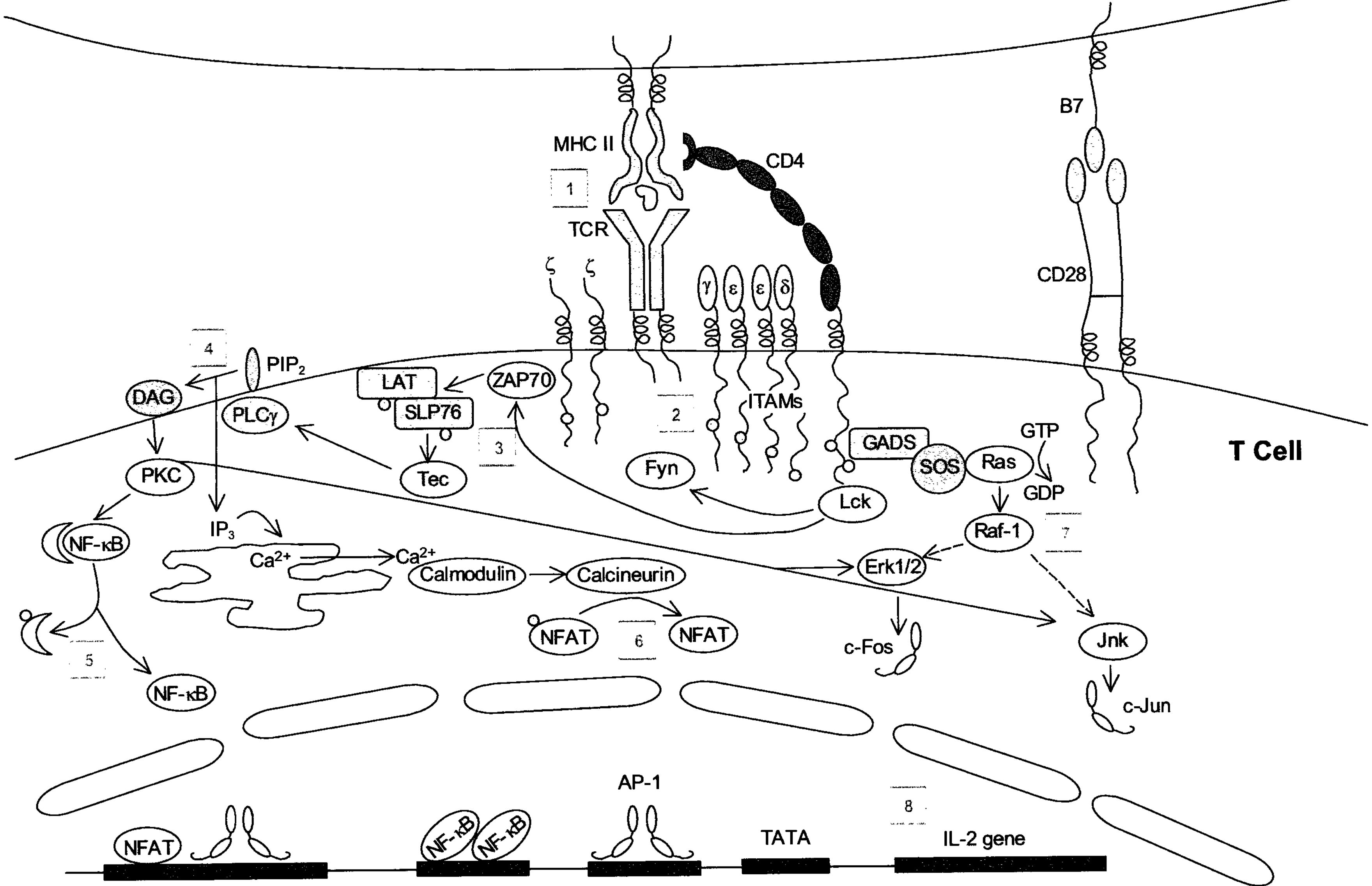
CD8 T cells are also able to regulate the immune response by producing cytokines. Recently it has become clear that effector CD8 T cells can, in addition to their familiar cytolytic function, also respond to antigen by secreting cytokines typical of either T_H1 or T_H2 cells and have been termed T_C1 or T_C2 cells[61]. The main cytokine released by CD8 effector T cells, however, is IFNγ[59].

1.8 T lymphocyte activation upregulates a number of cell signalling cascades

T lymphocytes are activated in vivo when antigen, presented in the context of an MHC molecule by an APC, binds to the T cell receptor (TCR). This binding triggers a complex process of signal transduction propagated through several integrated pathways

that ultimately results in gene expression changes (Figure 6). The TCR has a structure similar to an immunoglobulin in that it is a multiprotein complex made up of a heterodimer of two clonally variable antigen binding chains - α and β chains in 95% of T cells[69]. The TCR itself has a short cytoplasmic domain and therefore the cytoplasmic domains of the accessory complex CD3 or the coreceptors CD4 and CD8 transduce signals. The CD3 complex contains distinct chains - γ, δ, and ε, which have extracellular immunoglobulin domains, and an intracytoplasmic homodimer ζ. These invariant accessory proteins also have a single (γ,δ and ε) or three (ζ) immunoreceptor tyrosine-based activation motifs (ITAMs) composed of two tyrosine residues separated by 9-12 amino acids[69]. When antigen binds, the tyrosines in these ITAMs become phosphorylated by receptor associated tyrosine kinases of the Src family including Fyn (associated with CD3ζ) and Lck (associated with CD4 and CD8 coreceptors). The phosphorylated ITAMs are able to bind and transphosphorylate the tandem SH2 domains of ZAP70, another protein tyrosine kinase. The activation of ZAP70 requires not only binding and phosphorylation by the ITAMs but also phosphorylation by Lck, underscoring the important role of CD4 and CD8 in the signal transduction cascade following antigenic stimulation[59]. ZAP70 and its phosphorylated substrates, LAT (linker of activation in T cells) and SLP76, serve to propagate the signal from the cell membrane into the cytoplasm by binding proteins that contain SH2 and SH3 domains and, in so doing, activating the phospholipase C (PLCγ) and the MAP kinase pathways[69].

PLCγ is phosphorylated and activated by a family of Src-like tyrosine kinases called Tec kinases that contain SH2 and SH3 domains. Activated PLCγ cleaves


a.
Antigen Presenting Cell
T Cell
B7
CD28
MHC II
1
CD4
TCR
ζ
ζ
γ
ε
ε
δ
ITAMs
2
3
4
PIP2
DAG
PLCγ
LAT
SLP76
ZAP70
Tec
Fyn
Lck
GADS
SOS
Ras
GTP
GDP
Raf-1
7
Erk1/2
PKC
NF-κB
IP3
Ca2+
Ca2+
Calmodulin
Calcineurin
NFAT
6
NFAT
c-Fos
Jnk
c-Jun
5
NF-κB
AP-1
8
NFAT
NF-κB
NF-κB
TATA
IL-2 gene

b.

PMA

OKT3

Ionomycin

Figure 6. The cell signalling cascades triggered by T cell activation

When antigen is presented in the context of an MHC molecule and with the appropriate co-stimulatory signals (*box1*), these signals are transduced via the CD3 complex. This results in phosphorylation of the intracytoplasmic ITAMs by Fyn and Lck (*box2*), which in turn transphosphorylate ZAP70 (*box3*). ZAP70 phosphorylates LAT and SLP76 that bind Tec kinase resulting in the activation of PLC (*box4*). PLC cleaves PIP_2 into DAG - resulting in the activation of PKC and nuclear translocation of NFκB (*box5*), and IP_3 – releasing Ca^{2+} from the endoplasmic reticulum and triggering the calmodulin-calcineurin mediated nuclear translocation of NFAT(*box6*). The MAP kinase pathway is activated by phosphorylated LAT, which recruits GADS and SOS, ultimately binding and activating Ras (*box7*). This leads to a series of phosphorylations whereby Ras activates the transcription factors c-Fos and c-Jun. Together these transcription factors result in the upregulation of genes critical for T cell activation, such as IL-2. The overlay demonstrates where *in-vitro* activators (*green*) and inhibitors (*red*) exert their effects. (*orange dot indicates phosphorylation*)

phosphatidylinositol 4,5-bisphosphate to generate the second messengers diacyglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). Once formed these intracellular signalling molecules initiate two essential pathways. In one pathway, IP3 triggers a release of calcium from the endoplasmic reticulum and this intracellular calcium influx activates the calmodulin-dependent phosphatase, calcineurin. Activated calcineurin dephosphorylates the inactive form of the nuclear factor of activated T cells (NFAT), allowing it to translocate to the nucleus where it can act as a transcriptional regulatory protein for a number of genes, including IL-2[69]. In the other pathway, DAG activates the protein kinase C (PKC) which phosphorylates various substrates including IκB, ultimately leading to the release of another transcription factor, NF-κB[59].

The MAP kinase pathway results in the upregulation of c-Jun and c-Fos, which combine as a heterodimer to form the transcription factor AP-1. This pathway is initiated by phosphorylated LAT, which recruits GADS, an adapter protein with an SH2 domain flanked by SH3 domains. This complex binds the guanine-nucleotide exchange factor SOS, which in turn binds to Ras and displaces GDP, allowing GTP to bind in its place thereby activating Ras. The GTP bound form of Ras can then activate Raf, a serine/threonine kinase that acts as the MAP kinase kinase kinase in this pathway. Through a chain of phosphorylation Raf activates the MAP kinase kinases Mek1 and Mek2, which then activate either the MAP kinases Erk1 and Erk2 (extracellular-regulated kinase) or Jnk (c-Jun N-terminal kinase). The Erk MAP kinases are activated when the cascade is initiated through ligation of the TCR and they in turn activate the transcription factor Elk which upregulates the synthesis of c-Fos. The Jnk MAP kinase, that phosphorylates the transcription factor c-Jun, is activated following ligation of the co-

receptor CD28, once again underscoring the importance of co-receptor stimulation for optimal T cell activation[59]. PKC has also been shown to activate the MAP kinases, an example of crosstalk between the cell signalling pathways[59].

Synergism of the above pathways results in the activation of transcription factors necessary for the upregulation of cytokine genes, including IL-2, IL-4 and IFNγ, which are themselves synthesized and secreted to produce a second wave of signalling via cytokine receptors. Activation of each pathway independently may result in the activation of only a subset of cytokine genes and this can be exploited to investigate the relative importance of each pathway for cytokine expression.

1.9 T lymphocytes can be activated in-vitro using various monoclonal antibodies, pharmacological agents, and cytokines

Antigen-induced activation of T lymphocytes is difficult to study because the frequency of antigen-specific responding T cells is less than one in 10^6 cells[61]. Several experimental methods of *in-vitro* T cell activation are used because they permit the study of a larger, more homogeneous population of activated T lymphocytes. Moreover, a number of the experimental activators trigger only a subset of the cell signalling pathways involved in T cell activation, which permits dissection of these pathways for study.

T lymphocyte stimulation with superantigens and monoclonal antibodies are most similar to stimulation with conventional antibodies. Superantigens, such as toxic shock syndrome toxin, are a group of viral and bacterial product that can activate up to 20% of T cells in a population because they interact with the TCR outside of the peptide-MHC binding site and therefore these antigens do not require antigen processing[61].

Monoclonal antibodies (mAb), such as anti-CD3 and anti-CD28 interact with specific cell surface molecules and mimic the agonist or antagonist effects of this binding. OKT3 is a murine anti-CD3 mAb that possesses potent T cell activating and suppressing properties, resulting from its ability to cross link the CD3 receptor (Figure 6, *overlay*) and initiate the activation-induced cell signalling cascades (activation) and subsequently trigger activation-induced cell death (immunosuppression)[70]. The latter effect is the rationale for its use as a therapeutic immunosuppressive for solid organ transplant recipients whereas the former effect results in the side effect known as the "cytokine release syndrome"[71]. Treatment of T cells *in-vitro* results in cell proliferation, the expression of activation markers CD69 and CD25, and the production of cytokines – IL-2, IFNγ, IL10, and TNFα[72]. The end result is skewing towards the TH1 response with >25% of cells producing IFNγ and 1.5% producing IL-4[73].

Pharmacological agents can trigger a subset of the intracellular events involved in T cell activation and have been used as probes to address the importance of each of these events in initiating cellular activation responses. Examples include the calcium ionophores, such as ionomycin (Io), which increase cytoplasmic free calcium leading to the activation of NFAT and the phorbol esters, such as phorbol 12-myristate 13-acetate, which activate PKC, resulting in the nuclear translocation of NFκB and c-Jun[61] (Figure 6, *overlay*). PMA is similar to OKT3 in that it skews the response towards TH1 with only modest production of TH2 cytokines[73], but unlike OKT3, PMA is not mitogenic to isolated T cells and induces neither a proliferative response[74] nor the production of IL-2[75] whereas the combination of PMA and Io does induce a proliferative response with the production of IL-2[75].

Cytokines, such as IL-2 and IL-4 act on the IL-2 subfamily of class I cytokine receptors that share a common γ subunit. As previously described, the high affinity IL-2 receptor is trimeric, composed of α, β, and γ chains. It signals via Jak1 and Jak3, which activate STAT5a/b heterodimers that translocate to the nucleus and act as transcription factors[69]. IL-2 signalling is dependent on the presence of the high affinity IL-2 receptor and therefore *in-vitro* treatment of resting T cells with IL-2 alone is insufficient to mediate the biological effects of IL-2. Resting T cells require pre-treatment with either antigen or IL-1 to respond to IL-2[76]. IL-4 has a single functional form of its receptor - IL-4αγ. IL-4 also signals via Jak1 and Jak3, which activate STAT6[62]. As previously discussed, IL-4 is essential for the differentiation of the T_H2 subset.

Pharmacological inhibitors are also useful tools to study different signalling pathways and cytokines involved in T cell activation. One such agent is FK506, an immunosuppressant currently used post-transplantation with 100 times the potency of cyclosporine[77]. The therapeutic target is the FK-binding protein, to which it binds and forms a complex that inhibits the calcineurin-calmodulin dependent dephosphorylation of NFAT[77] (Figure 6, *overlay*). Interference at this single step results in suppression of T cell proliferation and transcriptional inhibition of the genes encoding IL-2, IL-4, IFNγ and several others[61].

1.10 Activated T lymphocytes have a down-modulated response to Type I interferons

Under normal physiological conditions, Type I IFNs are secreted at low levels in primary lymphoid organs but upon viral infections, they are rapidly produced in the blood[78, 79]. The major source of Type I IFNs are rare plasmacytoid cells, which

themselves respond to IFN by differentiating into specialised dendritic cells known as natural interferon-producing cells (IPC)[80, 81]. Importantly, IFNs exhibit modulatory effects on several elements of the Ag-specific adaptive immune response such as stimulating the cytotoxic activities of NK cells and macrophages[82] and directly contributing to the development of CD4 T lymphocytes along the T_H1-specific lineage[83].

Type I IFNs, as discussed in the first section, are also potent inhibitors of cell growth and proliferation and therefore this presents a biological puzzle – how can a host mount an immune response that involves T cell proliferation in the presence of the inhibitory effect of Type I IFNs? A recent study by Dondi et al.[84] has helped to resolve this paradox. It addressed the regulation of T cell function by Type I IFNs. While the entry of naive resting cells into the replication cycle following stimulation was delayed by IFNα, no effect was observed on activated T cells. Moreover, activated T cells demonstrated a 10-fold reduced inducibility of several ISGs, including MxA, 2'-5' OAS, and IRF7. This appeared to be a late post activation event, beginning 24 hours after and maximal at 72 hours after T cell activation. Despite a documented decrease in the cell surface expression of IFNAR1 during T cell activation, the poor transcriptional response to IFNα or IFNβ did not appear to result from impaired Jak/STAT signalling since the relative amount of phosphorylated STAT 1 and STAT 2 was not remarkably different in activated cells as compared to the naive precursors. However, the residual transcriptional response to IFN-α was demonstrated in this study to be sufficient to inhibit replication of VSV in activated T cells. This group concluded, therefore, that the changes occurring

upon activation appear to alter T cell responsiveness to the antiproliferative but not the antiviral effects of Type I IFNs.

This and other studies have also demonstrated a decrease in activation induced T cell apoptosis and moreover an increase in proliferation associated with Type I IFN treatment of activated T cells. Matikainen et al.[85] suggested IL-2 and IFNα have overlapping activities because treatment of T lymphocytes with either IL-2 or IFN-α, upregulated the IL-2 responsive genes, CD25, c-myc, and pim-1. Similarly, Marrack et al.[86] showed that both IL-2 and IFNα/β were able to promote survival of activated T cells and inhibit apoptosis by a small but significant amount.

Several theories exist to explain this down modulated response to Type 1 IFN in activated T cells. As described in a previous section, activation towards the T_H1 phenotype upregulates the expression of the IL-12β1 receptor. Both IL-12 and IFNAR depend on signalling via Jak proteins and therefore if receptor numbers exceed the amount of available Jak, cross-interference might occur. Indeed it has been shown that, when ectopically expressed in human fibroblasts, the IL-12β1 receptor chain down-modulated IFN-α signalling, demonstrated by a reduction in the transcriptional response to IFN-α as well as reduced STAT and Jak kinase activation[87]. Similarly, the up-regulation of the IL-12β1 in activated T cells might account for their down modulated IFN response. However, the lack of impairment of STAT activation or of ISGF3 formation in activated T cells, as demonstrated by Dondi et al.[84] argues for the involvement of other mechanisms. Another theory implicates the suppressor of cytokine signalling (SOCS) 1 which is known to be upregulated during T cell activation[88] and is a direct inhibitor of IFN-mediated Jak/STAT signalling. SOCS1 exerts its effect by

binding Jaks and inhibiting their tyrosine kinase activities and the subsequent phosphorylation and nuclear translocation of STAT1[89]. Once again the demonstrated lack of impairment of STAT activation argues against this theory.

A third theory is more plausible - IRF-4 (also known as ICSAT, interferon consensus sequence binding protein for activated T cells) and IRF-8 (also known as interferon consensus sequence binding protein) are myeloid and lymphoid specific IRFs. They are only expressed in T cells following activation [90-92] and are capable of exerting repressive effects on several ISGs[90, 93-96].

The expression of IRF-8 is upregulated by IFNγ dependent STAT1 phosphorylation, nuclear translocation, and binding to an ISRE similar to the GAS element[92] - IFNα/β do not induce its expression[97]. IFNγ signalling can only account for part of this induction because activated T cells from $IFN\gamma^{-/-}$ mice still express IRF-8, although to a lesser degree[92]. Under experimental conditions IRF-8 is expressed following stimulation with anti-CD3 antibodies or mitogens and is detected in both T_H1 and T_H2 polarized cells[92]. IRF-4 is also expressed following activation of T lymphocytes with a variety of stimuli, including anti-CD3 antibodies, mitogens, PMA and Io or IL-4[98]. Importantly, IRF-4 deficient mice exhibit an almost complete block in the secretion of cytokines following stimulation with mitogens[91], placing IRF-4 at the center of the T lymphocyte response to activation. The expression of IRF-4 is regulated by NF-κB, NFAT and Sp-1 during activation and can be completely blocked in the presence of FK506[99].

IRF-8 and IRF-4 have DNA-binding domains and IRF association domains and are capable of mediating transcriptional activation and repression depending on their

interactions with other proteins. Binding of either IRF-4 or IRF-8 to the Ets transcription factor PU.1/Spi-1 results in the formation of a complex responsible for the transactivation of a number of genes critical for B cell and myeloid lineage development respectively[98]. PU.1 is not, however expressed in either resting or activated T cells and therefore IRF-4 and IRF-8 do not mediate transcriptional activation by this mechanism in T cells. Both IRF-4 and IRF-8 function as transcriptional repressors when bound to ISRE DNA elements. IRF-8 repressed expression of a chloramphenicol acetyltransferase reporter driven by the ISREs of the MHC class I, 2'-5' OAS, GBP-1, and ISG15 genes in IFN-treated cells[93] and this repression was dependent on the presence of the ISRE in the promoter. The repressive activity of IRF-8 appears to be enhanced by binding to IRF-1 and IRF-2[94] suggesting that IRF-8 may act to block IRF-1 mediated transcriptional activation. Indeed, IRF-8 prevented the IRF-1-mediated induction of reporter genes from MHC class I and IFNβ ISREs in the absence of IFN[95]. IRF-4 has also been shown to bind to ISREs and repress IRF-1 and IFNα mediated transcription from the promoters of 2'-5'OAS and IFNβ in a luciferase reporter system[90]. IRF-4 may also interact with other IRF members – Rosenbauer et al.[96] reported that IRF-4, IRF-8 and IRF-2 may interact to negatively regulate ISG15 expression. The binding of IRF-8 to IRF-4 suggests that these related proteins have similar but non-redundant roles in the cell. The induction of IRF-4 and IRF-8 in response to lymphocyte activation would therefore allow not only upregulation of lymphocyte-specific pathways, but simultaneously protect antigen-specific lymphocytes from the antiproliferative effects of Type I IFNs.

Overall it appears that the ability of activated T cells to respond to Type I IFNs is impaired and this effect is most likely responsible for the selective clonal expansion of T

lymphocytes in response to viral infection. Indeed, in the absence of a down modulated IFN-response the host might have increased susceptibility to viruses, including VSV.

1.11 The difference in susceptibility to VSV between resting and activated T lymphocytes may be attributable to changes in the IFN pathway

This thesis is an attempt to elucidate the molecular mechanisms responsible for the unique resistance of resting T lymphocytes to productive infection with VSV and their transformation to highly susceptible cells during the process of activation. The hypothesis from the outset was that resting T cells were resistant to VSV because they responded to infection with an effective IFN mediated antiviral response whereas activated T cells lost this ability during the process of activation. By examining each step in the viral life cycle of VSV – viral entry and endosome acidification, transcription, translation, and particle assembly and release –comparing resting to activated T cells, we determined that the block to VSV replication in resting T cells is at the level of mRNA. In order to define the components of activation that were essential for rendering T cells permissive to VSV, various *in-vitro* T cell activators, including OKT3, PMA and ionomycin, and inhibitors, such as FK506 and actinomycin D were exploited because of their differing abilities to stimulate or inhibit proliferation and cytokine production. These results indicated that although activators that induced both proliferation and IFNγ production rendered T cells most permissive for VSV infection, neither proliferation nor IFNγ were essential for this process and that viral transcription during activation was an absolute requirement for this process. Fusion of resting and activated T cells was used to establish that the resistant phenotype was dominant over the susceptible phenotype, suggesting that resting T cells are in a constitutively active antiviral state. Finally the IFN responsiveness of resting and

activated T cells was investigated by evaluating the ability of resting and activated T cells to respond to and produce Type I IFNs. Although activated T cells produce IFNα in response to VSV infection they have a down modulated response to its antiviral effect. On the other hand, resting T cells produce no IFN in response to VSV infection. A microarray analysis comparing resting and activated T cells during infection demonstrated that IFN-stimulated genes were down regulated during the process of activation. The mechanisms by which this may be occurring and the implications for cellular and host immunity are discussed.

2. MATERIALS AND METHODS

2.1 T lymphocyte purification and malignant T cell lines

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy donors by density gradient centrifugation with Ficoll Lymphocyte Separation Medium (ICN Biomedicals, Aurora, OH). T cells were isolated by immunomagnetic positive selection with anti-$CD3^+$ conjugated magnetic beads (Miltenyi Biotec, Auburn, CA). The purity of the T-cell population was > 95% positive as verified by flow cytometry with a PE-Cy5 conjugated anti-$CD3^+$ mAb as described below. After purification, T cells were cultured in IMDM (Gibco BRL, Grand Island, NY) supplemented with 10% FBS (Medicorp, Montreal, QC), 10 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL) and α-TG (Gibco BRL). Peripheral leukocytes (including neutrophils) were isolated from heparinized venous blood by incubation for 20 minutes on ice with erythrocyte lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl, 0.1 mM EDTA at pH 8.0), washed with PBS and cultured in the same media as T cells. Activation was achieved by culturing isolated T cells for 36 hours or for the indicated periods of

time with either 10 ηg/ml PMA (Sigma-Aldrich, St. Louis, MO), 1 μg/ml Io (Sigma-Aldrich), 100 U/ml human rIL-2 (Peprotech, Ottawa, ON), 20 ηg/ml human rIL-4 (Peprotech), 50 μg/ml LPS (Sigma-Aldrich) and 100 ηg/ml OKT3 (Muromonab, Ortho Biotech, Toronto,ON). FK506 (Tacrolimus, Fugisawa Healthcare Inc., Deerfield, IL) was used at a final concentration of 1 μM, actinomycin D (Sigma-Aldrich) was used at a final concentration of 5 μg/ml, cyclohexamide was used at a final concentration of 50 μg/ml, and Interferon α (Intron A, Schering, Pointe-Claire, QC) was used at a final concentration of 1000 IU/ml. Jurkat T-cells were cultured in IMDM as above.

2.2 Flow cytometry

For immunostaining, 0.1–0.5 x 10^6 cells were suspended in 100 μl of cold FACS buffer (PBS supplemented with 0.05% BSA (Medicorp) and 0.01% sodium azide (BDH Inc., Toronto, ON) and incubated with fluoresceinated or phycoerythrinated monoclonal antibodies (mAb) for 30 min on ice followed by a single wash with and resuspension in 300 μl of FACS buffer. Conjugated mAbs (Caltag Laboratories, Burlingame, CA) used (and their isotype controls) included: PE-conjugated CD25 (IgG2a), PE-Cy5 conjugated CD69 (IgG2a), CD3 (IgG2a) and PE-Texas Red conjugated CD4 (IgG2a), CD8 (IgG2a). Forward and side scatter and three colors of fluorescence were measured for each sample on a FACScan flow cytometer (BD Biosciences, San Jose, CA). Ten thousand events were analyzed in each sample and the data were analyzed using CellQuest software 3.3 (BD Biosciences).

2.3 VSV infection

A recombinant VSV expressing EGFP was constructed previously in our lab by sub cloning the EGFP ORF from pEGFP-N1 (Clontech, BD Biosciences) into the cloning site

introduced between the G and L genes of VSV in the genome vector pVSV-XN[100]. This recombinant genome was rescued using standard techniques[100] to generate a replication competent, GFP-expressing VSV clone of the Indiana serotype. The naturally occurring interferon inducing mutant (AV-1) has a naturally occurring mutation in the matrix gene (M51R) and a GFP expressing virus was constructed in a similar manner. The VSV-GFP virus was used throughout this study as the wild type virus and the AV1-GFP was used as the interferon-inducing mutant. Both viruses were propagated in Vero cells grown in 150 mm polystyrene plates, filtered with a 0.2 μm pore Nalgene filter (Nalg Nunc, Rochester, NY), concentrated by centrifugation at 14,000 rpm, resuspended in PBS and titred on Vero cells.

Infections of suspension cells were carried out in small volumes of media at cell concentrations >1 x 10^7/ml for 45 minutes at 37°C with occasional mixing, washed twice with 10 ml of PBS and then resuspended into flasks with IMDM at a concentration of 5-10 x 10^6 cells/ml. Unless otherwise specified the moi used in all experiments was 10 PFU/cell.

2.4 Viral plaque assay

The titre of VSV used in the experiments was determined by standard plaque assay on Vero cells with 1% agarose-1× DMEM (Gibco) with 10% FCS overlays. The virus was titred in duplicate in 10 fold increments. Viral production (PFU/cell) was assayed 24 hours after infection of T cells infected at a moi of 10 incubated at a concentration of 5 x 10^6 cells/ml. Supernatants were collected and titred by standard plaque assay as described above.

2.5 Western blot analysis

Each sample of 5 x 10^6 cells was washed with cold PBS and pelleted by centrifugation at 1000 rpm for 10 minutes. Cells were lysed in 60 ul lysis buffer (20mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton-X, and 5% glycerol) supplemented with Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Laval, QC), sodium orthovanadate (Aldrich, Milwaukee, WI), sodium fluoride (Anachemia, Montreal, QC) and sodium pyrophosphate (BDH Inc., Toronto, ON) for 20 min on ice, sonicated at 35% with micro tip (Fisher 300 Sonic Dismembranator, Fisher Scientific, Ottawa, ON) and centrifuged at 14,000 rpm at 4°C for 5 min to remove cellular debris . Protein concentration was quantified using the bicinchoninic acid protein assay (Pierce, Rockford, IL) using albumin as a standard. Twenty micrograms of protein lysate was mixed with 5X SDS sample buffer (60mM Tris HCl, pH 6.8, 2.5% glycerol, 2% SDS, 14.4M β-mercaptoethanol, and 0.1% bromophenol blue) supplemented with DTT for each sample. Proteins were separated by SDS-PAGE (10% acrylamide), transferred to a nitrocellulose membrane (Hybond C+, Amersham Biosciences, Piscataway, NJ) at 400 mA for 1 hour and blocked in 5% skim milk and TBST (10mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween-20) overnight at 4°C. The membrane was incubated with a primary antibody diluted in 5% skim milk in TBST for two hours at room temperature and an HRP-conjugated secondary antibody for 45 min at room temperature. Each incubation was followed by three 7 min washes with TBST to remove unbound antibody. A chemiluminescent substrate (Kirkegaard and Perry Laboratories, Guelph, ON) was applied to the membrane for 1 minute followed by exposure to X-ray film (Kodak Canada Inc., Toronto, ON). The primary antibodies used included: IRF-4 (sc-6059,

Santa Cruz, Santa Cruz, CA) at 1/200, Stat1α p91(sc345, Santa Cruz) at 1/300, Phospho-Stat1 (Tyr 701) (Cell Signalling, Beverly, MA) at 1/1000, VSV (polyclonal directed against the wild type Indiana VSV) at 1/5000, ISG20 (generous gift from Dr. Nadir Mechti, Universite Montpellier, France) at 1/2000, and Actin (sc-8432, Santa Cruz) at 1/5000.

2.6 Northern blot analysis

Total RNA was isolated using the Qiagen RNeasy Mini kit, as per the manufacturer's instructions (Qiagen, Mississauga, Canada). For Northern blotting, 5μg of purified RNA from each sample was heated at 65°C for 30 min to denature and loaded onto 1% agarose-5% formaldehyde denaturing gel, after which electrophoresis was performed at 80 V for 3h. The RNA was transferred to a nitrocellulose membrane (Hybond-N, Amersham Biosciences) overnight by capillary blotting. The blots were prehybridized for 2 hrs at 42°C in 15 ml of hybridization solution containing 50% formamide, 20x SSPE, 50x Denhardt solution, 10% SDS, and 250 μg/ml denatured salmon sperm DNA. The full-length VSV-N cDNA probe was labelled with [α-32P]dCTP using the Megaprime DNA labelling system (Amersham International, Bukinghamshire, England) and added to the Hybe solution for a 1 hour hybridization at 42°C. The membranes were washed three times for 15 min each time at room temperature in 2x SSC (1x SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0)/0.1% SDS, followed by twice for 15 min each time in 0.2x SSC/0.1% SDS at 65°C. The blot was developed for 45 min and quantified by a Phosphorimager:SI (Molecular Dynamics, Amersham) using Image QuanT 5.2 (Molecular Dynamics, Amersham). The sizes of the

mRNAs were determined by reference to 18S and 28S rRNA, which were visualized by ethidium bromide staining.

2.7 Electron Microscopy

For synchronized infection, 10^6 isolated T cells were incubated in 50 μl of IMDM with 10% FCS and VSV-GFP at a moi of 5000 PFU /cell for 20 min at 4°C. This permitted viral binding to the cell surface. The cells were then incubated for 20 minutes at 37°C, followed by a wash in 10 ml of PBS and resuspension in 1.6% glutaraldehyde. All subsequent processing and preparation of the EM slides was performed by Peter Ripstein at the Ottawa Civic Hospital (Department of Pathology).

2.8 Acradine Orange and DAPI staining

Isolated T cells were incubated with 0.6M of acradine orange (Sigma-Aldrich) for 20 minutes at 37°C. The cells were then washed with 10 ml of PBS and resuspended in PBS at a concentration of 10^8 cells/ml. A single drop of cell suspension was placed on a microscope slide, covered with a cover slip and examined fresh using a Zeiss Axioskop2 fluorescence microscope (Carl Zeiss, Inc., NY, NY). Images were captured with a Nikon Coolpix 990 camera (Nikon, Mississauga, ON). To verify the presence of double nuclei in the cells following cell fusion, unstained fused cells were washed three times in cold PBS, fixed in 4% paraformaldehyde for 1 hour at room temperature, washed three times for 10 minute in PBS and stained with 4', 6-diamidino-2-phenylindole (DAPI) (1 μl in 50 ml PBS) for 5 minutes at room temperature. The cells were subsequently washed twice in cold PBS and mounted on microscope slides with coverslips and examined fresh using the Zeiss Axioskop2 fluorescence microscope. Images were captured with a CCD camera (Carl Zeiss Inc.).

2.9 Cell Fusion

Lipophillic membrane dyes, dialkylcarbocyanines -DiI and DiD (Molecular Probes, Eugene, OR) - were used to stain the plasma membrane of resting and activated cells respectively. The cells were incubated at a concentration of 25 x 10^6 cells/ml at a final concentration of 1μg/ml of lipophillic dye for 15 minutes at 37°C. The cells were then washed twice in 10 ml warm PBS and resuspended in IMDM with 10% FCS. Cell membrane staining was verified by flow cytometry – resting cells stained with DiI (blue) had a shifted fluorescence on FL2, activated cells stained with DiD (*pink*) had a shifted fluorescence on FL3. Cellular fusion was performed as follows: Resting and activated cells were mixed in a single 15 ml tube and spun down at 1000 rpm to pellet the cells. The cells were then washed with 10 ml warm PBS and repelleted. PEG 4000 (50% vol/vol in PBS, Sigma-Aldrich) was added to the cells in a drop wise fashion (20 μl). The cells were tapped gently for 1.5 minutes, then "wiggled" at 37°C for 1.5 minutes and then spun down at 1000 rpm. The PEG was diluted with 5 ml of IMDM and the cells were again pelleted at 1000 rpm. The supernatant was removed and the cells were resuspended in IMDM with 10% FCS. Fusion of activated and resting cells was verified by flow cytometry – fused cells had a shifted fluorescence on both FL2 and FL3. The mixed resting and activated cells were treated in the same fashion but PBS instead of PEG 4000 was added to these cells.

2.10 Interferon ELISA Assay

Cell culture supernatants from either resting T cells or T cells activated for 36 hours with PMA and Io and cultured at a density of 5 x 10^6 cells/ml were collected 24 hrs following infection with VSV or AV1-VSV at a moi of 10 PFU/cell. Interferon-α and β

levels were measured using the Human Interferon-Alpha ELISA kit (PBL Biomedical, Piscataway, NJ) and the Human Interferon-β ELISA kit (Fujirebio Inc., Wilmington, DE) respectively, as per manufacturer's directions. Briefly, 100 μl of supernatant was incubated in a 96-well microtiter plate along with blanks and standards supplied by manufacturer. All samples and standards were processed in duplicate as per manufacturer's instructions and then read on a DYNEX plate reader (Revelation 3.04, Chantilly, USA) at 450nm. Sample concentrations of IFN were determined following interpolation of the curve generated by known concentrations of standard recombinant protein.

2.11 Microarray

A total of 4 x 10^8 T-cells, collected and isolated as described above were cultured in the presence or absence of PMA and Io for 36 hours and either mock treated or infected with wild type VSV-GFP at an moi of 10 PFU/cell as described above. Total RNA from 10^8 cells per sample was extracted as described above and precipitated with 95% ethanol in the presence of 0.3 M sodium acetate (Merk KGa, Damstadt, Germany), and 1μl of GlycoBlue (Ambion, Austin, TX). The pellet was washed with 75% ethanol, dried, and resuspended in water to a concentration of 2 μg/μl total RNA. RNA quality was verified using a 2100 Bioanalyser and interpreted with Bio Sizing version A.02.12 software (Agilent Technologies, Mississauga, ON). Twenty micrograms of RNA per sample was processed by The Ottawa Genome Center (Ottawa, ON) according to the manufacturers' standard protocol (Affymetrix, Santa Clara, CA) and hybridized to an Affymetrix HG-U133A chip, representing ~33,000 known human genes.

The fluorescence intensities of all DNA microarray chips were normalized using a scaling factor of 1500 and subsequent gene expression data were analyzed using two independent methods. The first method involved pairwise comparative analyses done directly in the Microarray Suite software 4.0 (Affymetrix) with either the infected T cells as the experimental chip file and uninfected cells as the baseline or the activated T cells as the experimental file and the resting T cells as the baseline. This software provided a difference call of "I" for increased, "D" for decreased, and "NC" for no change based on the average difference change (intensity change) and background noise in the microarray. GeneSpring expression analysis software version 5.01 (Silicon Genetics, Redwood City, CA) was used for the second method. Prior to analysis chips were normalized to the 100 normalization control genes present on each HG-U133 chip. Genes were defined as differentially expressed if the average expression level changed at least 2-fold in one of the treatments compared with that from matched uninfected or unactivated cells and were called "Present" on at least one chip by Microarray Suite.

2.12 Quantitative PCR

One microgram of total RNA was reverse transcribed in a 50 μl reaction containing 10 μl of First Strand Buffer (Gibco BRL), 10mM of each dNTP, 25 ū of RNAse inhibitor activity (Gibco BRL), 5mM DTT, 0.5 μg of oligo-dt primers (Gibco BRL) and 1μl of Superscript II (Gibco BRL). Quantitative PCR was performed using the Roche FastStart kit (Roche Diagnostics, Laval, Canada) in triplicate on each sample for each primer set using Roche Lightcycler technology (Roche Diagnostics). Crossing points were converted to absolute quantities based on standard curves generated for each primer set amplicon by quantifying serial dilutions of known amounts of product. All

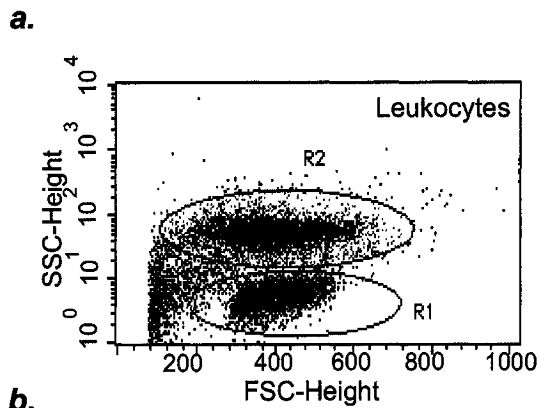
signals were subsequently normalized to β-actin. The primers used to amplify were synthesized by Sigma-Genosys (Cambridgeshire, UK) - β-actin: sense 5'- AAGTA CTCCGTGTGGATCGG-3', antisense 5'- CACCTTCACCGTTCCAGTTT -3', IL-2: sense 5'-ACCTCAACTCCTGCCACAAT-3', antisense 5' GCCTTCTTGGGCAT GTAAAA-3', IRF-4: sense 5'-GTCCTGAGCGAAAACAGGAG-3', antisense 5'-ACCCAAGACTCCCACAGTTG-3'

3. RESULTS

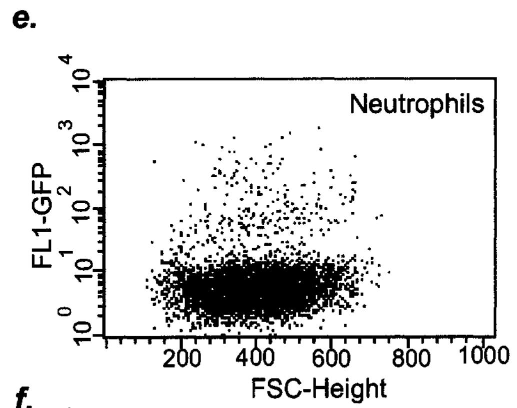
3.1 Peripheral Blood Lymphocytes are Resistant to Infection with VSV whereas Monocytes are Susceptible to VSV Infection

The replication of VSV has been previously shown to be restricted in murine splenocytes and human peripheral lymphocytes when assayed by infectious center and viral plaque assays[48, 57, 101]. To verify this resistance to VSV infection, peripheral leukocytes isolated from human venous blood following erythrocyte lysis were infected with VSV-GFP virus at a moi of 10 PFU/cell. The GFP expressing virus allows simultaneous quantification of the number of cells productively infected with VSV and identification of the infected subpopulation of leukocytes using B cell (CD19), T cell (CD4 and CD8) and monocyte (CD14) specific antibodies (Figure 7b-d and f-h). Neutrophils are easily distinguished based on size as determined by forward and side scatter (Figure 7a R2 and e). Flow cytometric analysis of GFP expression performed at 8, and 24 hours demonstrated restricted viral replication in B and T lymphocytes at both time points (Figure 8a). Monocytes were susceptible to VSV infection early with 17% of cells expressing GFP at 8 hours and 13% at 24 hours (Figure 8a). At 24 hours less than 30% of monocytes were functional to exclude propidium iodide, whereas 100% of the B

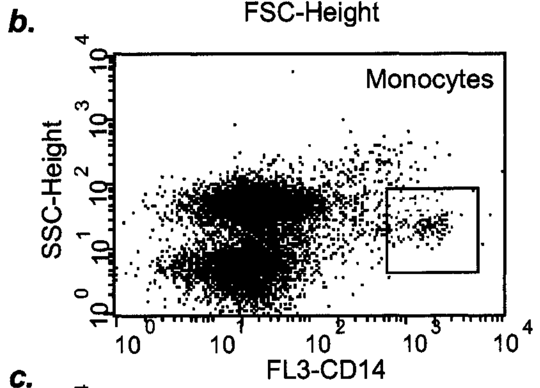
a.



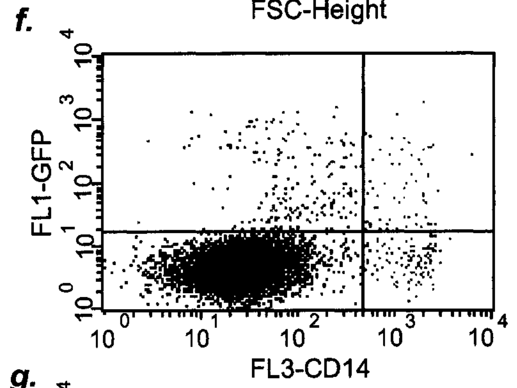
e.



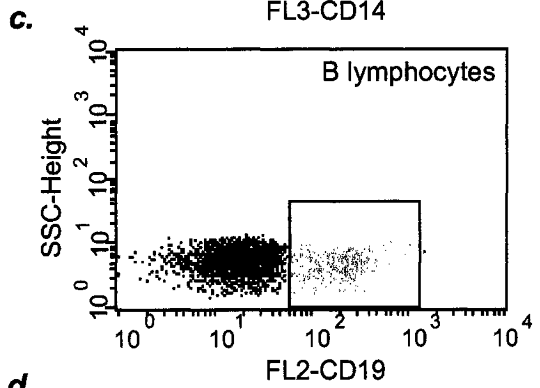
b.



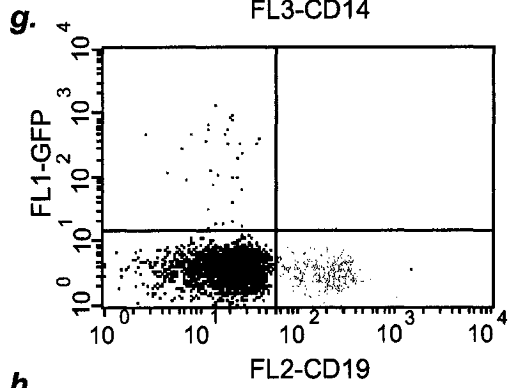
f.



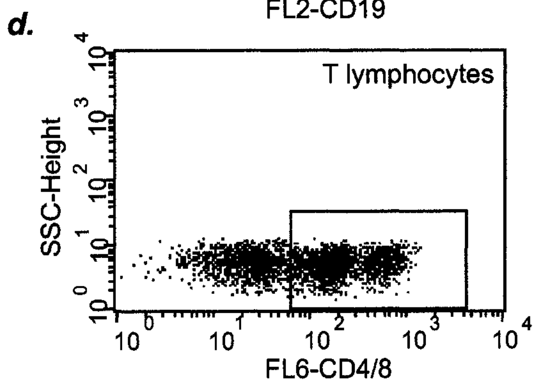
c.



g.



d.



h.

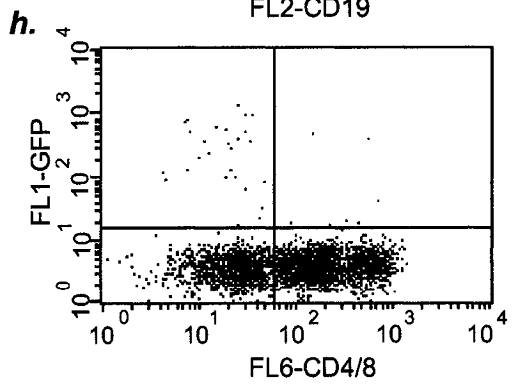
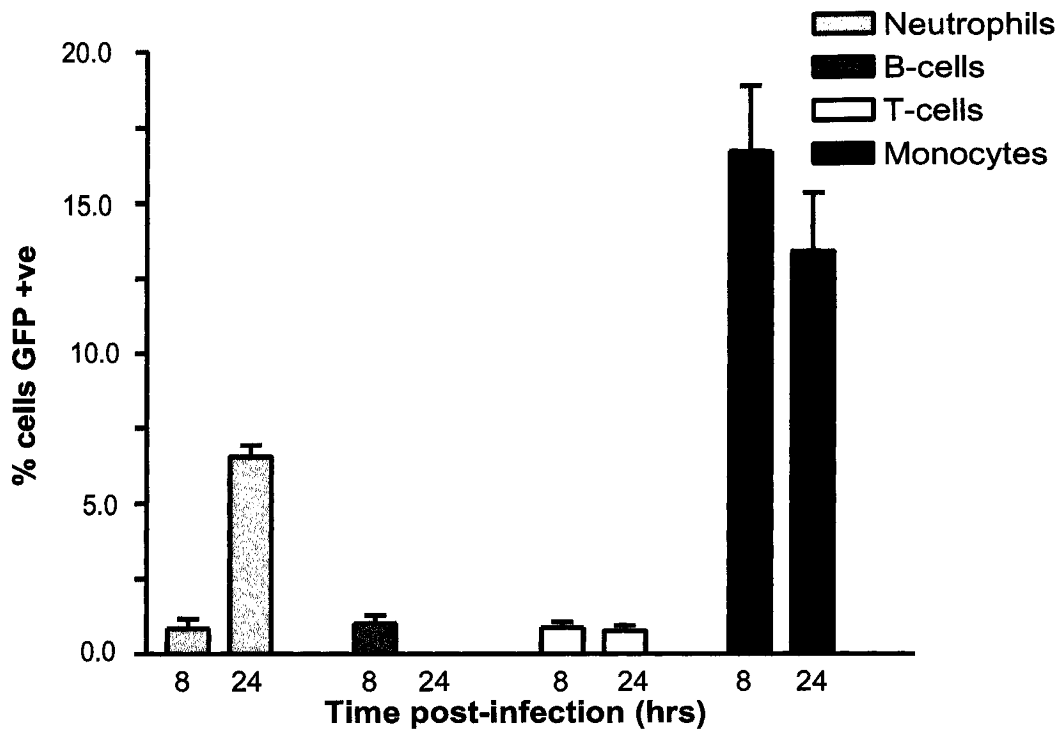


Figure 7. GFP expression in infected PBMC

PBMC were infected with VSV-GFP at a moi of 10 PFU/cell and GFP expression was analysed by flow cytometry at 12 hours post-infection. Cell identity was determined by staining with fluorochrome conjugated mAb as follows: T cells – CD4 and CD8 (d and h), B cells- CD19 (c and g) and Monocytes - CD14 (b and f). Neutrophils were gated based on their larger size and granularity by FSC and SSC (a). These flow cytometry plots demonstrate a representative experiment. Green colour represents GFP expression and VSV infection. Plots were gated as follows: No gate (a), gated on R1 (b-d, g and h), gated on R2 (e).


a.
Neutrophils
B-cells
T-cells
Monocytes
20.0
15.0
10.0
5.0
0.0
% cells GFP +ve
8
24
8
24
8
24
8
24
Time post-infection (hrs)

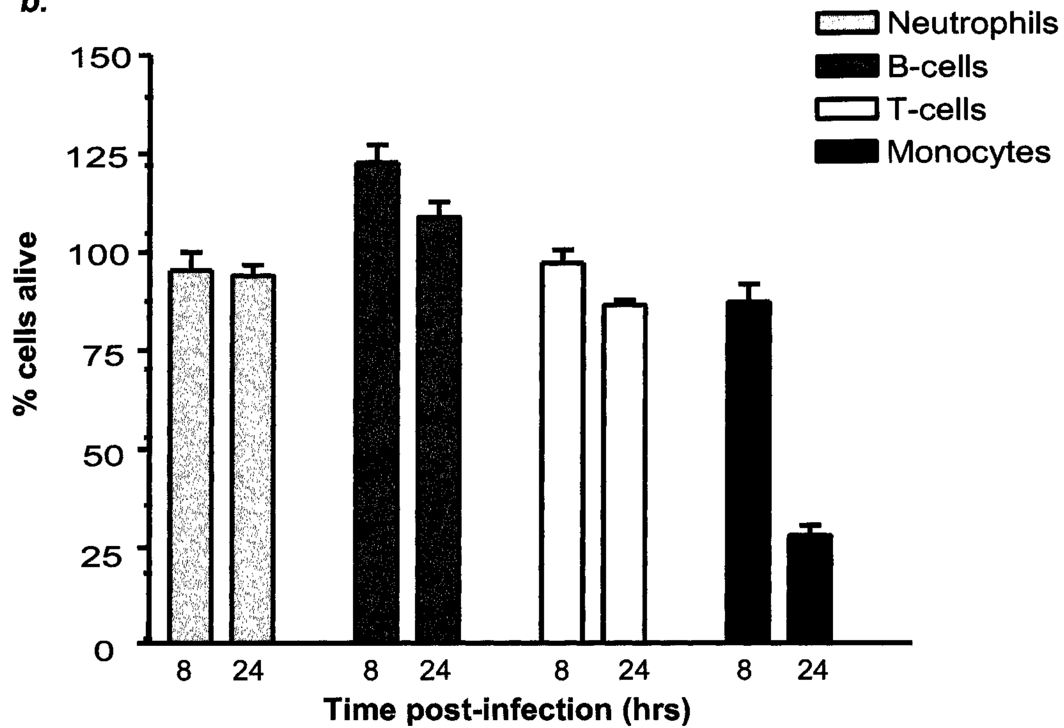

b.
Neutrophils
B-cells
T-cells
Monocytes
150
125
100
75
50
25
0
% cells alive
8
24
8
24
8
24
8
24
Time post-infection (hrs)

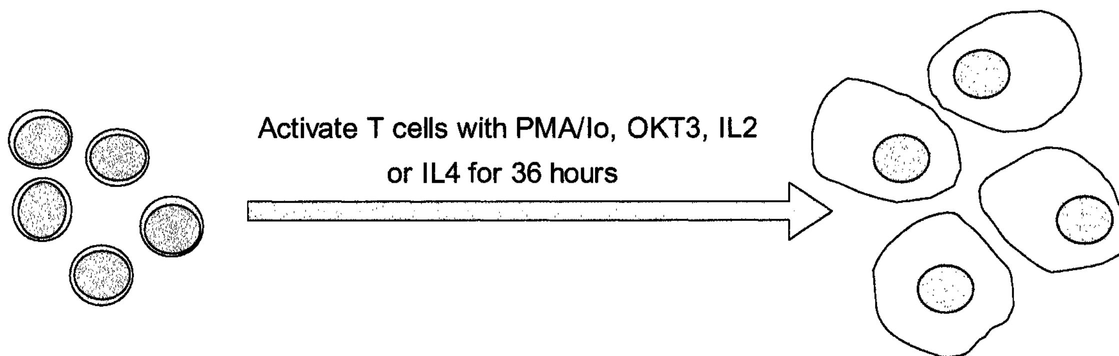
Figure 8. Lymphocytes are resistant and monocytes are susceptible to VSV infection

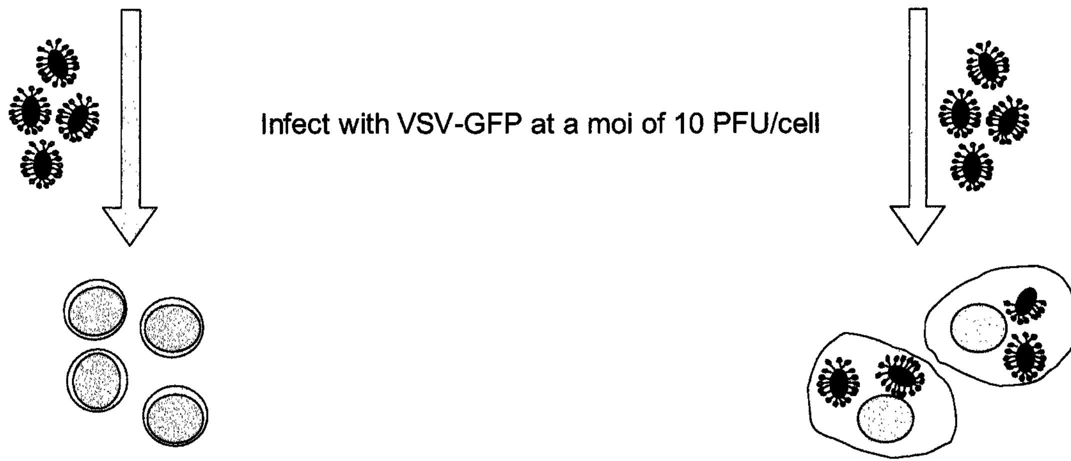
Infected PBMC were analysed for GFP expression and propidium-iodide (PI) staining at 12 and 24 hours post-infection with VSV-GFP at a moi of 10 PFU/cell. The percentage of GFP-expressing cells of each subtype is plotted at each time point (a). The number of cells functional to exclude PI was determined in the infected sample for each cell type and expressed as a percentage of cells excluding PI in the mock infected sample (b). Data are representative of three separate experiments.

and T lymphocytes excluded PI at the same time point (Figure 8b). This suggests that the infected monocytes are dying either by apoptosis or by a cytopathic effect secondary to viral infection. Neutrophils are relatively resistant to VSV infection with only 6% infected at 24 hours (Figure 8a).

3.2 Resting T lymphocytes do not support a productive infection with VSV

The extraordinary resistance to VSV displayed by T lymphocytes was further studied using purified CD3 T cells isolated from PBMC by separation on magnetic beads as described in the methods section. The general experimental design used for this and all subsequent experiments is outline in Figure 9. Isolated T-cells were resistant to VSV infection with less than 10% of cells expressing GFP when infected with VSV-GFP at infective multiplicities of 0.1, 10 and 1000 PFU/cell (Figure 10f and data not shown). Human Immunodeficiency Virus has been previously shown to preferentially infect activated or memory T cells[61]. In order to determine if a distinct population could account for the small but reproducible percentage of infected resting T lymphocytes, isolated CD3 cells were infected at a moi of 10 PFU/cell and at 12 hours post infection were tagged with fluorescent antibodies against CD69 (an early cell surface marker for activation), CD25 (the high affinity IL-2 receptor α), CD45Ro (a marker for memory T lymphocytes), CD 45Ra (a marker for naïve T lymphocytes), CD4 and CD8 (markers for helper and cytotoxic T lymphocytes respectively), and CD3 (pan T cell marker). The infected subpopulation was CD45 Ra expressing naïve T lymphocytes (Figure 10d) and not CD45Ro (Figure 10c), CD69 (Figure 10a) or CD25 (Figure 10b) expressing, suggesting that, unlike HIV, VSV does not infect activated or memory T lymphocytes. Moreover there was an equal proportion of CD4 (Figure 10e, *left*) and CD8 T


Activate T cells with PMA/Io, OKT3, IL2
or IL4 for 36 hours


Infect with VSV-GFP at a moi of 10 PFU/cell

Harvest cells at 12 or 24 hours post-infection

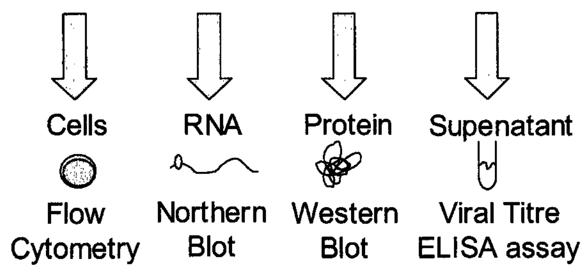

Cells
RNA
Protein
Supenatant
Flow Cytometry
Northern Blot
Western Blot
Viral Titre ELISA assay

Figure 9. Experimental design

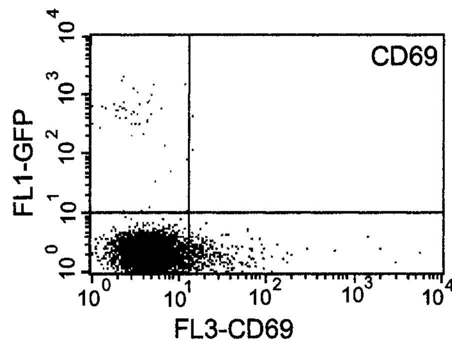
Isolated CD3 T cells were incubated in the presence or absence of activators, including PMA/Io, OKT3, IL-2 or IL-4 for 36 hours followed by infected with VSV-GFP at a moi of 10 PFU/cell. Cells were harvested at 12 hours post infection for flow cytometry, or mRNA and protein were extracted for Northern and Western blotting respectively. Viral supernatants were collected at 24 hours post-infection.

lymphocytes infected (Figure 10e, *right*). This method of detecting a subpopulation of infected resting T cells is limited because infected cells with a high level of GFP expression may loose a number of their cell surface markers. This appears to occur with CD3 where 97% of cells express CD3 in the uninfected population (Figure 10g *left*) but only 95% of cells in the population infected with VSV for 12 hours (Figure 10g *right*). Infected cells that have a high GFP expression and have lost surface expression of CD3 may account for the missing 2% of cells (Figure 10f).

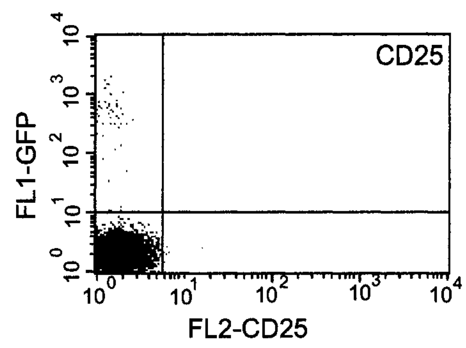
3.3 The block to VSV infection in T lymphocytes can be released by *in-vitro* activation

Pharmacological agents, such as PMA and Io, artificially activate T lymphocytes, resulting in a cascade of events that signal cell proliferation, cell morphology change and the expression of activation-induced genes. As expected, PMA and Io stimulation of T lymphocytes for 24-36 hours resulted in an activated phenotype which included an increase in cell size, as demonstrated by a rightward shift in the forward and side scatter measured by flow cytometry (Figure 11a), and the increased expression of both an early and late cell surface marker for activation, CD69 and CD25 respectively (Figure 11b-c). When infected with VSV at a moi of 10, PMA/Io activated T cells were sensitive to VSV infection with >70% of cells expressing GFP at 12 hours and 95% staining with PI by 48 hours indicating VSV susceptibility in the entire population of cells (Figure 11d-e, representative experiment). Activation of T cells with PMA and Io resulted in less than 15% of cells staining with PI establishing VSV as the cause of cell death in the activated and infected T cells population.

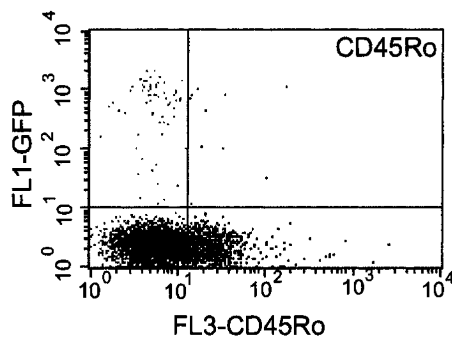
a.



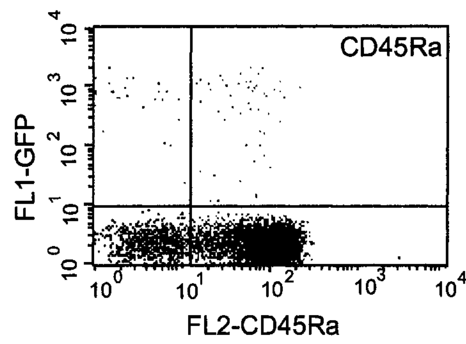
b.



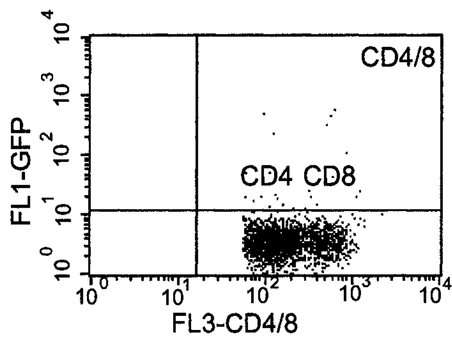
c.



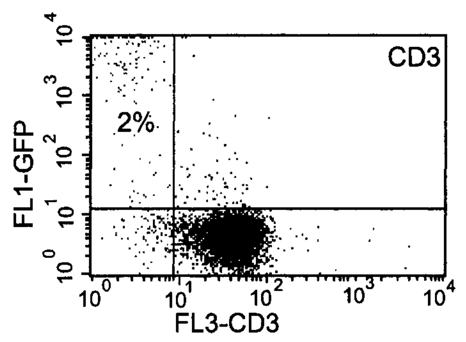
d.



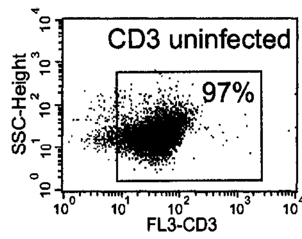
e.



f.



g.



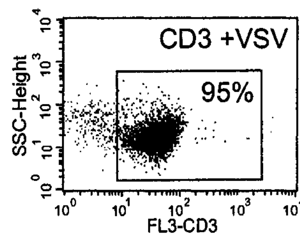
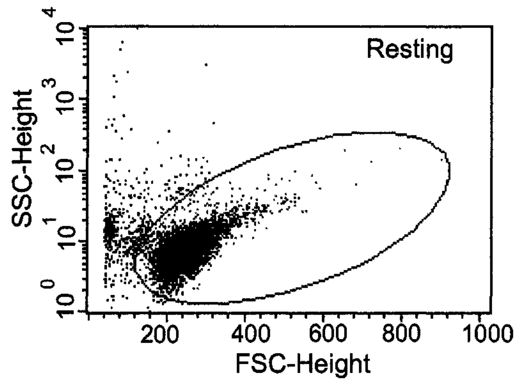
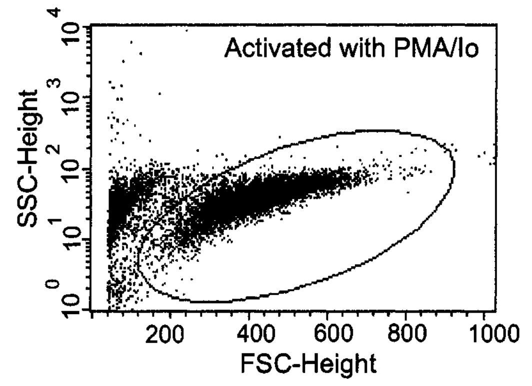


Figure 10. VSV infects a small number of naïve resting T cell

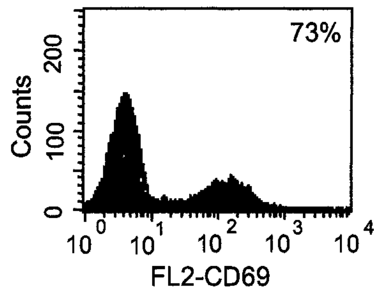
Isolated CD3 T cells were infected with VSV-GFP at a moi of 10 PFU/cell and GFP expression was analysed by flow cytometry at 12 hours post-infection. Cell identity was determined by staining with fluorochrome conjugated mAb as follows: T cells – CD3 (f), activated T cells – CD69 (a) and CD25 (b), memory T cells- CD45Ro (c), naïve T cells – CD45Ra (d), cytotoxic T cells – CD8 (e, *right*) and helper T cells – CD4 (e, *left*). These flow cytometry plots demonstrate a representative experiment.

a.

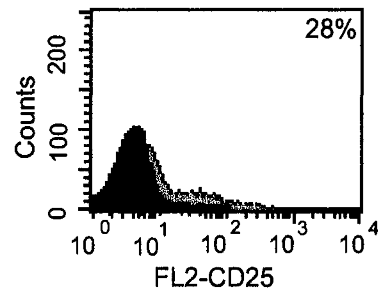




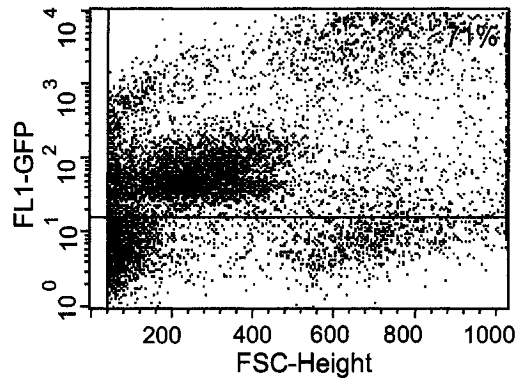
b.



c.



d.



e.

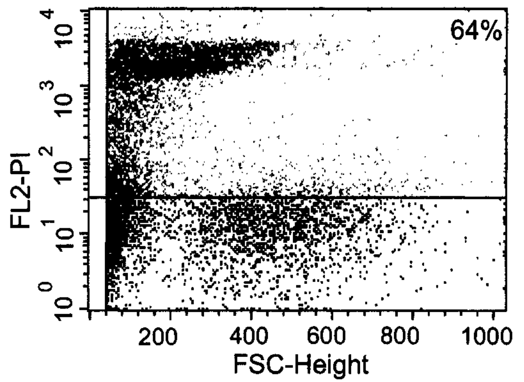


Figure 11. Activated T cells increase cell size, express cell surface markers of activation and are rendered susceptible to VSV infection

Resting and PMA/Io activated T cells were analysed by flow cytometry to assess their size, by forward and side scatter (a) and their expression of the activation induced markers, CD69 (b, *purple*) and CD25 (c, *orange*). Activated T cells were infected with VSV-GFP and a moi of 10 PFU/cell and the expression of GFP (d, *green*) and PI staining (e, *red*) was analysed by flow cytometry. These flow cytometry plots demonstrate a representative experiment.

3.4 Activation by mitogens, calcium ionophores and monoclonal anti-CD3 antibody but not the cytokines IL-2 and IL-4 render T lymphocytes susceptible to VSV

A variety of agents exist that enable the *in-vitro* study of the process of T lymphocyte activation or of specific components that contribute to this process. These include superantigens, monoclonal antibodies against distinct subunits of the TCR or co-receptors, recombinant cytokines, such as interleukins, and pharmacological agents including ionomycin, which increases cytoplasmic free calcium and PMA, which activates PKC. In order to establish which component(s) of the T cell activation signalling pathway were required to render T cells permissive for VSV a number of available agents were evaluated.

T lymphocytes were activated *in-vitro* for 36 hours with PMA, Io, a combination of PMA and Io, anti-CD3 mAb (OKT3), IL-2 or IL-4 and then infected with VSV at an moi of 10 PFU/cell followed by flow cytometry analysisfor the expression of GFP (Figure 12a). Activation with PMA alone, PMA and Io, or OKT3 rendered between 40-75% of T cells permissive for VSV infection whereas 95% of cells treated with IL-2 or IL-4 were still resistant to VSV infection. Activation with either PMA and Io or OKT3 resulted in expression of CD69 in 73% and 71% of cells respectively and expression of CD25 in 28% and 30% respectively. Activation with the interleukins 2 and 4 did not result in expression of CD69 and only induced the expression of CD25 in 6.5% and 7% of cells respectively (data not shown). The relative quantity of viral protein, determined by Western blotting with a polyclonal antibody to VSV, is in agreement with the flow cytometry data (Figure 12b). Lipopolysacharide is a known B cell superantigen and was

a.

% cells GFP +ve

75.0
50.0
25.0
0.0

Resting
IL-2
IL-4
OKT-3
PMA
Io
PMA/Io

***in-vitro* Activation**

b.

uninfected
Resting
IL-2
IL-4
OKT-3
LPS
PMA
Ionomycin
VSV-GFP

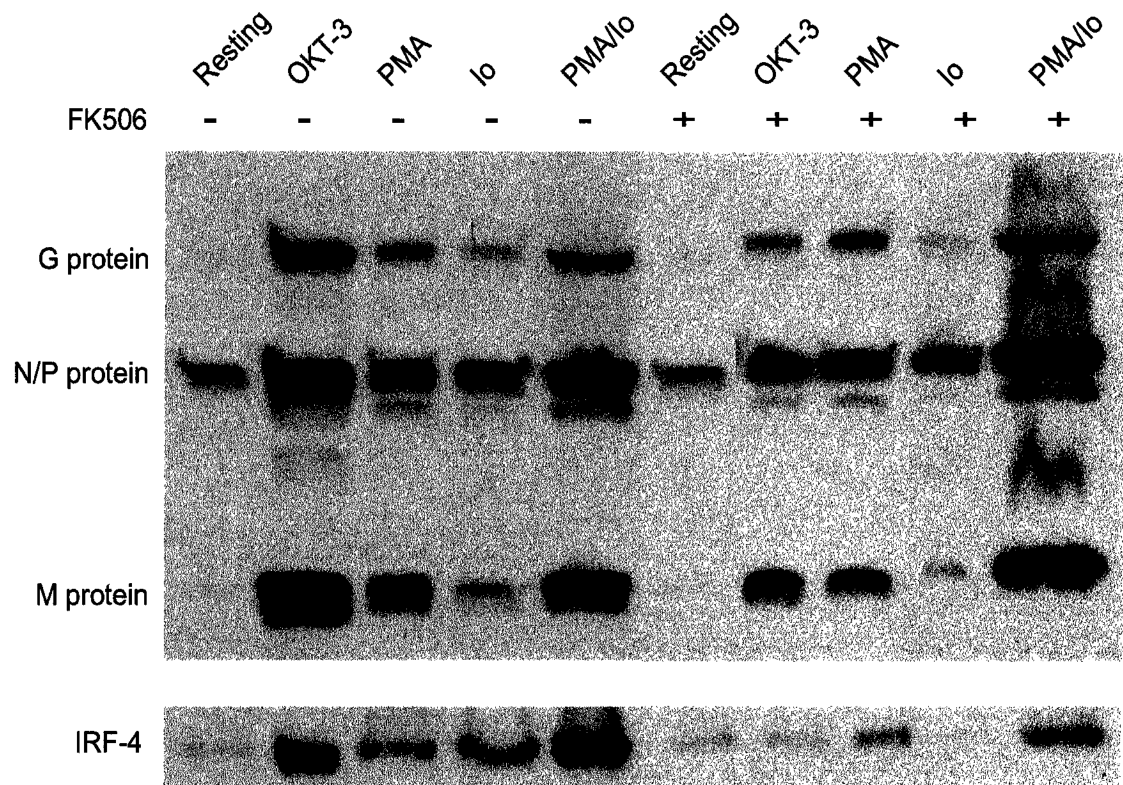
L protein
G protein
N/P protein
M protein
β-actin

Figure 12. A number of *in-vitro* activators render T cells susceptible to VSV

Isolated CD3 T lymphocytes were incubated with PMA, Io, a combination of PMA/Io, OKT3, LPS, IL-2 and IL-4 or mock activated for 36 hours followed by infection with VSV-GFP at a moi of 10 PFU/cell. At 12 hours post-infection cells were analysed for the expression of GFP or the quantity of viral protein in the cell lysate. The percentage of GFP-expressing cells is plotted against the activator used (a). Data are representative of three separate experiments. The quantity of viral protein in 20 μg of cells lysate was detected by immunoblotting with a polyclonal anti-VSV antibody (b). β-actin is used as a loading control (b, *bottom*).

not expected to activate T cells or render them susceptible to VSV infection.

The immunosuppressant FK506 has been shown *in-vitro* to block cellular proliferation and the production of a number of proinflammatory cytokines, including IL-2, IL-4 and IFN-γ through its ability to block the calmodulin dependent dephosphorylation of NFAT. Activation of T cells for 36 hours with a number of activators in the presence and absence of FK506, followed by infection with VSV-GFP at a moi of 10 PFU/cell was performed to determine if this component of the cell signalling cascade and the downstream cytokines were essential for T cell activation-induced susceptibility to VSV. Activation with OKT3 in the presence of FK506 resulted in an approximately 3 fold reduction in the quantity of viral protein produced at 12 hours (Figure 13a), as determined by Western blotting but this effect was not seen when the percentage of infected cells was measured by GFP expression (Figure 13b). By contrast activation with PMA and Io in the presence of FK506 did not result in a significant decrease in viral protein production or the percentage of GFP expressing viraly infected cells (Figure 13 a-b). The immunosuppressant effects of FK506 were verified by demonstrating no change in the forward and side scatter profile of cells activated in the presence of FK506 (data not shown) and by probing cell lysates for IRF-4, a protein whose upregulation during T cell activation is dependent upon the transcription factor NFAT[99] (Figure 13a, *bottom*). The reduction in IRF-4 expression with FK506 was more complete in cells activated with OKT3 as compared to PMA and Io.


a.
infected with VSV at moi=10PFU/cell
Resting
OKT-3
PMA
Io
PMA/Io
Resting
OKT-3
PMA
Io
PMA/Io
FK506
-
-
-
-
-
+
+
+
+
+
G protein
N/P protein
M protein
IRF-4

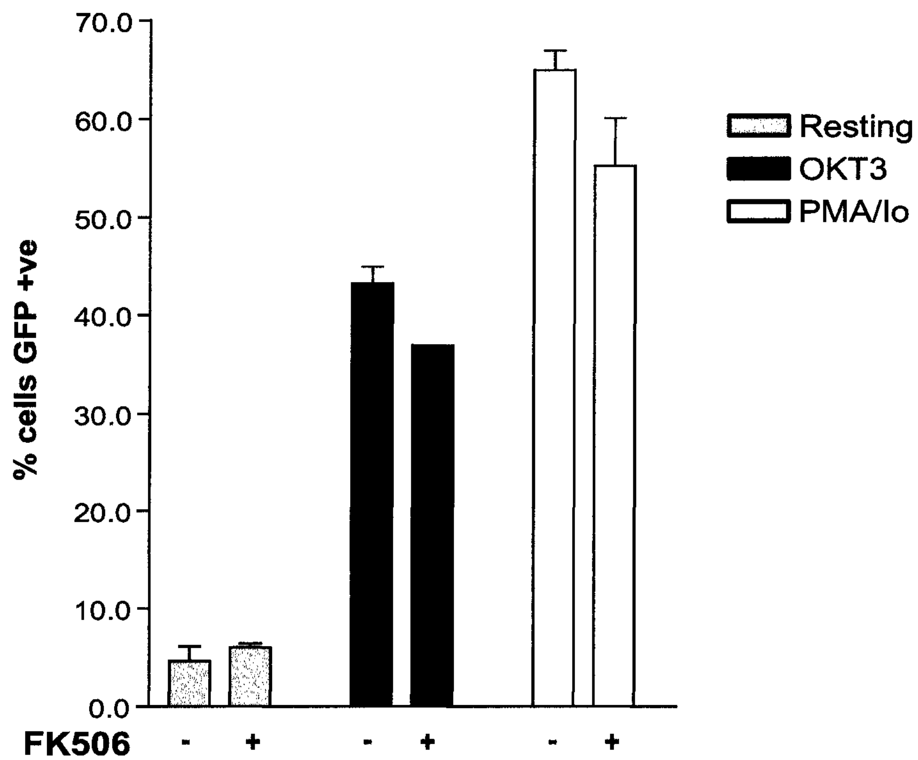
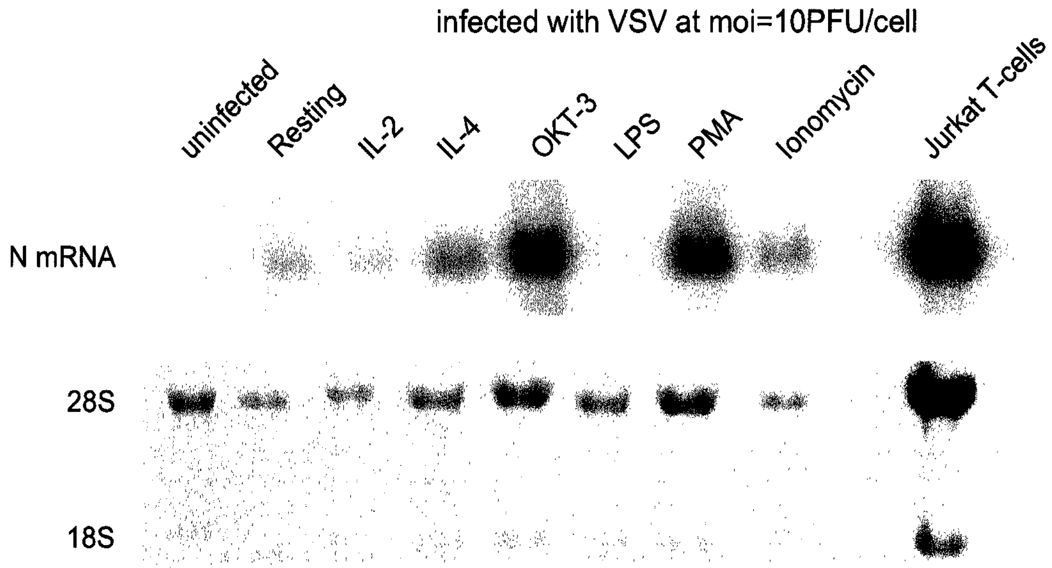

b.
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% cells GFP +ve
Resting
OKT3
PMA/Io
FK506
-
+
-
+
-
+

Figure 13. Activation of T cells in the presence of FK506 does not inhibit activation induced susceptibility to VSV

Isolated CD3 T lymphocytes were incubated with PMA, Io, a combination of PMA/Io, and OKT3 or mock activated for 36 hours in the presence or absence of FK506, followed by infection with VSV-GFP at a moi of 10 PFU/cell. At 12 hours post-infection cells were analysed for the expression of GFP and the quantity of viral protein in the cell lysate. The percentage of GFP-expressing cells is plotted against the activator used (b). Data are representative of two separate experiments. The quantity of viral protein in 20 μg of cells lysate was detected by immunoblotting with a polyclonal anti-VSV antibody (a) and the expression of IRF-4 in 20 μg of the same lysate was detected using a monoclonal antibody against IRF-4 (a, *bottom*).

a.



b.

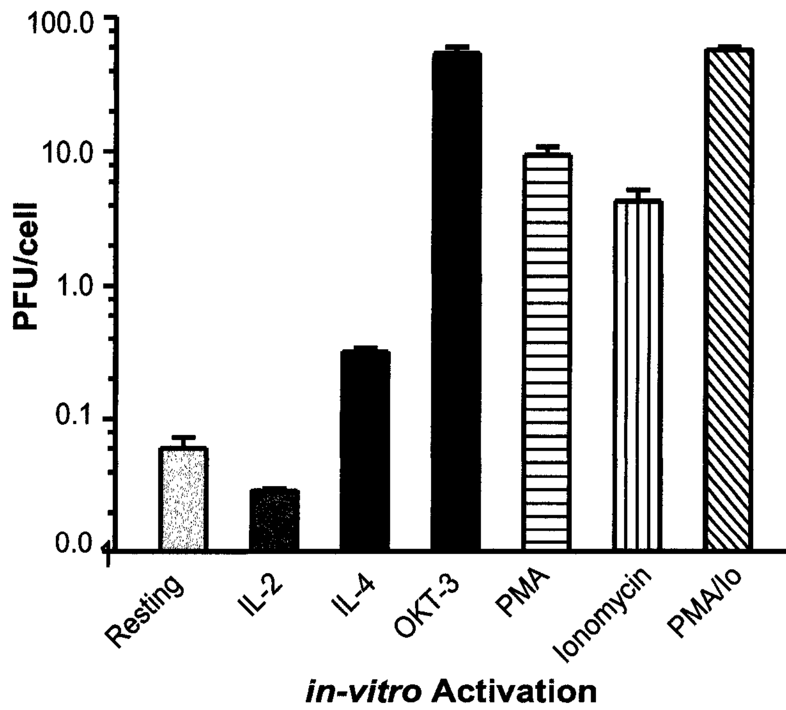


Figure 14. Viral replication is restricted at the level of mRNA in resting T cells

Isolated CD3 T lymphocytes were incubated with PMA, Io, a combination of PMA/Io, OKT3, LPS, IL-2 and IL-4 or mock activated for 36 hours followed by infection with VSV-GFP at a moi of 10 PFU/cell. At 12 hours post-infection cells were analysed for quantity of viral mRNA by Northern blot with a cDNA probe against the mRNA of the VSV N protein (a). The quantity of viral mRNA was compared to the amount of 28S ribosomal RNA determined by ethidium bromide staining (a, *bottom*). At 24 hours post-infection the supernatant was collected and viral titres were determined by plaque assay. The number of PFU per cell in the supernatant is plotted against the activator used (b). Data are representative of two separate experiments.

3.5 The block to viral replication is released at the level of transcription, translation, and viral assembly and release during T lymphocyte activation

In order to determine at what stage(s) in the viral life cycle VSV (Figure 2) was restricted in the resting T-cell, analysis of viral mRNA transcription, viral protein synthesis and infectious particle production was analysed. Viral mRNA for the VSV N protein was detected in total RNA from all infected T cell populations, including resting T cells, at 8 hours post infection (Figure 14a). T cells activated with PMA and OKT3 prior to infection had a more than 20 fold increase in the amount of N mRNA as compared to resting T cells when quantified by densiometry whereas activation with Io and IL-4 resulted in a 2 fold increase, and IL-2 did not result in an increase in the amount of VSV N mRNA over the resting T cell. Viral mRNA quantity was corrected for the amount of 18S ribosomal RNA determined by ethidium bromide staining (Figure 14a, *bottom*).

The ratios of viral protein synthesis at 24 hours post infection closely correlated to the ratios determined for viral mRNA transcription (Figure 12b and 14). Viral protein could be detected in all infected T cell populations with the most abundant expression in T cells activated with PMA or OKT3, followed by T cells activated with Io or IL-4. The least abundant expression of viral protein was found in resting T cells or T cells activated with IL-2 or the B lymphocyte superantigen LPS. Taken together with the ratio of viral mRNA expression and the proportion of GFP expressing cells, this data suggests that the block to viral replication occurs at or before the level of viral RNA transcription, including a block to viral particle entry (adsorption, endocytosis, endosome trafficking and acidification and viral uncoating), inhibition of viral RNA transcription or viral

mRNA degradation. Alternatively, the data could be explained by hypothesizing a block to viral protein synthesis because translation of the RNA polymerase proteins is essential for propagation of viral transcription and exponential amplification of viral protein synthesis.

Infectious viral particle production by activated T cells was determined by viral plaque assay of the supernatant collected at 24 hours post infection from equivalent numbers of resting and activated T-cells. Viral titres also correlated with the flow cytometry, mRNA and protein data but the magnitude of difference between the resting and activated population was greater by an order of 100 for cells activated with OKT3 and PMA/Io (Figure 14b). Whereas activation with OKT3 resulted in a 10-20 fold increase in the number of infected cells over resting cells (measured by flow cytometry and viral mRNA expression), the viral titre was 1000 fold increased. The same difference in magnitude was observed for cells activated with PMA and Io. Activation with PMA alone or IL-4 resulted in a 9 fold increase and Io alone in a 4 fold increase in viral particle production over the resting T cell population. The difference in the magnitude of increase in viral titre compared to RNA and protein synthesis measured in T cells activated with OKT3 and PMA/Io may reflect amplification in viral replication that occurred between 8-12 hours, when the RNA and protein levels were assayed, and 24 hours when the viral titres were measured. Equally, it could represent a second block to viral replication at the level of viral assembly and release.

3.6 Viral entry and endosome acidification are not impaired in resting T lymphocytes

Since the block to viral replication in resting T cells appears to occur at or before the level of viral RNA transcription, we investigated the process of viral entry and endocytosis in resting T cells and T cells activated with a combination of PMA and Io. VSV enters cells by binding to the phosphatidylserine receptor and subsequently triggering receptor-mediated endocytosis. Viral uncoating in the endosome is necessary for release of the negative strand RNA into the cytoplasm and this process depends upon endosomal acidification (Figure 2).

The quantity of viral protein within resting and PMA/Io activated T cells was determined following a one hour infection with VSV at a moi of 10 PFU/cell in the presence of cyclohexamide to prevent new protein synthesis. The cells were washed four times with large volumes of PBS to remove any unbound virus from the supernatant before pelleting and lysing the cells. The amount of viral protein detected within resting and activated cells was similar (Figure 15).

This assay was not able to differentiate between viral particles bound to the cell surface and those internalized and found within endosomes. In order to address this question, viral particle entry was directly observed by electron microscopy following infection of isolated T cells at a moi of 4500 PFU/cell (Figure 16). Viral particles were observed attached to the cell surface, in coated pits and within endosomes in both the resting (Figure 16a and b, *endosome*) and activated (Figure 16c, *endosome* and d, *coated pit*) T cells. Quantification of the number of particles endocytosed per cell was not

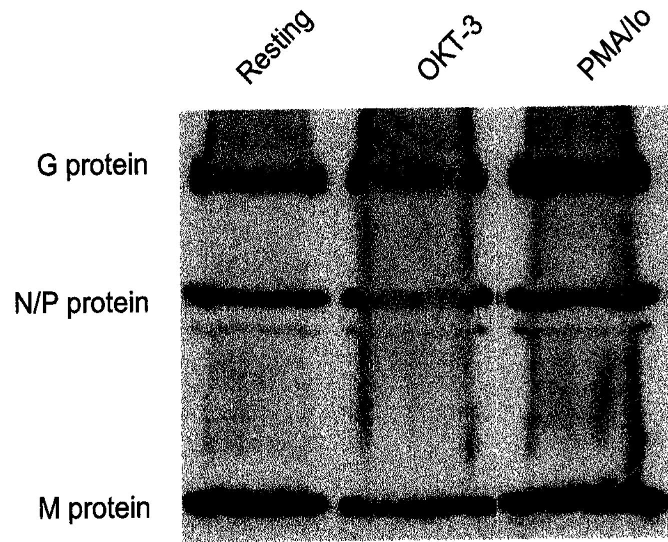
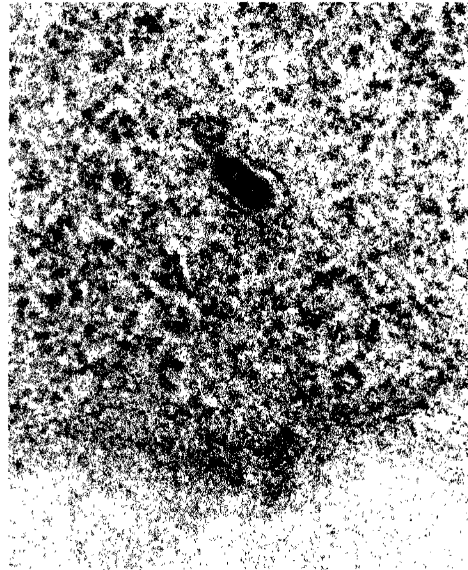

VSV particle entry at 1 hour
Resting
OKT-3
PMA/Io
G protein
N/P protein
M protein

Figure 15. Viral entry is not impaired in resting T lymphocytes – Western blot

Isolated CD3 T lymphocytes were incubated with PMA/Io or mock activated for 36 hours followed by a one hour infection with VSV-GFP at a moi of 10 PFU/cell in the presence of cyclohexamide (50 μg/ml) to prevent new protein synthesis. The quantity of viral protein in 20 μg of cells lysate was detected by immunoblotting with a polyclonal anti-VSV antibody.

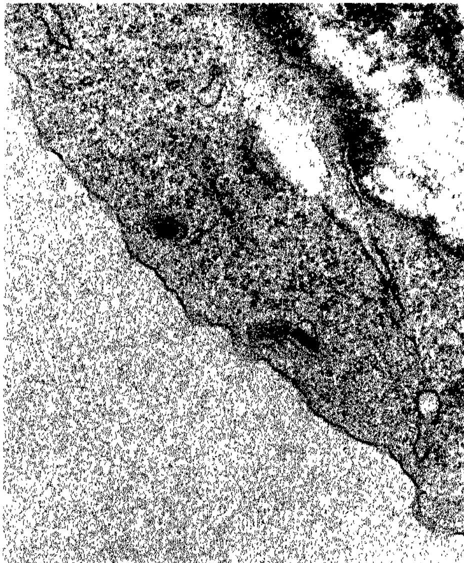
a.

b.



Resting T cell

c.



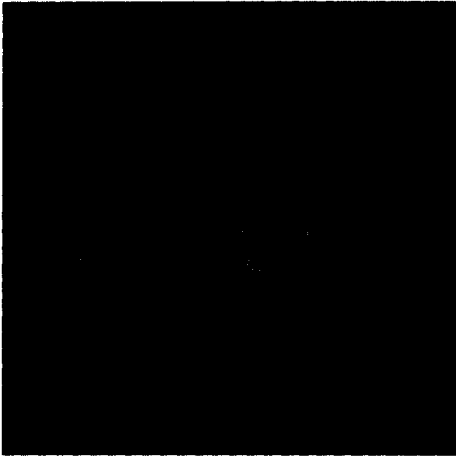
d.

Activated T cell

Figure 16. Viral entry is not impaired in resting T lymphocytes – Electron Microscopy

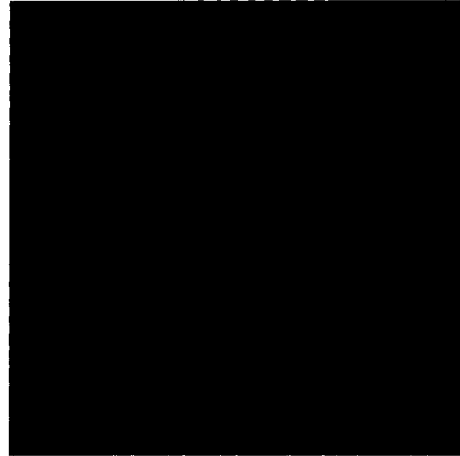
Isolated CD3 T lymphocytes were incubated with PMA/Io or mock activated for 36 hours followed by a 20 minute infection with VSV-GFP at a moi of 4500 PFU/cell. The cells were fixed in glutaraldehyde and examined by electron microscopy for the presence of viral particle within endosomes and coated pits.

a.



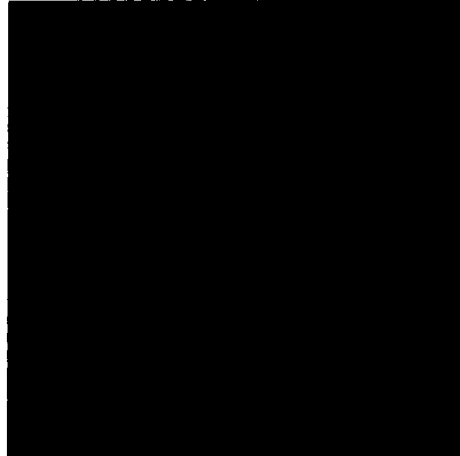
Activated T cell

b.



Resting T cell

c.



Resting T cell (10X)

Figure 17. Endosomal acidification is not impaired in resting T cells

Isolated CD3 T lymphocytes were incubated with PMA/Io or mock activated for 36 hours followed by a one hour infection with VSV-GFP at a moi of 10 PFU/cell. The cells were incubated with acradine orange (0.6M) and viewed by fluorescent microscopy for the presence acidified endosomes within the cytoplasm.

possible because of the low frequency of viral particles within both the activated and resting T cells (between 0 and 2 particles per cell).

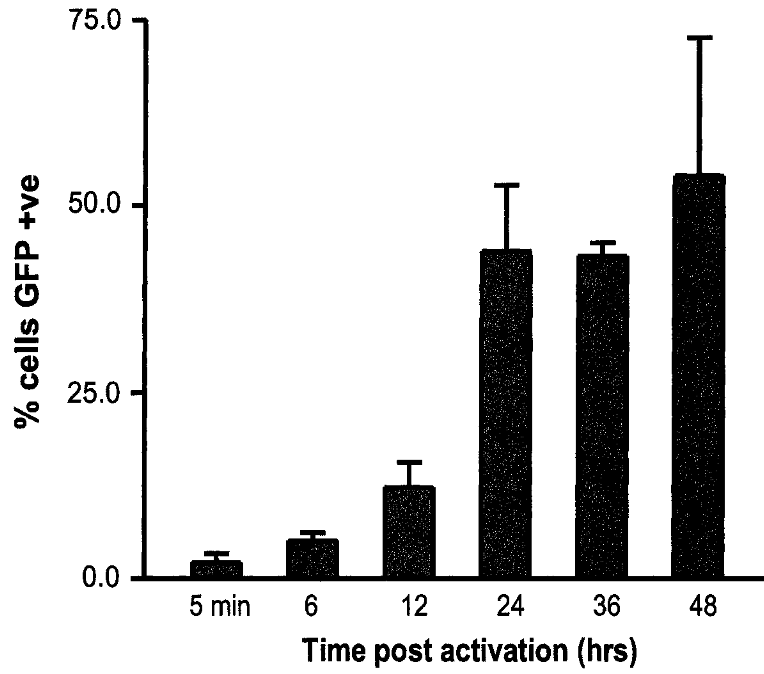
Endosomal acidification and trafficking were evaluated using acridine orange, an agent that fluoresces green at a neutral pH and orange at an acidic pH. Resting T cells and T cells activated with PMA and Io for 36 hours were exposed to acridine orange for 20 minutes prior to infection or 1 hour post infection with VSV at a moi of 10 PFU/cell. As seen in Figure 17, no defects in endosomal trafficking or acidification were demonstrated in the resting T cell (Figure 17a), as compared to activated (Figure 17b and c) T cells, in the presence of VSV.

Although this data is not quantitative and cannot rule out a block to viral entry in resting T cells it does suggest that viral entry and uncoating are not responsible for restricted viral replication in resting T cells.

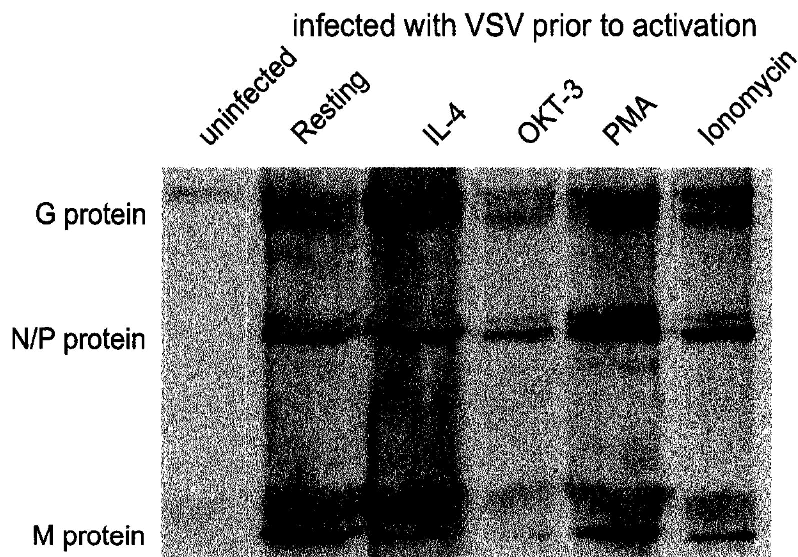
3.7 Activation renders T lymphocytes permissive to VSV infection in a time dependent manner

T cell activation involves several phases of response that include immediate post-translation modifications as part of several signal transductions cascades followed by the transcriptional up regulation of "immediate early activation genes", within minutes to hours, and the subsequent sequential upregulation of a number of cytokine genes[61]. In order to determine which phase(s) of T cell activation mediates susceptibility, T cells were infected with VSV-GFP at various time points following activation and infected cells were quantified by GFP expression using flow cytometry (Figure 18a) at 12 hours post infection. Activation for 5 minutes prior to infection did not induce viral susceptibility suggesting that the immediate post-translational modifications themselves

a.



b.



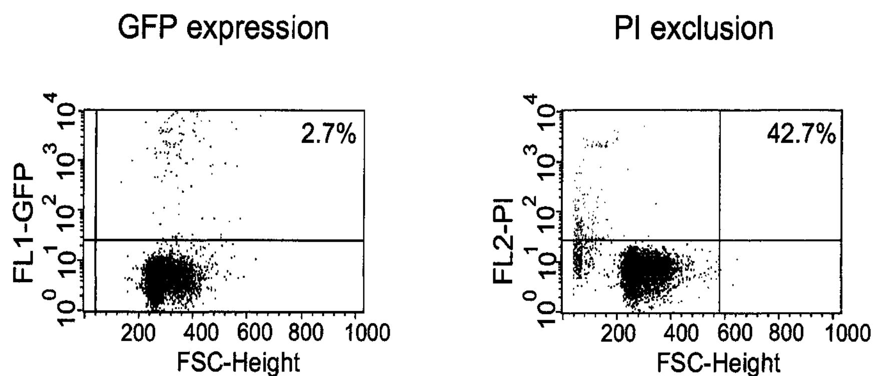
were insufficient to render T cells permissive for VSV infection. Activation for 6 and 12 hours prior to infection resulted in a small but significant increase in the percentage of virally infected cells (5% and 12% respectively) suggesting that up-regulation of the early activation genes is not responsible for activation mediated susceptibility in T lymphocytes. Activation for 24 hours prior to infection increased the number of virally infected cells to 44% and further activation for 36 and 48 hours did not further enhance viral susceptibility. It appears that relatively late events during activation are necessary for the development of activation-induced susceptibility in T cells.

In order to determine if the block to viral replication in resting T cells is reversible or irreversible, resting T cells were pre-infected with VSV at a moi of 10 PFU/cell for one hour followed by activation for 36 hours with IL-4, OKT3, PMA and Io. Following activation for 36 hours there were no T cells expressing GFP by flow cytometry (data not shown) and no increase in viral protein production with any activator as demonstrated by Western blotting (Figure 18b). This suggests that the block to viral replication is an irreversible process, such as one that prevents uptake of VSV or eliminates intracellular VSV particles, as opposed to a reversible a process where VSV replication is statically inhibited at a given step.

3.8 Cellular transcription during activation is an absolute requirement to rendering T cells susceptible to VSV infection

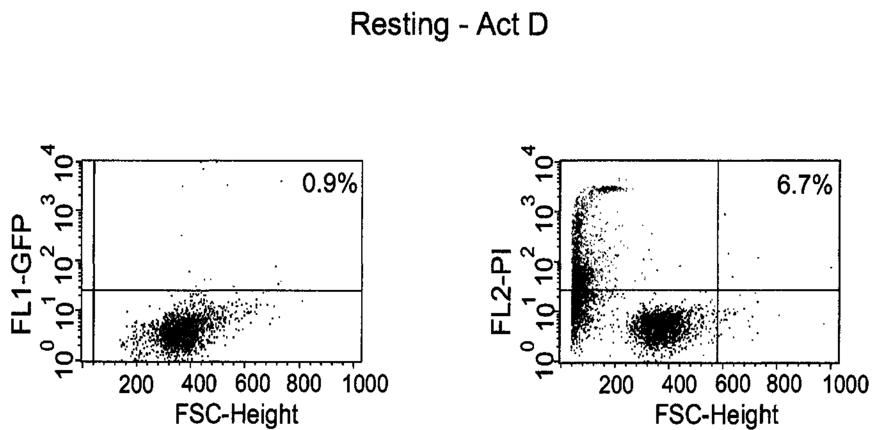
Because T cells were only permissive for VSV replication following 24 hours or more of activation with PMA/Io, we hypothesized that cellular transcription is necessary to render T cells susceptible during *in-vitro* activation. To test this hypothesis, T lymphocytes were infected with VSV-GFP following incubation for 36 hours with and

a.



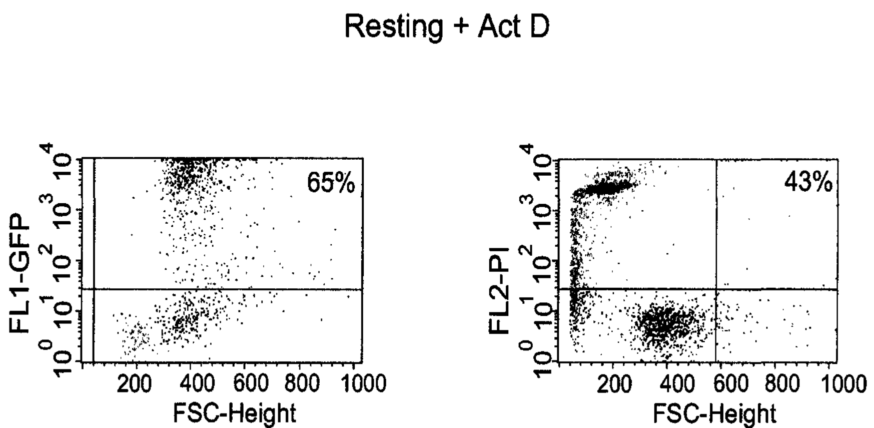
Resting - Act D

b.



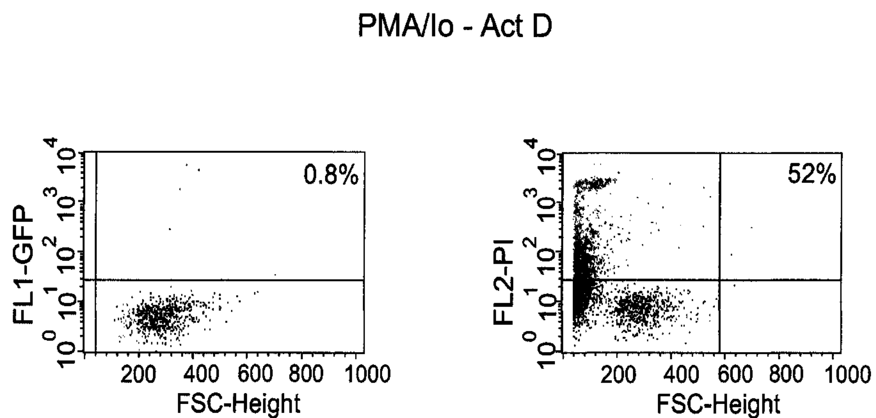
Resting + Act D

c.



PMA/Io - Act D

d.

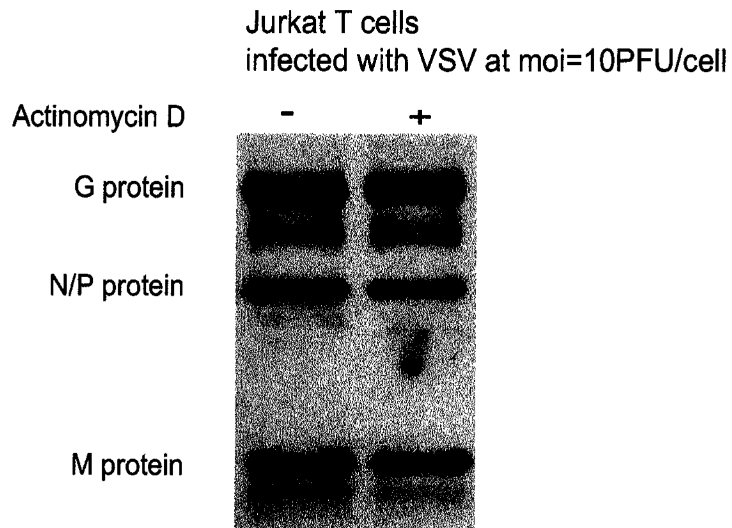


PMA/Io + Act D

Figure 19. Actinomycin D can inhibit activation induced T cells susceptibility to VSV – Flow cytometry

Isolated CD3 T lymphocytes were incubated with PMA/Io or mock activated for 36 hours in the presence or absence of actinomycin D (5 μg/ml), followed by infection with VSV-GFP at a moi of 10 PFU/cell. At 12 hours post-infection cells were analysed for the expression of GFP and staining with PI. The inset number represents the percentage of cells expressing GFP (*left*) or staining with PI (*right*). These flow cytometry plots demonstrate a representative experiment.

a.



b.

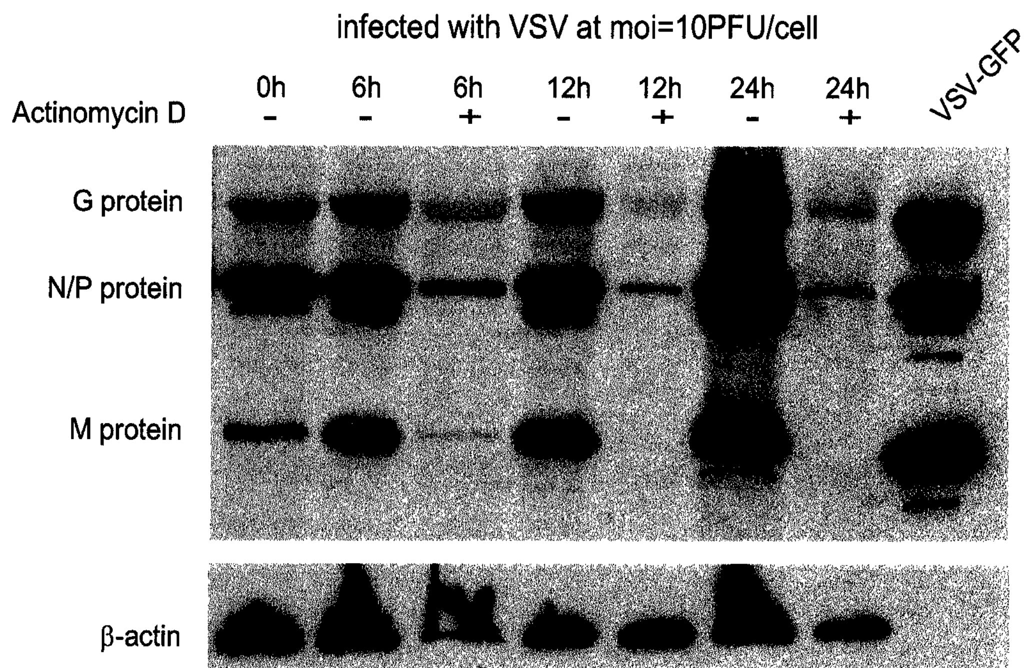


Figure 20. Actinomycin D can inhibit activation induced T cells susceptibility to VSV – Western blot

The same experimental design was used as in Figure 19. At 12 hours post-infection cell lysates were collected and the quantity of viral protein in 20 μg of cell lysate was determined by immunoblotting with a polyclonal anti-VSV antibody (b). β-actin is used as a loading control. In a separate experiment, susceptible Jurkat T cells were infected with VSV-GFP at a moi of 10 PFU/cell in the absence or presence of actinomycin D and cell lysates were collected at 8 hours post infection. The quanity of viral protein detected is equal verifying that actinomycin D does not inhibit viral transcription (a).

without PMA/Io in the presence or absence of actinomycin D. Actinomycin D interferes with DNA-dependent RNA synthesis by forming a stable complex with DNA and blocking the movement of RNA polymerase. It therefore selectively inhibits cellular transcription with no effect on the RNA-dependent VSV polymerase[102, 103]. The specificity of action of actinomycin D was verified by demonstrating no difference in viral translation in a population of susceptible Jurkat T cells in the presence or absence of actinomycin D (Figure 20a).

Flow cytometry at 12 hours post infection for GFP expression and PI exclusion permitted quantification of viral protein production and cell viability respectively (Figure 19 a-d). The number of resting T lymphocytes expressing GFP following VSV infection was similar between cells infected in the presence (Figure 19b) and in the absence (Figure 19a) of actinomycin D (0.9% vs. 2.7%). PI staining, however, was different between these two populations (42.7% vs. 6.7%) reflecting the cellular toxicicity resulting from 36 hours of treatment with actinomycin D. By contrast, T lymphocytes activated with PMA/Io for 36 hours demonstrated a significant difference in GFP expression between cells activated in the absence of actinomycin D (65.5%) and cells activated in the presence of actinomycin D (0.8%), whereas the number of T cells excluding PI was similar (43% vs. 52%). A Western blot from a replicate experiment, probed with a polyclonal anti-VSV antibody, confirmed the results obtained by flow cytometry (Figure 20b). These results establish cellular transcription as necessary for the development of VSV susceptibility in T lymphocyte during the process of activation.

3.9 Fusion of activated T lymphocytes with resting T lymphocytes results in cells that are resistant to VSV infection

The above results indicate that the difference in susceptibility to VSV between resting and activated T lymphocytes is dependent upon the transcription of one or several factors. This could reflect a state whereby resting T cells possess an "inhibitor" to VSV replication that is itself down modulated by this transcribed factor during activation or, alternatively, resting T cells may lack this factor, which is critical for VSV replication. To differentiate between these two possibilities, resting and activated T cells were fused prior to infection with VSV. We hypothesized that if the process of activation results in transcription of a factor that down modulates the antiviral state then resting T cells should be able to compensate for the defect found in activated cells by replacing the "inhibitor" to viral replication and the fused cells should be resistant to infection with VSV. On the other hand if activation results in transcription of a factor essential for viral replication then resting T cells should not be able to inhibit viral replication occurring in activated cells and the fused cells should be permissive for infection with VSV.

Resting T cells and T cells activated with PMA/Io for 36 hours were stained with the lipophillic dyes DiI and DiD respectively. DiD gives the cell membrane a blue colour and is detected as increased fluorescence on FL-3 and DiI a pink colour, which is detected on FL-2. Resting and activated T cells were then fused with PEG 4000 and the fusion was verified by flow cytometry. Fused cells were detected by the cell surface expression of both membrane dyes (Figure 22, *left*, R1) and their larger size was confirmed by multicolour gating (purple cells) on a side scatter (SSC) vs. FL-2 plot (Figure22, *right-purple*). The fused cell population represented between 10-20% of the

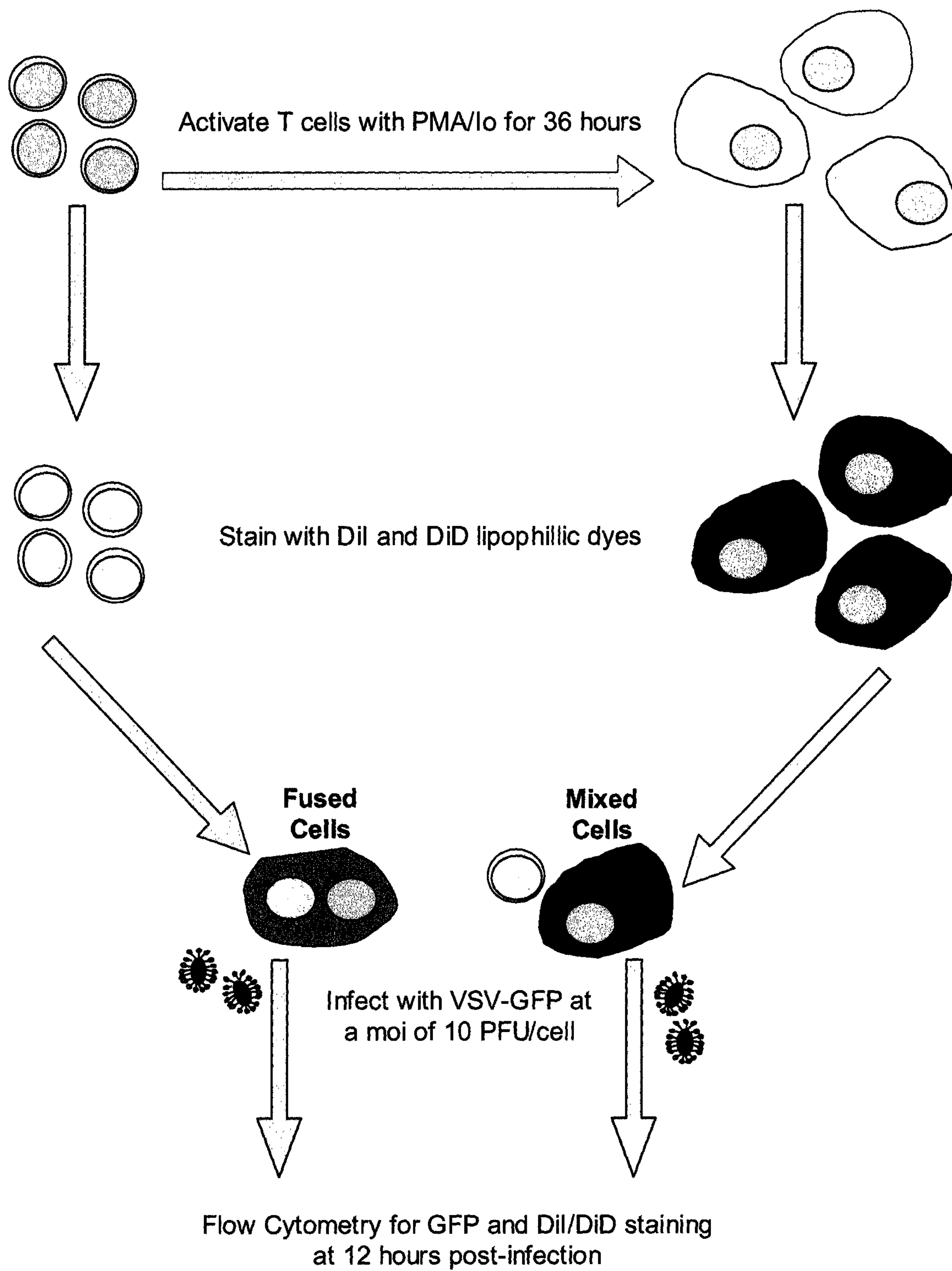
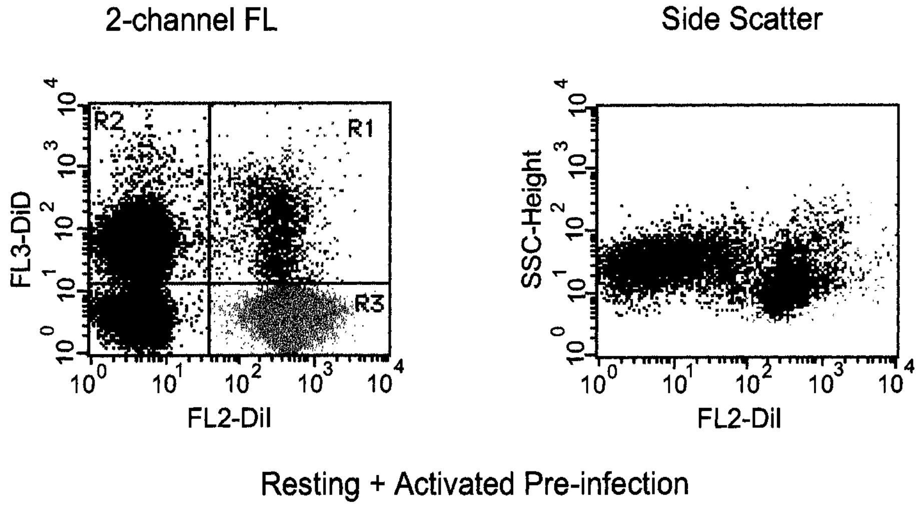

Activate T cells with PMA/Io for 36 hours
Stain with DiI and DiD lipophilic dyes
Fused
Cells
Mixed
Cells
Infect with VSV-GFP at
a moi of 10 PFU/cell
Flow Cytometry for GFP and DiI/DiD staining
at 12 hours post-infection

Figure 21. Experimental design for fusion of resting and activated T cells

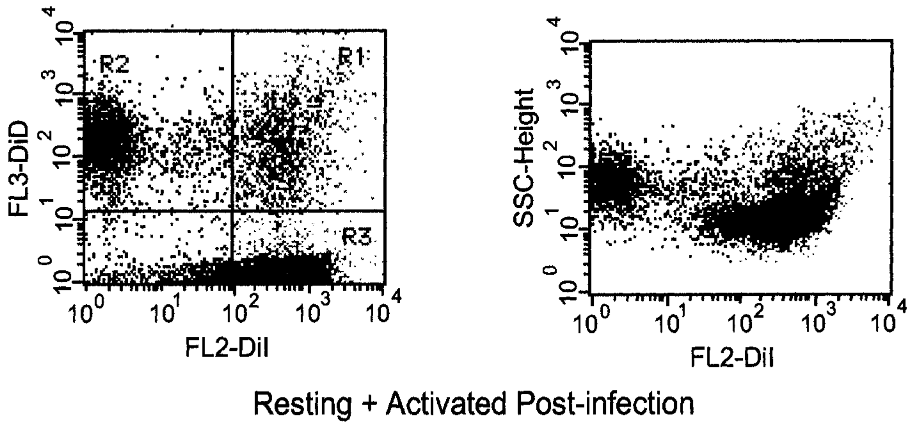
Isolated CD3 T lymphocytes were incubated with PMA/Io or mock activated for 36 hours followed by staining with the lipophillic dyes DiD (*blue*) and DiI (*pink*) respectively. Cells were then fused with PEG 4000 or mixed only and then infected with VSV-GFP at a moi of 10 PFU/cell. Modifications to this experimental design included fusion following infection of activated T cells for one hour and fusion of resting T cells to Jurkat T cells.

a.



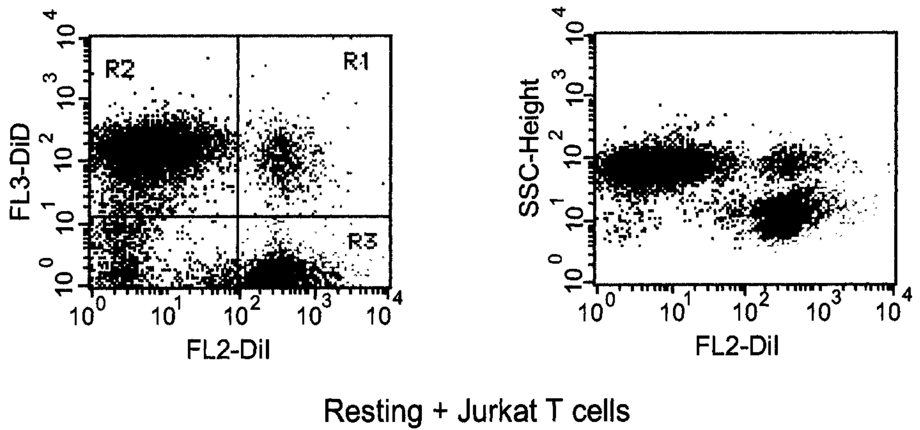
Resting + Activated Pre-infection

b.



Resting + Activated Post-infection

c.

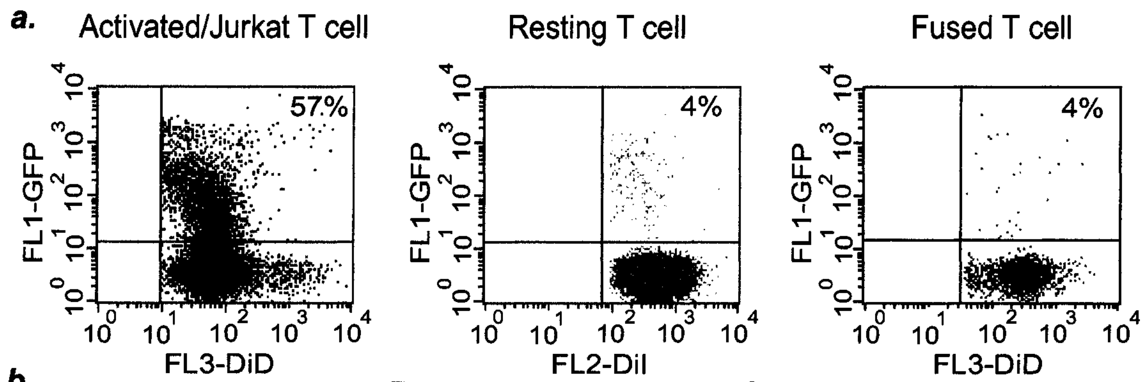


Resting + Jurkat T cells

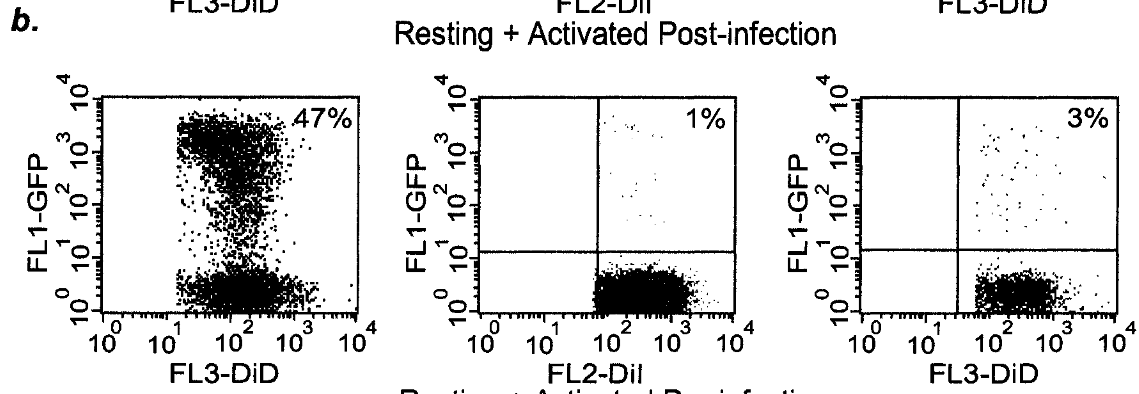
Figure 22. Fusion of resting and activated T cells results in fused cells that are larger and express two membrane dyes

Isolated CD3 T lymphocytes were incubated with PMA/Io or mock activated for 36 hours followed by staining with the lipophillic dyes DiD (*blue*) and DiI (*pink*) respectively. The fused cells (*purple*) posess both the DiD and DiI within their membranes and therefore fluoresce on both FL-3 and FL-2 channels (*left*). They are larger in size as demonstrated by their scatter on SCC-Height (*right*). The flow cytometry plots represent fusion of resting and activated T cells pre-infection (a), post-infection (b) and fusion of resting and Jurkat T cells (c).

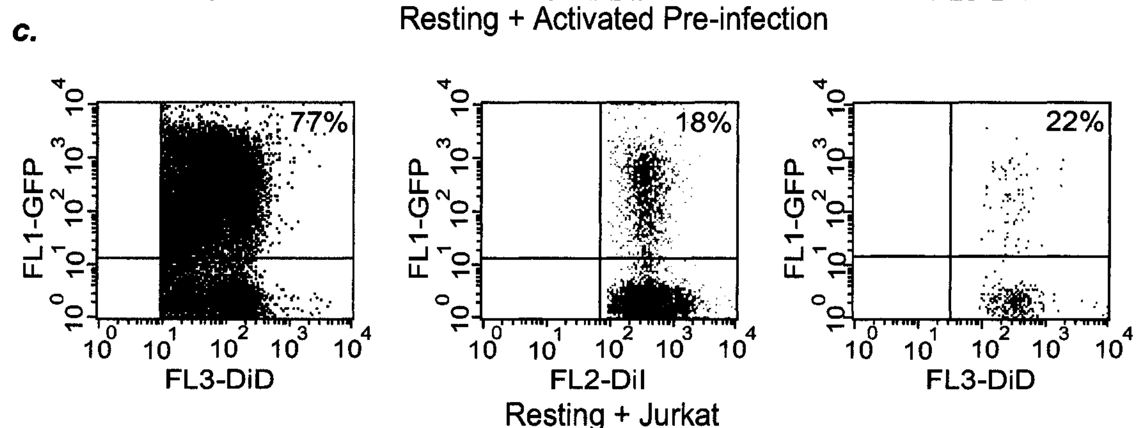
a.



b. Resting + Activated Post-infection

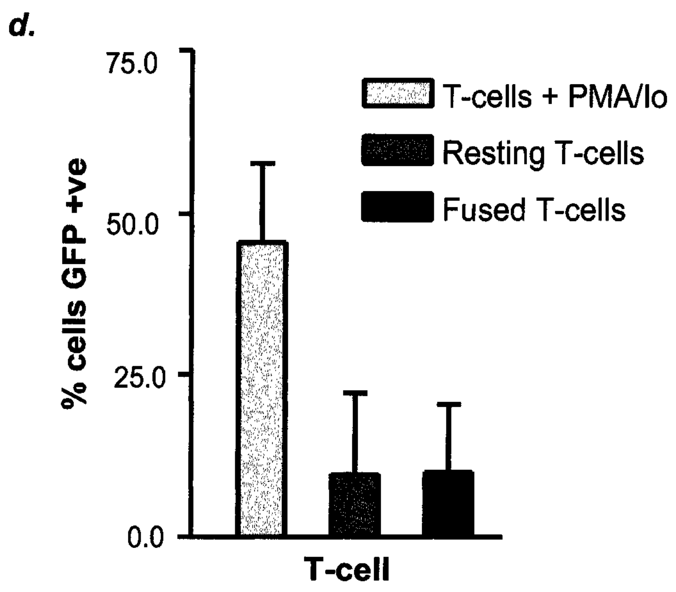


c. Resting + Activated Pre-infection

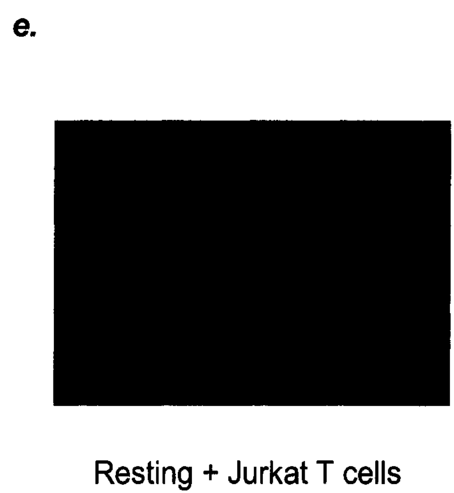


Resting + Jurkat

d.



e.



Resting + Jurkat T cells

Figure 23. Fusion of resting and activated T cells results in a cell resistant to VSV

Fused T cells in culture with non-fused resting and activated T cells were infected with VSV-GFP at a moi of 10 PFU/cell and at 12 hours post-infection cells were evaluated for GFP-expression. The inset denotes the percentage of cells expressing GFP. The flow cytometry plots represent fusion of resting and activated T cells pre-infection (a), post-infection (b) and fusion of resting and Jurkat T cells (c). The percentage of GFP-expressing cells in the resting, activated and fused cells is plotted for three separate experiments (d). The presence of fused cells was verified by using DAPI staining to visualize two cellular nuclei by microscopy (e).

total population of T cells. Fused cells were also verified by using DAPI staining to visualize two cellular nuclei by microscopy (Figure 23e). As a control stained resting and activated cells were mixed in the absence of PEG 4000 (data not shown). The mixed population and fused population were infected at a moi of 10 PFU/cell and flow cytometry was performed at 12 hours post infection. The experimental design is outlined in Figure 21.

As expected, activated T cells (DiD, *blue*) were highly infected with 57% GFP positive (Figure 23a, *left*) whereas resting T cells (DiI, *pink*) were relatively resistant with 4% GFP positive (Figure 23a, *middle*) and these results were similar to the mixed cells (60% and 4% respectively, data not shown). Fused T cells (*purple*) had a phenotype similar to resting T cells with 4% GFP positive (Figure 23a, *right*). The results from replicate experiments are presented in Figure 23d. In order to verify that the process of fusion itself did not render cells resistant to VSV, PMA/Io activated T cells were stained with either DiD or DiI and then fused in an identical manner prior to infection. In this experiment the fused cells had the same proportion of GFP expressing cells, approximately 60%, as the mixed cells (data not shown) confirming that the process of fusion did not itself render cells resistant to VSV.

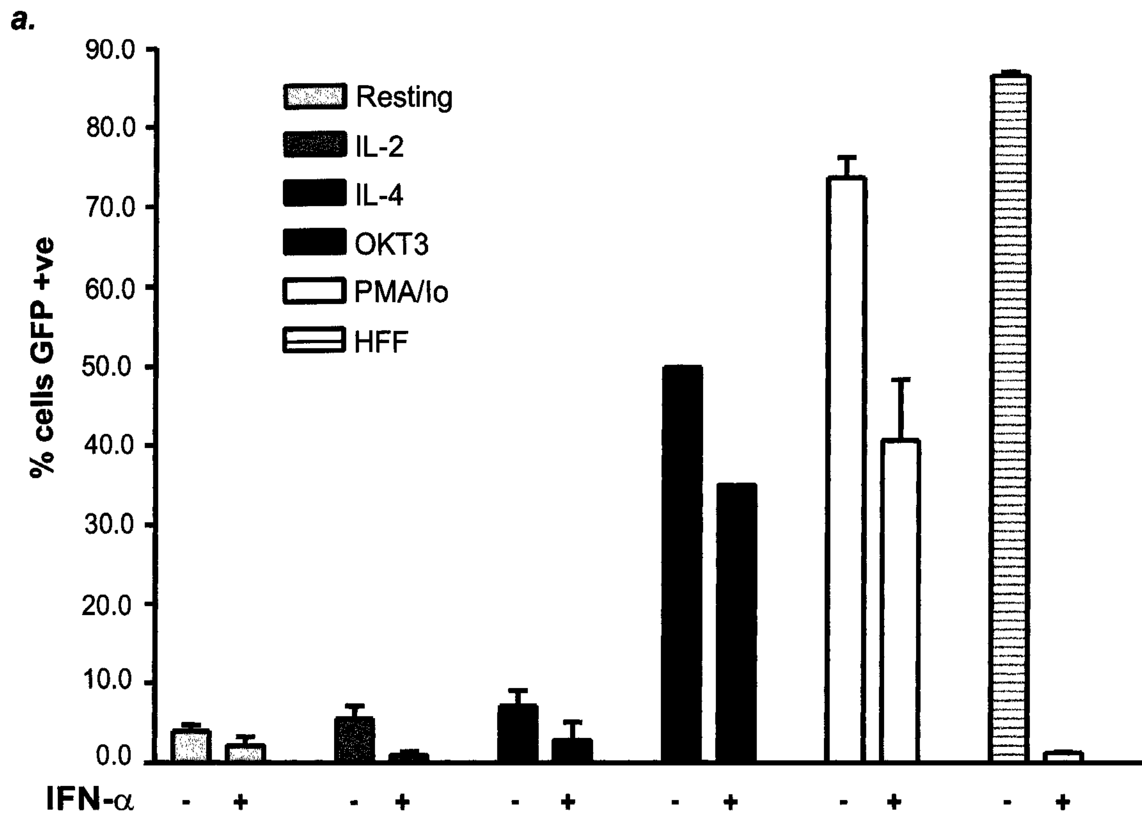
To establish whether the block to viral replication demonstrated in the fused cells occurred at the level of viral entry, the experiment was repeated with the activated T cell population infected for 1 hour at a moi of 10 PFU/cell prior to fusion with uninfected resting T cells. The rationale being that prior incubation with VSV would ensure viral entry in the activated T cells prior to fusion with the resting T cells. The results were similar to those seen for T cells infected post fusion with only 3% of fused cells

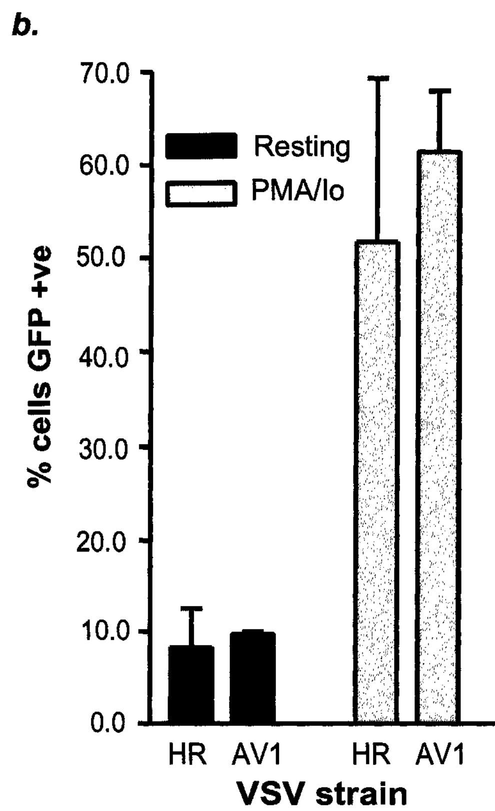
expressing GFP at 12 hours post infection (Figure 23b, *right*). On the basis of these results it appears that viral entry is not the site for restricted viral replication in resting T cells but that this block must occur at a later step in the viral life cycle. Of interest, resting T cells were also able to restrict VSV replication when fused to a susceptible malignant Jurkat T cell line (Figure 23c)

3.10 Activated T lymphocytes have a down modulated antiviral response to IFN

Type I interferons, including IFNα and IFNβ, are powerful antiviral and immunoregulatory cytokines that are rapidly produced during viral infection. In particular, IFNs exert a potent antiproliferative effect on most cells and therefore it seems paradoxical that clonal expansion of effector T cells can occur during an infection in the presence of large amounts of IFN. Recent evidence indicates that type I IFN responsiveness is down modulated during T cell activation and that activated T cells are resistant to the antiproliferative effects of type I IFNs[84]. This would permit the growth of activated T cells in the presence of this cytokine. This study was not, however, able to demonstrate a reduced sensitivity to the antiviral effect of IFNα following infection with VSV[84].

We evaluated the antiviral effect of IFNα in resting and activated T cells by pre-treating these cells, during the final 12 hours of activation, with 1000 IU/ml of recombinant human IFNα. The cells were subsequently infected with VSV-GFP and flow cytometry was performed at 12 hours (Figure 24a). In the absence of IFNα, the percentage of infected T cells was 50% when activated with OKT3 and 73.5% when activated with PMA/Io. These percentages decreased to 35% and 40.7% respectively with IFN-α pre-treatment. By comparison, 86.5% of normal human foreskin fibroblasts were


a.
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% cells GFP +ve
Resting
IL-2
IL-4
OKT3
PMA/Io
HFF
IFN-α
- + - + - + - + - + - +


b.
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% cells GFP +ve
Resting
PMA/Io
HR AV1 HR AV1
VSV strain

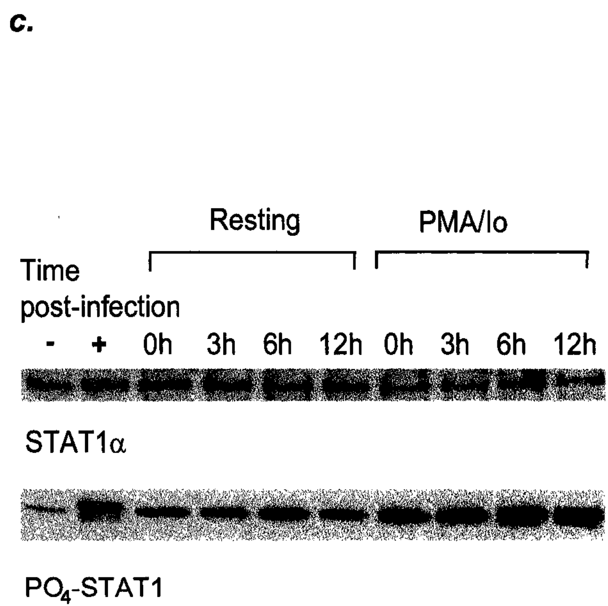

c.
Resting
PMA/Io
Time
post-infection
- + 0h 3h 6h 12h 0h 3h 6h 12h
STAT1α
PO4-STAT1

Figure 24. Activated T cells are only partially protected by IFNα

Isolated CD3 T lymphocytes were incubated with PMA/Io or mock activated for 36 hours. During the last 12 hours cells were also incubated with or without IFNα (1000 IU/ml). The cells were then infected with VSV-GFP at a moi of 10 PFU/cell. At 12 hours post-infection cells were analysed for the expression of GFP and the percentage of GFP-expressing cells is plotted against the activator and the treatment given (a). In a separate experiment, isolated CD3 T lymphocytes, incubated with PMA/Io or mock activated for 36 hours, were infected with either VSV-GFP or the IFN-inducing mutant AV1-VSV-GFP at a moi of 10 PFU/cell. The percentage of GFP-expressing resting and activated cells at 12 hours post-infection is plotted against the virus used (b). Data are representative of two separate experiments. In a third experiment, isolated CD3 T lymphocytes, incubated with PMA/Io or mock activated for 36 hours, were infected with VSV-GFP at a moi of 10 PFU/cell and cell lysates were collected at various time intervals just prior to and following infection. The quantity of STAT1α protein and phosphorylated-STAT1 were determined by immunoblotting (c) 20 μg of protein lysate with a non-phospho-specific (*top*) and phospho-specific (*bottom*) STAT1 antibody respectively.

infected in the absence of IFNα but only 1.3% were infected following a 12 hour pre-treatment with IFNα. These results indicate that activated T cells have a down modulated response to the antiviral effects of type I IFNs, because IFNα only partially protected activated T lymphocytes from VSV when compared to normal human foreskin fibroblasts.

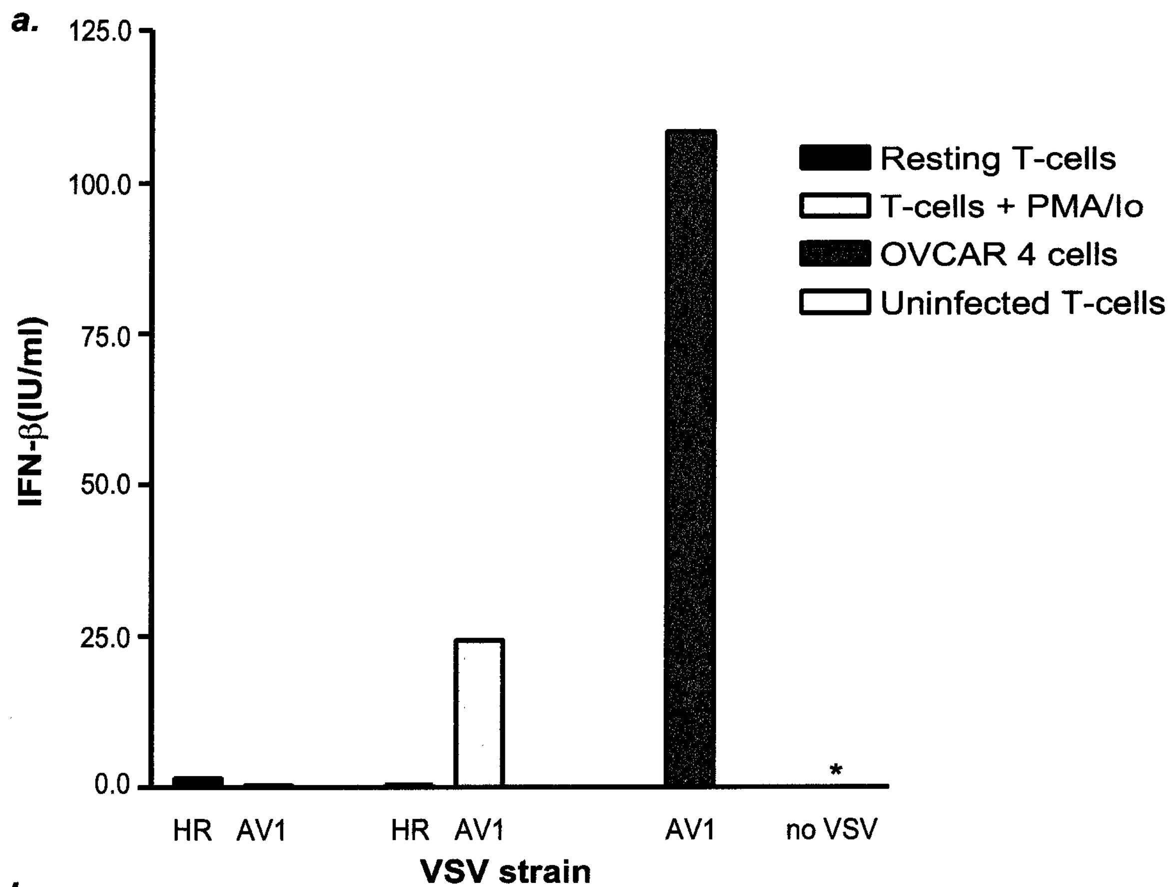
To further investigate this phenomenon, we used an IFN-inducing strain of VSV, AV1-VSV. The M protein of the wild type VSV (HR-VSV) has been shown in our lab to block the nuclear export of IFNβ and therefore prevent the synthesis and secretion of IFNβ and IFNα. A single amino acid substitution in the C-terminal of the M protein abrogates this effect and results in an IFN-inducing mutant, AV1, which has been demonstrated to be attenuated in IFN responsive cells lines and *in-vivo*. We reasoned that if activated T cells were capable of producing and responding to IFNβ then they should be less permissive for infection with AV1-VSV as compared to wild type VSV. Resting T cells and T cells activated with PMA/Io for 36 hours were infected at a moi of 10 PFU/cell with the GFP-expressing variant of either HR-VSV or AV1-VSV. Flow cytometry was performed at 12 hours and supernatants were collected at 24 hours for an IFNα/β ELISA discussed below. The results from the flow cytometry for GFP demonstrated no difference in VSV infection between activated T cells infected with HR-VSV or the IFN-inducing mutant AV1-VSV (Figure 24b). This corroborates the above result which points to a down modulated antiviral response to type I IFNs in activated T cells.

STAT1 signalling, via phosphorylation and nuclear translocation, is essential for IFN-mediated activity and therefore we examined the protein expression and tyrosine

phosphorylation of STAT1 during infection with HR-VSV in resting and activated T cells. The expression of STAT1, detected using an anti-STAT1α Ab, was unchanged following both activation and infection (Figure 24c, *top*). The expression of the phosphorylated form of STAT1, detected using a phospho-specific STAT1 Ab, did not decrease during activation but appeared to increase minimally. During infection of resting T cells the expression of phospho-STAT1 did not change significantly (Figure 24c, *bottom*), as expected because wild type VSV blocks the production of IFNα and β. This agrees with previously published data that demonstrated no impairment in STAT1 signalling following IFNα treatment of activated T cells[84].

3.11 Resting T cells do not produce Type I interferons in response to infection with VSV

Because activated T cells appear to have a down modulated antiviral response to type I IFNs, we wanted to evaluate the ability of resting and activated T cells to produce IFN-α and β in response to infection with VSV. As described above, supernatants from resting and activated T cells infected with either HR-VSV or AV1-VSV were assayed for IFN-β and α by ELISA (Figure 25a-b). The ovarian carcinoma cell line, OVCAR 4, infected with AV1-VSV was used as a positive control because it has been previously demonstrated in our lab to produce IFNα and β following infection. Surprisingly, neither IFNα nor β was produced in resting T cells infected with either strain of VSV. This might suggest that the block to viral replication that occurs in resting T cells is sufficiently early or complete that the antiviral IFN response is not triggered, or alternatively, it could indicate that the resting cell is incapable of producing IFNα. It is interesting that uninfected resting T cells appeared to produce a baseline level of IFNα,


a.
125.0
100.0
75.0
50.0
25.0
0.0
IFN-β(IU/ml)
Resting T-cells
T-cells + PMA/Io
OVCAR 4 cells
Uninfected T-cells
*
HR
AV1
HR
AV1
AV1
no VSV
VSV strain

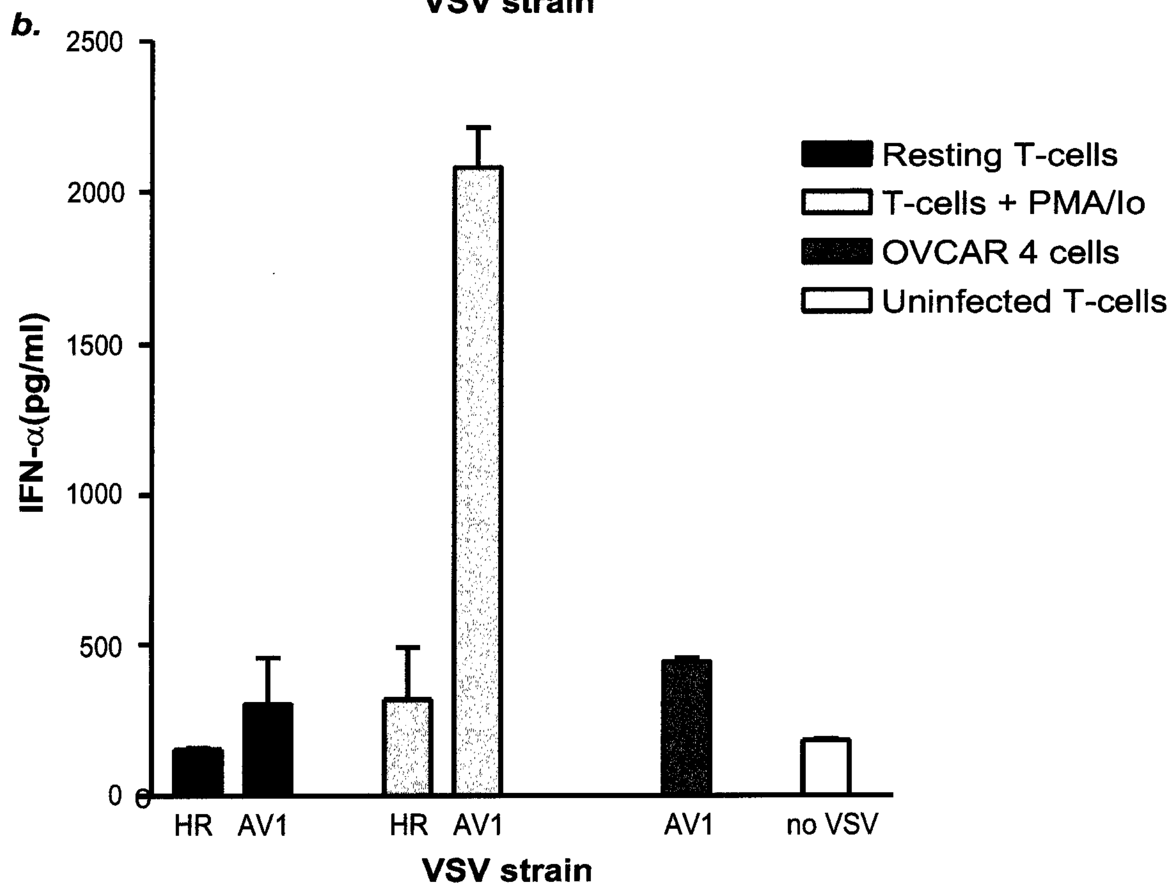

b.
2500
2000
1500
1000
500
0
IFN-α(pg/ml)
Resting T-cells
T-cells + PMA/Io
OVCAR 4 cells
Uninfected T-cells
HR
AV1
HR
AV1
AV1
no VSV
VSV strain

Figure 25. IFN α and β production by resting and activated T cells

Isolated CD3 T lymphocytes, incubated with PMA/Io or mock activated for 36 hours, were infected with VSV-GFP at a moi of 10 PFU/cell. At 24 hours post-infection supernatants were collected from equal numbers of cells and IFNα (b) and β (a) ELISA assays were performed. The absolute amount of IFN produced is plotted against the cell type and virus used. OVCAR4 cells were used as a positive control and were infected for the same time period at the same moi. Data are representative of two separate samples.

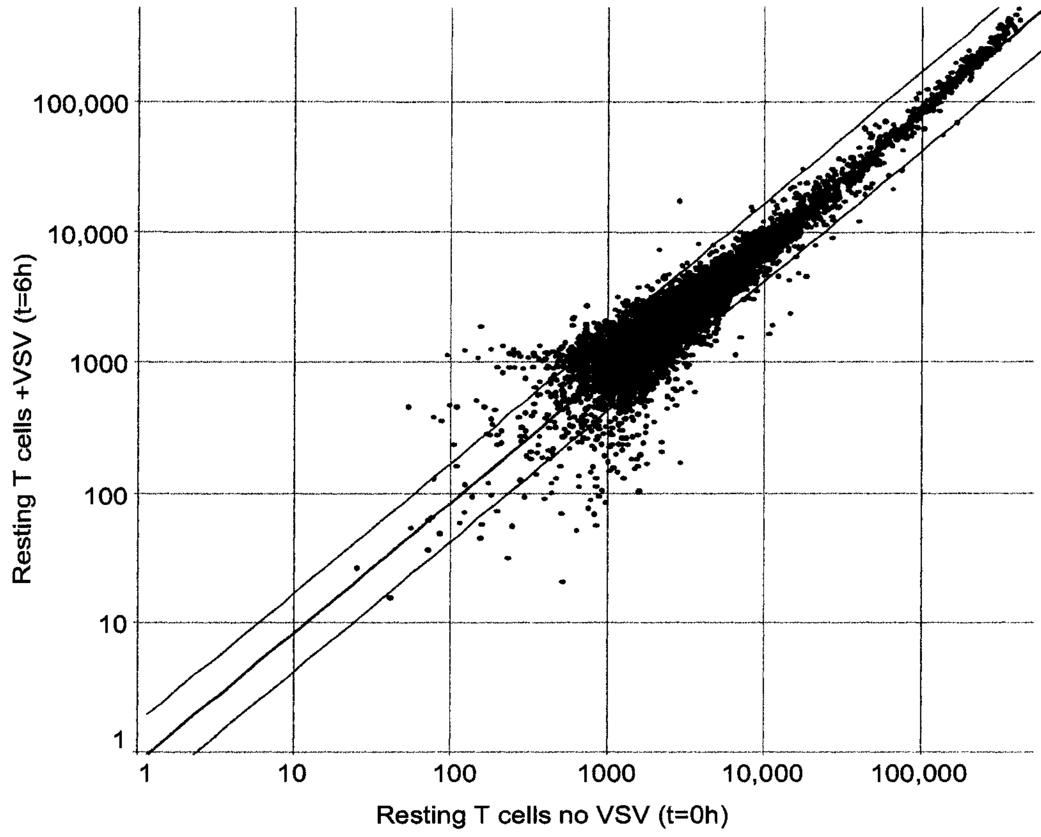
which may represent constitutive IFN signalling, but this has not been demonstrated in previous studies[81].

Activated T cells produced both IFNα and β in response to AV1-VSV but none in response to HR-VSV as expected since wild type VSV blocks IFNβ mRNA nuclear export. Relative to the OVCAR4 cells however, activated T cells produced more IFNα and less IFNβ. This may reflect the constitutive expression of IRF-7 found in lymphocytes. IRF-7 is a transcription factor for IFNα and its transcription is triggered during viral infection by IFNβ signalling[17]. Its constitutive expression in T lymphocytes may allow them to circumvent the production of IFNβ and produce IFNα directly.

3.12 Microarray gene expression profile demonstrates few changes in gene expression during infection of resting T cells with VSV

The IFN response pathway (Figure 3) has many downstream effects that induce the transcription of more than 300 ISGs. In order to evaluate these genes during the process of activation and infection, a gene expression microarray was performed. Resting T cells and T cells activated for 36 hours with PMA and Io were infected with VSV-GFP for 6 hours. Messenger RNA from these samples was isolated at 0 hours and 6 hours and compared using four Affymetrix (Santa Clara) gene chips. The data was evaluated with both a gene to gene and spot to spot comparison. Gene changes that occurred during infection in the resting T cells (Figure 26a) and in the PMA/Io activated T cells (Figure 26b) were minimal, with only 489 and 593 genes changing more than 2 fold in these samples respectively. This stands in striking contrast to gene expression arrays from various other normal and cancer cell lines performed in our lab, which have demonstrated

a.



b.

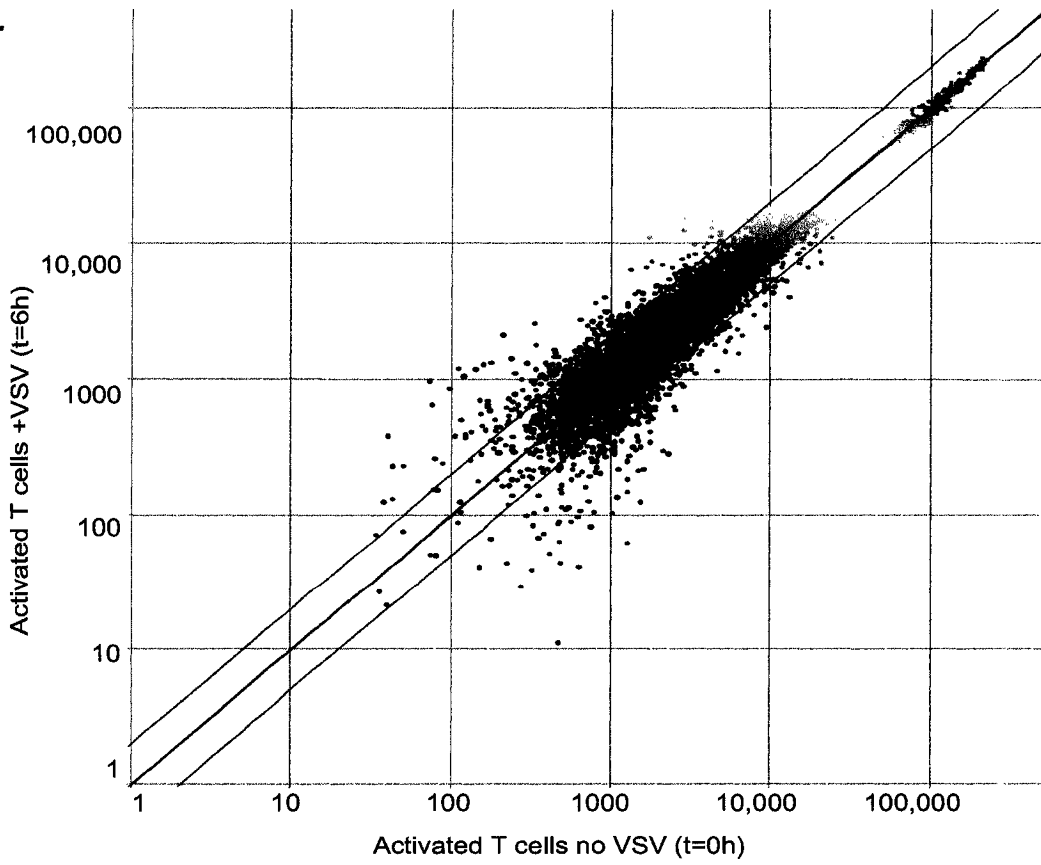
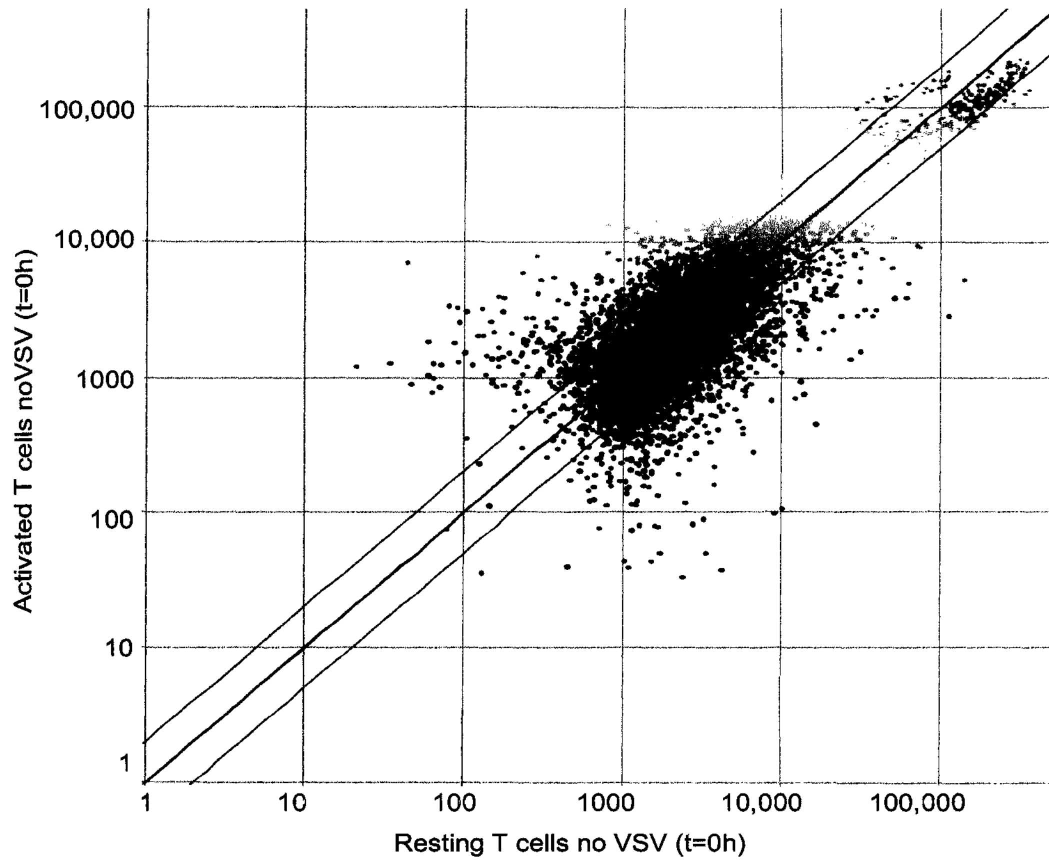


Figure 26. Gene expression changes during VSV infection in resting and activated T cells

Isolated CD3 T lymphocytes, incubated with PMA/Io or mock activated for 36 hours, were infected with VSV-GFP at a moi of 10 PFU/cell and mRNA was isolated at 6 hours post-infection for a microarray gene expression analysis. The signal intensity of mRNAs present in at least one sample, scaled and normalized, in uninfected T cells (x-axis) is plotted against VSV infected T cells (y-axis) for resting (a) and activated (b) cells. The upper and lower green lines represent 2-fold changes in either direction. The dot colour denotes the expression level in the uninfected sample – red dots have a high expression level, blue dots have a low expression level and yellow dots and intermediate expression level.


a.
100,000
10,000
1000
100
10
1
Activated T cells noVSV (t=0h)
1
10
100
1000
10,000
100,000
Resting T cells no VSV (t=0h)

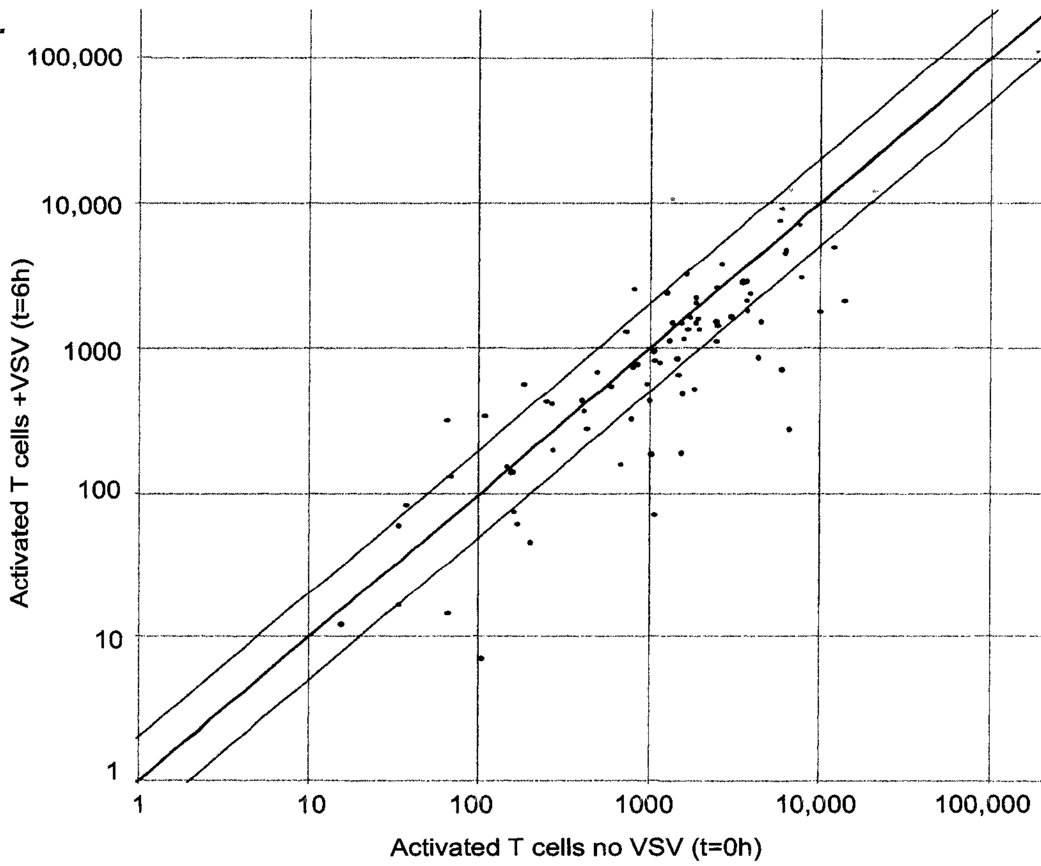

b.
100,000
10,000
1000
100
10
1
Activated T cells +VSV (t=6h)
1
10
100
1000
10,000
100,000
Activated T cells no VSV (t=0h)

Figure 27. Gene expression changes during T cell activation

The same experimental design was used as in Figure 26. The signal intensity of mRNAs present in at least one sample, scaled and normalized, in resting T cells (x-axis) is plotted against activated T cells (y-axis) (a). A list of IFN related genes and ISGs was generated from the literature and the expression levels of these genes was evaluated during activation in an analogous plot (b). The upper and lower green lines represent 2-fold changes in either direction. The dot colour denotes the expression level in the uninfected sample – red dots have a high expression level, blue dots have a low expression level and yellow dots and intermediate expression level.

Resting T lymphocytes

Expression down regulated during VSV infection		*Fold Change*
BCL3	anti-apoptotic,member of the NFκB family, mediates activated T cell survival	9.85
hCREM	cyclic AMP-responsive element modulator	9.85
SCYA4	small inducible cytokine 4A, homologous to MIP-1β, proinflammatory cytokine upregulated during influenza infection and mediates leukocyte migration	8.57
SCYC2	chemokine (C motif) ligand 2, monocyte chemoattractant protein-1	7.46
SCYA3	homologous to MIP-1α, proinflammatory cytokine upregulated during influenza infection	6.96
RABGGTa	Rab geranylgeranyltransferase, alpha subunit	5.66
IL-8	Interleukin 8, proinflammatory cytokine upregulated during influenza infection and mediates leukocyte migration	3.03

Expression upregulated during VSV infection		*Fold Change*
Hs.174303 EST	EST	24.25
CLIC3	chloride intracellular channel 3	12.13
SS18	synovial sarcoma translocation, chrom 18	8.00
NMA	putative transmembrane protein	5.28
cathepsin L	cysteine proteinase, may play a role in virion uncoating	4.00
Melanoma antigen, familyD2	alias breast cancer associated protein 1 and HCC associated protein	3.03

Activated T lymphocytes

Expression downregulated during VSV infection		*Fold Change*
nudix	nucleoside diphosphate linked moiety X	4.00
GLUT-3	glucose transporter	3.73
CD69	activation induced cell surface molecule	3.03
GLUT-14	glucose transporter	2.83
GEM	GTP-binding protein overexpressed in skeletal muscle	2.83

Expression upregulated during VSV infection		*Fold Change*
HSP-70	Heat shock protein 70, ER protein chaperone, upregulated in response the ER stress and the unfolded protein response	7.46
GBP-1	Guanylate binding protein 1	3.73
eIF5	Eukaryotic initiation factor 5	3.73
grp78	BiP, ER protein chaperone, upregulated in response the ER stress and the unfolded protein response	3.48
NUP98	nucleoporin 98, nucleoporin, target for the VSV M protein which block nucleocytoplasmic export of selected mRNAs	3.25

Table 1. Microarray: Selected genes upregulated and down regulated during T cells infection with VSV

Selected genes that were upregulated or down regulated greater than 2 fold during VSV infection in both the spot-to-spot and normalized gene-to-gene comparison are listed. The fold change represents the fold change from the spot-to-spot comparison.

a large number of gene changes during infection (e.g. 6,845 genes changed greater than 2 fold in OVCAR4 cells infected with HR-VSV for 6 hours). The most significant gene changes that occurred during infection are listed in Table 1.

By contrast the process of activation, evaluated by comparing resting and activated T cells at 0 hours prior to infection, resulted in a large number of changes in gene expression with 1,140 genes increasing and 1,278 genes decreasing more than 2 fold (Figure 27a). The upregulation of IL-2 (158 fold), IFNγ (30 fold), IL-12Rβ (6 fold) and lymphotaxin α (6.5 fold), with no increase in IL-4 (absent) verify previous published data demonstrating that activation with PMA and Io results in the generation of predominantly T_H1 and T_C1 cells[73].

3.13 IFN related genes are down regulated in T cells during activation

A list of ISGs was generated from the literature[14, 17] and their profile of expression change was evaluated during T cell activation (Figure 27b). Four ISGs were up-regulated more than 2 fold during activation with PMA and Io whereas 14 ISGs were down regulated. The upregulated genes included IRF-4 (4.6 fold), IRF-8 (6 fold) and SOCS1 (2 fold) – known inhibitors of the IFN signalling pathway- and IFNγ –previously demonstrated enhance to VSV replication in T cells[39]. The down regulated genes include important regulators of the IFN meditated antiviral response including ISG20, MxA, 2'5'-OAS, GBP-1, IRF1 and STAT1 (Table 2). These results are consistent with a down modulation of the response to IFNα in activated T cells.

ISG20 was explored further as an interesting candidate gene because it is constitutively expressed in PBMC[29], it mediates an alternate antiviral pathway that can confer resistance to VSV infection in the absence of IFN[28] and it blocks VSV

Resting PMA/Io

Time post-infection 0h 3h 6h 12h 0h 3h 6h 12h

ISG20

β-actin

Figure 28. ISG20 is constitutively expressed in resting T cells and its expression is down regulated during T cell activation

Isolated CD3 T lymphocytes, incubated with PMA/Io or mock activated for 36 hours, were infected with VSV-GFP at a moi of 10 PFU/cell and cell lysates were collected at various time intervals just prior to and following infection. The quantity of ISG20 protein was determined by immunoblotting 20 μg of protein lysate with a monoclonal anti-ISG20 antibody. β-actin is used as a loading control.

Expression upregulated during T cell activation		*Fold Change*
IL-2	interleukin-2	157.67
granzyme B	proteolytic serine esterase, critical for cytolytic activity	73.52
VEGF	vascular endothelial growth factor	42.22
IFNγ		**26.86**
SCYA3	homologous to MIP-1α, proinflammatory cytokine upregulated during influenza infection	25.99
SCYC20	chemokine (C-C motif, ligand 20), MIP-3α	21.11
IRF-8	**interferon regulatory factor 8 (ICSBP)**	**6.06**
IRF-4	**interferon regulatory factor 4 (ICSAT)**	**4.59**
IRDR2	**interferon-related developmental regulator 2**	**2.14**

Expression down regulated during T cell activation		*Fold Change*
GAC1	human glioma amplified on chrom 1 protein precursor	157.59
Hs.277431	hypothetical protein	147.03
Hs.296687	probable thromboxane A_2 isoform β	73.52
RASGRP2	Ras guanyl releasing protein 2	55.72
KLF-2	Kruppel-like factor 2	51.98
SGKL	serumglucocorticoid regulated kinase	48.50
ISG20	**interferon-stimulated gene 20 kDa (HEM45)**	**11.31**
IFRG28	**interferon responsive protein 28**	**11.31**
RI58	**retinoic acid and IFN inducible protein 58**	**10.56**
GBP-1	**guanylate binding protein 1**	**8.00**
Mx1	**myxovirus (influenza) resistance 1**	**3.73**
IFI-6-16	**interferon α inducible protein 6-16**	**3.48**
ISG15	**interferon-stimulated protein, 15 kDa**	**3.03**
IRF-1	**interferon regulatory factor 1**	**2.83**
IRF-7	**interferon regulatory factor 7**	**2.64**
IRF-9	**interferon regulatory factor 9 (p48)**	**2.46**
IFIT1	**interferon-induced protein with tertricopeptide repeats 4**	**2.30**
STAT1	**signal transducer and activator of transcription 1**	**2.14**
2'-5' OAS	**2'-5' oligoadenylate synthetase**	**2.00**
PML-1	**promyelocytic leukemia protein**	**2.00**

Table 2. Microarray: Selected genes upregulated and down regulated during T cells activation with PMA and Io

Selected genes that were upregulated or down regulated greater than 2 fold during T cell activation with PMA and Io in both the spot-to-spot and normalized gene-to-gene comparison are listed. The fold change represents the fold change from the spot-to-spot comparison. Genes highlighted in bold represent important ISGs.

replication at the level of mRNA because it is an RNA exonuclease[28]. The expression of ISG20 was evaluated in both resting and PMA/Io activated T cells before and during infection with VSV at a moi of 10 PFU/cell (Figure 28). The protein level of ISG20 was reduced more than 2 fold during the process of T cell activation and did not increase during infection in either cell type. These results are consistent with the microarray data that demonstrate a substantial reduction in mRNA expression of ISG20 in activated as compared to resting T cells.

4. DISCUSSION

4.1 Lymphocytes are resistant to infection with VSV but monocytes are sensitive

Although B and T lymphocytes are very resistant to VSV infection, CD14 mononuclear cells are quite sensitive. Blood mononuclear cells expressing CD14 may be monocytes or circulating dendritic cells (DC) which represent 2% and 0.1% of circulating blood leukocytes respectively[69]. Both cell types are considered "sentinel" cells because they are the first cells to detect infection and present antigen to lymphocytes. Monocytes circulate in the blood for a short time before migrating into tissue and differentiating into macrophages - effective phagocytic and antigen presenting cells. Tissue macrophages cannot return to the blood but remain in the tissue through which they can wander with ameboid movement. DCs may reside in the tissue, such as interstitial DC, or they may circulate in the blood and lymph, known as blood DC or "veiled" cells respectively[59]. DCs function as APCs – they capture antigen in the tissue, migrate into the blood or lymph and circulate to various lymph organs to present antigen to B and T cells. Unlike macrophages, these immature DCs can migrate in and out of the circulation throughout

their lives. Monocytes and DCs are though to be derived from the same lineage but whether macrophages and DCs can interconvert *in-vivo* is a still unresolved question[69].

Monocytes and DCs have both been shown to be productively infected with VSV[104, 105]. In theory viral replication may be required in these APC for efficient antigen presentation and the release of appropriate cytokines to initiate an acquired immune response. In the case of dendritic cells, Ludewig et al.[105] demonstrated that in the presence of an intact IFN system, DCs contained the spread of VSV infection and migrated to secondary lymphoid organs where they released low levels of live virus. This led to a rapid B cell mediated neutralizing IgM response followed by T_H cell mediated isotype switching and the resultant IgG response. Interestingly, UV inactivated virus did not induce anti-VSV neutralizing antibodies of either type. This data emphasizes the importance of a productive infection of DCs and release of live virus for the activation of B cells and T_H cells. Similarly, monocytes and macrophages infected with influenza-A require a productive infection in order to release essential proinflammatory cytokines, including macrophage inflammatory protein 1a (MIP-1α) and interleukin-8 (IL-8)[106]. Monocytes fail to produce any cytokines following infection with heat inactivated influenza-A[107].

Studies of murine *in-vivo* VSV-GFP infection in our laboratory have demonstrated productive VSV infection (determined by GFP expression) solely in the lymph nodes of a non-tumour bearing animal. The nature of these infected cells is still under investigation but we have speculated that these are DC that have migrated to secondary lymphoid organs to release low levels of VSV to B and T cells.

4.2 The block to VSV replication appears to be at the level of transcription in resting T cells

A systematic evaluation of each step in the life cycle of VSV (Figure 2) was undertaken to determine the level at which VSV was restricted in resting T cells, using activated T cells as a comparison. The first step, viral entry, consists of viral adsorption to the ubiquitous phosphatidyl serine receptor, which is energy independent, followed by the energy dependent process of receptor-mediated endocytosis. Viral entry was similar between resting and activated T cells as detected by western blot for viral proteins after a one hour incubation with VSV at a moi of 10 PFU/cell and by electron microscopy following a 20 minute incubation at a moi of 4500 PFU/cell. Both of these assays are limited in sensitivity and it is questionable whether a difference would be detected even if one did exist. For the protein assay, the limit of detection was due to the presence of viral particles in the supernatant because, despite several large volume washes, it is uncertain whether more virus was present in the small volume of supernatant around the cells or within and bound to the cells. The electron microscopy circumvented this problem by allowing direct detection of VSV particles within the cell. It was also limited because only a single cross section of each cell could be examined and the majority of cells, in both the resting and the activated populations had no detectible viral particle in that cross section. The process of endocytosis and endosome acidification of acradine orange during VSV infection was not restricted in resting T cells but the endocytosis of VSV itself was not directly evaluated.

The best evidence to suggest that the process of viral entry is not responsible for the block to VSV replication in resting T cells comes from the fusion experiment of infected

activated T cells with uninfected resting T cells. The activated T cells were incubated with VSV for one hour prior to fusion with resting T cells. This was adequate time for viral entry as demonstrated by the 47% infection rate in the non-fused activated T cells. The fused cells were still resistant to VSV with only 3% productively infected, providing strong evidence that replication of VSV is restricted at a step beyond viral entry.

VSV replication was restricted as the level of viral mRNA production and, as previously discussed, this could represent a block at the level of viral transcription or translation. Differentiating between these two scenarios experimentally is complicated. One possibility would include blocking protein synthesis with an inhibitor, such as cyclohexamide, and comparing viral mRNA production during VSV infection of resting and activated T cells. If a significant difference was detected, it could be inferred that the block to viral replication was at the level of transcription and not secondary to protein synthesis. Unfortunately, detecting no difference does not necessarily indicate the corollary. Other experiments, such as VSV infection in the presence of a CKII inhibitor or infection with a genetically engineered VSV possessing a mutant L or P protein are similarly limited.

The discrepancy between resting and activated T cells at the level of viral assembly and release appeared to be even greater than at the RNA and protein level. As mentioned, this most likely reflects the different time points at which that these two variables were measured. Because VSV replication has an exponential phase, measuring parameters at 12 and 24 hours will invariably lead to different results and a comparison of viral protein production and PFU/cell, both performed at 12 hours, would clarify the existence of a second block at the level of viral assembly and release, if one in fact exists.

4.3 The mitogenic response to *in-vitro* T cell activation is not necessary to render activated T cells permissive for VSV

The most effective activators for rendering isolated T cells permissive for VSV were OKT3 and a combination of PMA and Io. These activation conditions have different mechanisms of action but similar downstream effects – proliferation and increased metabolic activity[108], synthesis of IL-2 and CD25[75], and a shift towards the differentiation of T_H1 cells with synthesis of IFNγ[73, 109-111]. From our microarray data, the gene expression profile of isolated T cells activated with PMA and Io was consistent with these studies – IL-2 and IFNγ were upregulated and IL-4 was absent. In addition, T cell stimulation with IL-4 did not result in enhanced sensitivity to VSV. This raises the possibility that a T_H1 response is essential for sensitivity to VSV. However, in order to definitively establish that a T_H1 response is necessary, and a T_H2 response insufficient, for activation-induced T cells sensitivity, T cells would have to be activated under established polarizing condition, such as anti-CD3 Ab, IL-2, anti-IL-4 Ab and IL-12 for T_H1 and anti-CD3, IL-2 and IL-4 for T_H2. The activated T cell phenotype would have to be verified by ELISA assay for production of IFNγ in T_H1 cells and IL-4 in T_H2 cells. If only T_H1 polarized cells became permissive to VSV then one could conclude that the T_H1 cytokine profile is essential for sensitivity to VSV.

Other experimental evidence demonstrated, somewhat unexpectedly, that none of these downstream effects appeared to be essential in order to render T cells susceptible to VSV. PMA alone sensitized 52% of T cells despite the fact that it is non-mitogenic for isolated T cells[74] and it does not induce the synthesis of IL-2 or CD25[75]. This suggests that neither proliferation nor IL-2 signalling are absolute requirements for T cell

sensitivity. Moreover, activation with either PMA and Io or OKT3 in the presence of FK506 is reported to prevent proliferation, IL-2 signalling and the synthesis of IFNγ[61] but we have demonstrated that FK506 did not significantly alter T cell sensitivity to VSV. Although we did not measure cytokine production in T cells activated in the presence of FK506, we did demonstrate a block to IRF-4 expression, indicating that FK506 was active in this assay. The nature of the cytokine response that is required for sensitivity to VSV is still unclear.

4.4 The restrictive state of a resting T cell to VSV replication is dominant, irreversible and not dependent on transcription

Resting T cells can protect activated T cells following fusion. This indicates that the restrictive state is dominant over the permissive state and therefore resting T cells must possess a factor(s) that actively inhibits VSV replication. This 'inhibitory' factor(s) must be absent or inhibited in activated T cells. If resting T cells lacked a factor(s) that was necessary for VSV replication, such as CKII or translation initiation factors, then activated cells would supply this factor(s) during fusion and the fused cells would be permissive. For example, activated T cells have a much higher rate of cellular transcription or translation than resting T cells and a simple explanation would attribute the disparity in viral mRNA and protein production between these two cell types to this difference. This would impart a passive mechanism of viral resistance to the resting T cell and therefore fusion of a resting and activated T cell would be expected to result in a susceptible cell, or at the very least a cell with an intermediate phenotype.

The other possible explanation for the results of the fusion experiment is that a threshold amount of a factor(s) is required in activated T cells to render them permissive

to VSV and that fusion dilutes this factor to below the threshold. This seems unlikely because resting T cells are $1/10^{th}$ the size of activated T cells and therefore the threshold would have to be very close to the level of expression of this factor(s) in activated T cells.

When resting T cells are activated following a one hour infection with VSV, they cannot be rendered permissive. This indicates that the block to VSV replication is permanent and irreversible which could be explained by a block to viral entry or by degradation of VSV RNA or protein. Because our data is not suggestive of a block to viral entry, we have speculated that resting T cells have a mechanism to degrade viral proteins or RNA. This degradation may be responsible for the resistance to VSV but alternatively it may reflect a reversible block to viral RNA or protein synthesis where the half life of viral RNA and protein is shorter than the time required for reversal of this block to transcription or translation. Differentiating between these two possibilities experimentally would be difficult. Protein degradation, if it was mediated by the ubiquitin pathway, could be investigated as a mechanism by using a proteasome inhibitor, such as pyrrolidine dithiocarbamate, but there is no equivalent agent to evaluate RNA degradation.

Treatment of resting T cells with actinomycin D, a selective inhibitor of cellular transcription, does not impair their resistance to VSV. This suggests that the antiviral state of resting T cells is constitutively active and is not dependent on transcription following infection. This model is consistent with the finding that resting T cells produce very little IFNβ and α following infection with VSV. The constitutive expression of an 'inhibitory' factor(s) would halt VSV replication before the cell could respond through the IFN signalling pathway.

Taken together this data points towards a model whereby resting T cells constitutively express a factor(s) that actively inhibits VSV replication by inhibiting the process of viral transcription/translation or by degradation of viral RNA/protein (Figure 29).

4.5 The process(es) necessary to render T cells permissive for VSV during activation are time-dependent, and require transcription

The process of activation with PMA and Io required 24 hours and was inhibited by actinomycin D indicating that cellular transcription is absolutely necessary to render T cells permissive for VSV. As discussed previously, the results of the fusion experiment establish that activated T cells lack a factor(s) that inhibits VSV replication. Overall, the process of activation appears to involve the transcription of a second factor, which could exert its effect in several ways. This 'activation' factor could down regulate the expression of the 'inhibitory' factor present in resting T cells, it could block the activity of this 'inhibitory factor, by post-translational modification or by direct binding or it could selectively degrade the 'inhibitory' factor at the RNA or protein level (Figure 30). For the second two models one would have to assume that the inhibition or degradation of the inhibitory factor was too slow or incomplete to render fused cells susceptible to VSV – that is to say that the 'inhibitory' factor was able to restrict VSV replication before it could be degraded or its activity blocked.

4.6 Down modulation of the IFN response during T cell activation would fit this model

IFNα, even at high concentrations, did not completely protect activated T cells from VSV infection. This is unlike other normal cells which have been shown to be effectively

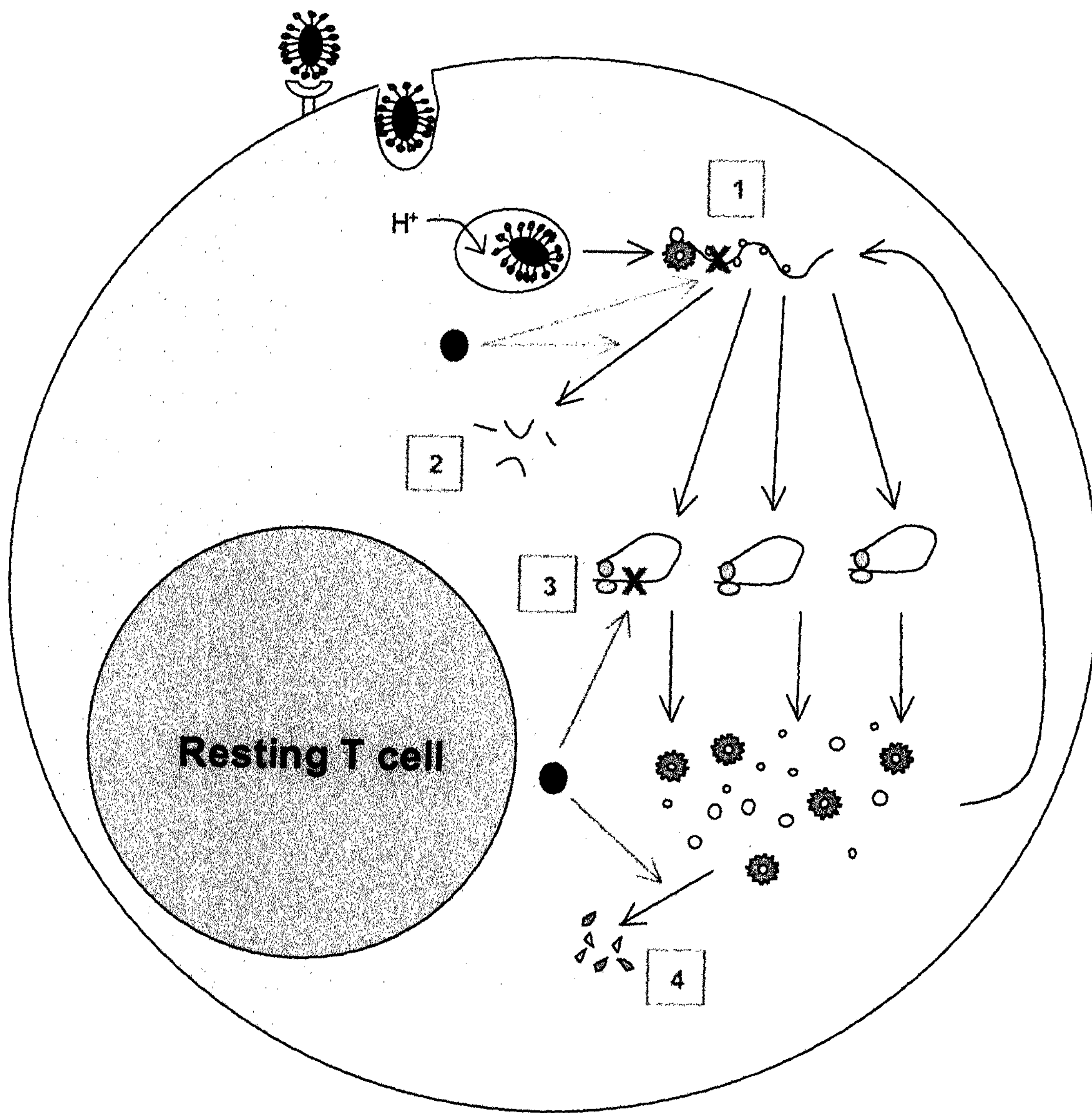

H+
1
2
3
Resting T cell
4

Figure 29. Resting T cells express an 'inhibitory' factor(s) that restricts VSV replication

A model of a resting T cell which proposes the constitutive expression of an 'inhibitory' factor that is able to restrict VSV replication at the level of mRNA expression. The 'inhibitory' factor is represented by the red circle. This factor could result in inhibition of viral transcription by the L and P proteins, such as Mx1 (*box1*), selective degradation of viral mRNAs, such as ISG20 or RNAse L (*box2*), inhibition of viral protein translation (*box3*), or selective degradation viral proteins, such as ubiquitin conjugating enzymes (*box4*).

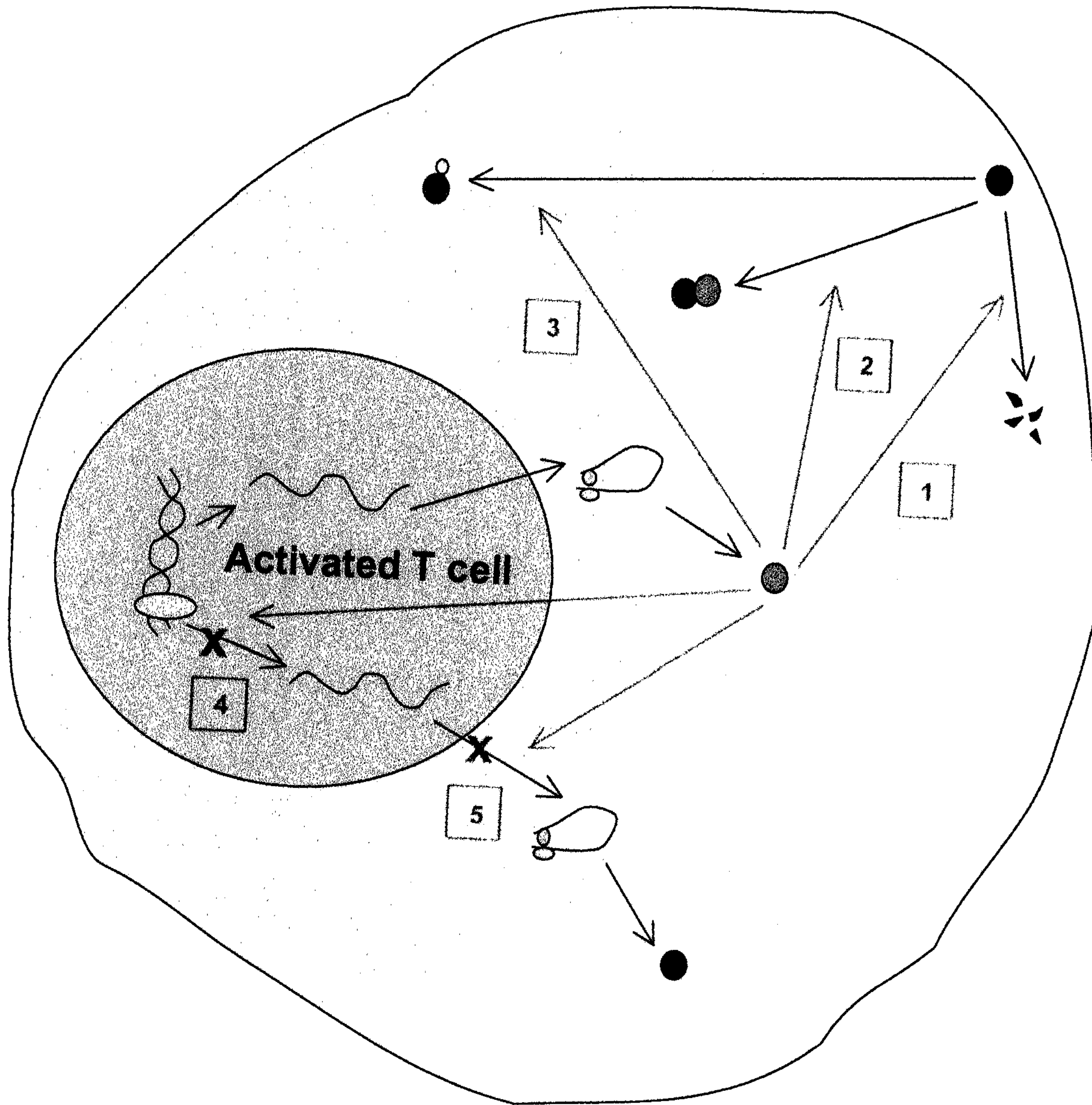

Activated T cell
1
2
3
4
5

Figure 30. T cells express an 'activation' factor(s) during activation that down modulates the antiviral state in resting T cells

A model of an activated T cell which proposes the activation induced expression of an 'activation' factor(s) that is able to down modulate the 'inhibitory' factor(s) present in resting T cells during the process of activation. The 'inhibitory' factor is represented by the red circle and the activation factor by the green circle. The factor could block the activity of the 'inhibitory' factor by selective degradation (*box1*), direct binding (*box2*), post-translational modification (*box3*), or by down regulating the expression of the 'inhibitory factor at the transcriptional (*box4*) or translational level (*box5*).

protected from VSV in the presence of IFNα both *in-vitro*[35] and *in-vivo* (unpublished data from our lab). In fact, this phenotype is reminiscent of a cancer cell that, as described earlier, down modulates a component of its IFN response pathway in order to escape the antiproliferative effects of IFN and undergo unregulated cellular growth and replication. It also makes sense that T cells would need to down modulate their IFN response during a viral infection, in order to proliferate in the presence of IFN. A number of theories as to the mechanism of IFN down modulation during T cell activation, presented in the introduction, can now be considered in light of our results.

IRF-4 and IRF-8 may represent the 'activating' factor whose activity inhibits transcription of the 'inhibitory' factor(s) (Figure 30, *box 4*). T cell activation induces upregulation of IRF-4 and IRF-8, both of which have been demonstrated to bind to ISREs promoters and repress IRF-1 transactivation of ISGs, such as 2',5'-OAS[90], ISG15[93], MHC class I[93, 95], GBP-1[93] and IFNβ[90, 95]. It seems reasonable to predict that upregulation of these IRFs would result in the down regulation of ISGs whose expression is controlled by an ISRE. Examples of such ISGs from our microarray analysis include IFIT1, ISG20, IFI-6-16, ISG15, GBP-1, Mx1, p48, IRF-1, and PML-1. Many of these factors are controlled by IRF-1 transactivation and therefore inhibition of IRF-1 activity by heterodimer formation with IRF-8 (and possibly IRF-4) represents another mechanism for blocking the 'inhibitory' factor(s) in resting T cells – direct binding (Figure 30, *box2*). It was surprising that blocking IRF-4 upregulation during activation with FK506 did not have a dramatic impact on VSV replication. It may be that because the ability of FK506 to block IRF-4 is not complete, as seen in the Western blot (Figure 13a, *bottom*), a sufficient amount of IRF-4 was produced to down modulate ISGs. Alternatively, IRF-8

may play a somewhat redundant role and expression of IRF-4 or IRF-8 is sufficient to mediate T cell susceptibility during activation. The protein level of IRF-8 following activation in the presence of FK506 was not evaluated but this would help to clarify the role of IRF-8 expression. Furthermore, other yet unidentified factors may be involved in the down regulation of ISGs during activation. To test these hypotheses an mRNA comparison of selected ISGs (microarray, Q-PCR or Northern analysis) of T cells resting and following activation in the presence or absence of FK506 would provide valuable information – our hypothesis would predict that these ISGs would still be down modulated in the presence of FK506.

A second mechanism could involve IFNγ signalling. Recall that during T_H1 differentiation these T cells synthesize and secrete IFNγ while simultaneously internalizing IFGRβ receptors and, in doing so, limiting the cells ability to respond to IFNγ[65, 66]. ISGs whose expression depends on IFNγ signalling, such as GBP-1[31] would therefore be down regulated.

SOCS1 is also upregulated during activation[88] and it fits the profile of a 'activation' factor that inhibits the 'inhibitory' factor by direct binding (Figure 30, *box 2*) because it binds to and inhibits tyrosine phosphorylation of STAT 1 by Jak[89]. However, as mentioned earlier, the lack of impairment in STAT1 signalling following activation indicates that this process is of dubious importance.

We were unable to establish the identity of any 'activating' factors that could modulate the 'inhibitory' factor in resting T cells by mRNA or protein degradation or post-translational modification. There are few published reports of endogenous inhibitors of IFN signalling and moreover our assay was designed to detect transcriptional changes

and therefore it is difficult to predict which of the factor(s) upregulated during activation could mediate their effects by these mechanisms.

4.7 Several candidate genes from the gene chip array denote classic and alternate IFN-mediated pathways

A number of ISGs, whose expression was demonstrated in the microarray analysis to be down regulated following activation, are potential candidates for the identity of the 'inhibitory' factor(s) in resting T cells. The expression of these ISGs is inhibited during activation suggesting that they may be constitutively active in resting T cells and therefore may be responsible for the antiviral state established in these cells. As speculated these factors could have one of several mechanisms of action – inhibition of viral transcription, viral RNA degradation, inhibition of viral translation or viral protein degradation.

Inhibition of viral transcription

Constitutive expression of proteins that inhibit VSV replication at the level of viral transcription include MxA and possibly GBP-1. Mx1 belongs to the dynamin family of GTPases, which bind and hydrolyse GTP[112]. It is located in the cytoplasm and has been detected in association with the endoplasmic reticulum[113]. Mx1 appears to have several mechanisms of antiviral activity including directly blocking viral protein trafficking of Thogota virus, inhibiting translation of measles virus proteins and inhibition of VSV at the transcriptional level. Mx1 has been shown to be constitutively expressed at a low level in lymphocytes[114], although its expression is higher in monocytes which are susceptible to VSV. The expression is induced with IFNα, but not IFNγ[114], and its promoter consists of two ISREs, which appear to bind the ISGF-3

complex[115]. The IFN-inducible guanylate- binding protein family, which includes GBP-1, also exhibits GTPase activity. IFNγ treatment results in a strong induction in GBP-1, which is dependent on the transcription factor IRF-1[116], whereas IFNα results in minimal induction. Constitutive expression of GBP-1 reduces viral titre whereas inhibiting its IFN mediated expression using anti-sense RNA increased the viral yield following IFNγ treatment, but not following treatment with IFNα[31]. The expression of GBP-1 in lymphocytes has not been investigated.

Both Mx1 and GBP-1 could represent 'inhibitory' factors in resting T cells (Figure 29, *box1*). Mx1 inhibits VSV replication at the level of transcription, effectively shutting down the virus before the production of viral proteins and dsRNA, which could trigger an IFN, mediated antiviral response. Although the antiviral mechanism of GBP-1 has not been elucidated, its GTPase activity suggests that it may also inhibit VSV at the level of transcription. Murine MxA has been shown to be constitutively present in lymphoid cells, further establishing it as a potential candidate. The down regulated expression of both genes during activation, demonstrated by the microarray analysis, is conceivable. The expression of both Mx1 and GBP-1 are regulated by transactivation of an ISREs present in both their promoters– the site of inhibition for IRF-4 and IRF-8 during activation. Finally, in the case of GBP-1, its expression is dependent on IFNγ and, as discussed earlier, activated T_H1 cells do not respond to IFNγ.

Viral RNA degradation

ISG20 and RNAseL, both RNAses regulated by IFN, are potential candidates for the 'inhibitory' factor in resting T cells because their constitutive expression would also inhibit VSV replication at the level of transcription (Figure 29, *box2*). As described in the

introduction, RNAse L mediates its antiviral activity through cleavage of single-stranded RNA. RNAse L is latent in the cytoplasm but is activated by the 2'-5' OAS pathway – 2-5' OAS are a family of enzymes whose synthesis is induced by IFNs but their enzymatic activity requires the presence of dsRNA[15]. Although constitutive expression of 2'-5' OAS in T lymphocytes has not been studied, both IRF-4 and IRF-8 have been shown to inhibit its expression[90, 93] and this could explain the down regulation of 2'-5' OAS during activation. ISG-20, on the other hand, has been reported to be constitutively expressed in T lymphocytes[29] and we have confirmed this by western blot. ISG20 is an IFN stimulated 3' to 5' exonuclease which has been recently shown to mediate an alternative antiviral pathway[28]. Over expression of ISG20 conferred resistance to VSV, even in the absence of IFN, by inhibiting VSV mRNA and protein accumulation, whereas the expression of a dominant negative mutant of ISG20 significantly reduced the ability of IFN to block VSV replication[28]. IFN-mediated induction of ISG20 is strictly dependent upon IRF-1 binding to an ISRE within the its promoter[30] providing a site for inhibition by IRF-4 and IRF-8.

Inhibition of viral protein synthesis

Interferon-induced protein with tetratricopeptides repeats, IFIT1 or p56, belongs to a family of closely related proteins from a genetic locus on chromosome 10q23-q24. IFIT1 is the most abundant of all IFN-induced proteins. It has been demonstrated to bind the p48 subunit of the translation initiation factor eIF-3 *in-vitro* and inhibit protein synthesis in a dose-dependent fashion[117]. Expression *in-vivo* of IFIT1, or its induction by IFN treatment, caused an inhibition in overall cellular protein synthesis and growth[15]. Constitutive expression of IFIT1 could therefore define an antiviral pathway

of translation inhibition. The physiological significance of this pathway is questionable however because although IFIT1 is the most abundant IFN-induced protein, it cannot completely inhibit protein synthesis because the cellular pool of eIF-3 is much higher[15]. Moreover, its role in the inhibition of VSV in resting T cells is unlikely because it is reported not to inhibit the replication of VSV[15].

Another IFN-induced protein that is known to inhibit VSV at the level of protein translation is PKR. PKR is a serine-threonine kinase that undergoes autophosphorylation upon activation by dsRNA, which enables it to phosphorylate the translation initiation factor eIF2α resulting in the effective shutdown of protein synthesis[17]. PKR expression did not change during activation by microarray analysis and more importantly co-infection of resting T cells with vaccinia virus - which encodes E3L, a potent inhibitor of PKR[118] - did not render these cells susceptible to VSV (unpublished data), suggesting that PKR is not an important modulator of either sensitivity or resistance in activated and resting T cells respectively. Indeed, PKR-/- T lymphocytes were as resistant to VSV as wild type T lymphocytes (unpublished data) further negating a role for PKR in T cell resistance.

Although we have not defined an example of translational regulation of VSV replication in resting T lymphocytes (Figure 29, *box3*) it is certainly possible that one will be elucidated in the future as the antiviral mechanisms of other ISGs, such as IFI 6-16, IFRG28, PML-1, and RI58 are better understood.

Viral protein degradation

Many viral antigens are processed by the ubiquitin (Ub) proteasome pathway before presentation on MHC molecules. This process, which occurs in APCs, involves

polyubiquitination of viral antigens that are subsequently targeted to the immunoproteasome – composed of subunits specializing in the generation of antigenic peptides – followed by translocation of peptides into the lumen of the endoplasmic reticulum by the transporter associated with antigen processing (TAP) for loading onto MHC class I molecules[119]. Ubiquitin conjugation is the rate-limiting step in this process. Nyman et al.[120] demonstrated that several ubiquitin conjugating enzymes – UbcH5, UbcH6 and UbcH8 – are upregulated in macrophages following IFNα treatment, leading them to speculate that IFNα facilitated antigen presentation by this mechanism. This study also demonstrated that the ubiquitin conjugating enzymes, UbcH5and UbcH8, were expressed at a low level in resting T cells and up regulated in response to IFNα[120]. Enhanced Ub conjugation of viral proteins could represent a mechanism for resting T cells resistance to VSV. Both UbcH6 and UbcH8 were highly expressed at the mRNA level in our microarray (signal intensities of >5,000 and >11,000 respectively) but their expression was not significantly changed during activation. Nevertheless, these or possibly other Ub conjugating enzymes are possible candidates for the 'inhibitory' factor present in resting T cells that mediate their antiviral effect through enhanced viral protein degradation (Figure 29, *box4*).

Another interesting gene whose expression was down regulated during T cell activation was ISG15. ISG15 is strongly induced after IFN treatment and viral infection[121]. Notably, the sequence of ISG15 protein possesses significant homology to the ubiquitin sequence and more remarkably, ISG15 was found to be conjugated to intracellular proteins via an isopeptide bond in a manner similar to Ub and other Ub-like proteins[122]. ISGylation occurs via a similar but distinct pathway compared with Ub

conjugation and the activating enzyme UBE1L has recently been identified[123]. In addition, similar to modification by other Ub-like proteins, the conjugation of ISG15 is reversible and is accomplished by a highly specific protease UBP43 which is also induced by IFN via IRF-3[124]. Malakhova et al.[125] demonstrated that mice lacking UBP43 were hypersensitive to IFN stimulation - IFNβ induced prolonged STAT1 tyrosine phosphorylation, DNA binding, and IFN-mediated gene activation[126]. More over STAT1, as well as Jak1, ERK1 and PLC have recently been identified as targets for ISGylation[127]. One might speculate that unopposed ISGylation of these and other targets regulates enzymatic or DNA binding activity and leads to enhanced IFN signalling and a more robust response. Of interest, both ISG15 and UBE1L were down regulated during T cell activation. It is not clear how ISG15 would fit into our model of a resistant resting T cell but perhaps further studies elucidating the role of ISG15 will lend themselves to an interesting hypothesis.

4.8 Resting T cells may be in a constitutive IFN-independent antiviral state

Activated T cells appear to have down modulated their antiviral response to type I IFNs but whether this translates into a constitutive expression of one or several components of the classic or alternative antiviral signalling pathways in resting T cells is still conjecture. Determining which proteins are constitutively expressed and active in resting T cells, such as ISG20, will help target those proteins of interest. Additionally, knock-out and knock-down studies using T cells from deficient mice or anti-sense RNA treatment of resting T cells may help to clarify the contribution, if any, of each ISG to the uniquely resistance state of resting T cells to VSV.

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Statement of Contribution of Collaborators

Brian Lichty cloned and rescued the wild type HR-VSV-GFP and Jenn Paterson cloned and rescued the AV1-VSV-GFP. Jaime Blais provided QPCR standard curves for β-actin and VSV-M and the N-cDNA construct for the Northern Blot was provides by Dave Stojdl. Leigh Miller provided several buffers for PBMC processing and FACS. Blood was provided by generous donation from members of the Bell and Atkins Labs.

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PSI Grant for Women in Surgery Study — 2003
First Place at Collins Surgical Day — 2003
Maclean-Muller Award for best Canadian research paper — 2003

PSI Award for Excellence in Resident Research — 2002
Research Award for Department of General Surgery Research Day — 2002
Outstanding Resident Research Award at Collins Surgical Day — 2001

Medical School Awards

Gold Medal in Medicine for the *highest standing throughout medical school* — 2000
The Bryan George Blair Memorial Prize in Internal Medicine
The Dr. Osler Briggs Dickinson Scholarship in Surgery
The Hannah Washburn Prize for the *proficiency in Medicine, Surgery and Obstetrics*

Edgar Forrester Scholarship for the *highest standing in the third year* — 1999
Reuben Wells Leonard Penultimate Year Scholarship
Thomas Gibson Scholarship for the highest overall standing in *Pharmacology*
Rattray Scholarship for the highest standing in *Physiology* in the third year
Soroptomist Foundation of Canada *National Scholarship*

Edgar Forrester Scholarship for the *highest standing in the second year* — 1998
BC Research Institute Summer Research Scholarship
First Place in the Clinical Sciences division at the BC Institute Research Day

Edgar Forrester Scholarship for the *highest standing in the first year* — 1997
Dr. John Matthews Prize for the *highest standing in Clinical Skills*
Murphy Memorial Scholarship for the highest standing in the final examinations

Graduation Honours 1996

Provost Scholar for ranking among the top twenty graduating students
Ethel F. Dixon Paul Award for the highest standing among female graduates
University of Toronto Scholar for the top one percent of graduates
University of Toronto Faculty Scholar

Research Experience and Publications

Prospective study of a dynamic method of abdominal wound closure — Dec 2002-present
University of Ottawa, Division of Plastic Surgery, Ottawa, Ontario
Supervisor: Dr. M. Allan, Associate Professor, Plastic Surgery

Presented at *Canadian Surgery Forum*, Vancouver, September 2003

A dynamic method for delayed primary closure of fasciotomy wounds — Sept 2002-Jan 2003
University of Ottawa, Division of Plastic Surgery, Ottawa, Ontario
Supervisor: Dr. M. Bell, Associate Professor, Plastic Surgery

Taylor R, Reitsma, B, Sarrazin S, Bell M. Early results using a dynamic method for delayed primary closure of fasciotomy wounds. *Amer J Surg*. (publication Nov 2003)

Barriers to selection of a career in general surgery among female medical students — Sept 2002-present
Queens University, Division of General Surgery, Kingston, Ontario
Supervisor: Dr. D. Poenaru, Associate Professor, General Surgery

Presented at *Canadian Surgery Forum*, Vancouver, September 2003

Prophylactic β-blockade for the prevention of perioperative myocardial infarction in high risk general surgery patients — Sept 2001-Sept 2002
University of Ottawa, Division of General Surgery, Ottawa, Ontario
Supervisor: Dr. G. Pagliarello, Associate Professor, General Surgery

Taylor R, Pagliarello G. Prophylactic β-blockade for the prevention of perioperative myocardial infarction in high risk general surgery patients. *Can J Surg*. 2003;46(3):216-22

Self Expanding Metal Stents for the palliation of malignant esophageal strictures — Aug 2000 - Sept 2001
University of Ottawa, Department of Thoracic Surgery, Ottawa, Ontario
Supervisor: Dr. Reza Mehran, Associate Professor, Thoracic Surgery

Presented at *Canadian Thoracic Surgery Society*, Quebec City, September 2001

The clinical use of Tamoxifen for breast cancer chemoprevention — April 2000 - Nov 2001
Queen's University, Department of General Surgery, Kingston, Ontario
Supervisor: Dr. Kenneth Taguchi, Associate Professor, General Surgery

Presented at *Canadian Surgery Forum*, Quebec City, September 2001

Taylor R, Taguchi K. Tamoxifen for breast cancer chemoprevention: a "paradigm shift" with an insignificant effect on clinical practice. *CMAJ.* (submitted July 2003)

Surgical treatment for infertility of fallopian tube etiology — April - Aug. 1998
BC Research Institute for Women's Health, Vancouver, British Columbia
Supervisor: Dr. Peter McComb, Program Director, Obstetrics and Gynecology

Taylor RC. Berkowitz J. McComb PF. Role of laparoscopic salpingostomy in the treatment of hydrosalpinx. *Fertility & Sterility. 75(3):594-600, 2001 Mar.*

Athletic Involvement

Ottawa Irish Women's Rugby Club 2001-2003
Eastern Ontario Regional Champions

Ottawa Carleton Ultimate Association 2000-2002
Recreational co-ed team

Queen's University
Women's Varsity Ultimate 1996- 1999
National Ultimate Championships in Edmonton, Alberta
Intramural Athletics 1996-1999
Athletic Representative for the Faculty of Medicine
First Year Representative for the Faculty of Medicine

University of Toronto
Women's Varsity Rugby 1994-1996
OWIAA silver medallists in Hamilton, Ontario
T-Holder Academic and Athletic Excellence Award
Intramural Athletics 1992-1996
Vice President of the St. Hilda's Athletic Association
Secretary of the St. Hilda's Athletic Association
Francis Endicote Award for outstanding contribution to intramural athletics
Female Athlete of the Year 1995

University Involvement

University of Ottawa 2000-2003
Surgical Post-Graduate Education Committee Representative
Christmas Rounds Coordinator

Queen's University 1996-1999
Vice President for the Medical School Class of 2000
Queen's Medicine Curriculum Review Committee
Aesculapian Society and *Medical House* Executive Member
Medical Variety Night Performer and Writer
Coffee House Organizer and Performer for *Interval House* Women's Shelter
Queen's Medicine *Sharing with the Future* Coordinator

University of Toronto and Trinity College 1992-1996
Academic Peer Counselor for Department of Pharmacology
Conversat Variety Night Director
Trinity Literary Debating Society Member
Trinity Science and Nature Club Secretary
St. Hilda's College Head of Second Year

Community Involvement

Volunteer at *Camp Trillium* for children with cancer 1999
Outdoor Leader for *Project DARE* youth correctional facility 1996
Wilderness Instructor at *Boundless Adventures* for populations at risk 1995
Science Instructor for *Innovators in the Schools* 1994
Program Facilitator at *Forward Avenue Emergency Housing Shelter* 1993