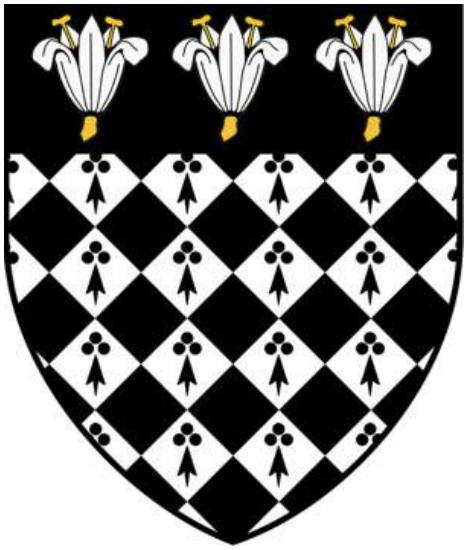
The Innate Immune Effector Cell Response Against HIV-1





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A thesis submitted for the degree of

Doctor of Philosophy

Trinity Term 2013

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Abstract

Since being identified as the cause of AIDS in 1983, HIV-1 infection has reached pandemic proportions. Despite public awareness about prevention, the growing incidence of HIV-1 infection and the limitations of current antiretroviral therapy underscore the imperative need for a vaccine. Understanding the basis of an immune response that controls infection or provides sterilizing immunity remains a major goal in the search for effective vaccines or immunotherapies. Research into correlates of immunity to HIV-1 have largely focused on $CD8^+$ T cells or neutralising antibodies (NAbs) but to date these responses have not proved effective in containing viral replication in vaccinees who become infected.

Natural killer cells (NKs), monocytes (MCs), and neutrophils (PMNs) are cells of the innate immune system with intrinsic cytotoxic function that can be enhanced by antibodies (Abs) in what is termed antibody-dependent cellular cytotoxicity (ADCC). In my studies I investigated the production of PMNs from human stem cells, the elimination of HIV-1 infected cells by these effector cells, the modulation of cellular cytotoxicity by Ab, and characterized how Abs facilitate a potent ADCC response. I developed a novel flow cytometry assay to measure cytotoxic activity against HIV-1 infected $CD4^+$ T cells. Using this, effector cells were shown to have different cytotoxic capacities which were enhanced by Ab. Comparing ADCC mediated by patient serum revealed that higher levels correlated with IgG binding to infected cells. I observed no correlation between serum-mediated ADCC and markers of disease progression including patient status, viral RNA load, $CD4^+$ T cell count, or NAb titers. The data presented here have implications for acquisition and control of early HIV-1 infection by NKs, MCs, and PMNs prior to activation of an adaptive immune response, at later stages in the presence of HIV-1-specific Abs, and are relevant to vaccine-induced anti-HIV-1 Ab-based effector mechanisms.

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For making me breakfast everyday, driving me to NIH and listening to all the children's books on CD during our daily 3 hour commute, giving me a hug when I ran down to your lab frustrated, for being my lunch buddy, picking me up at midnight or coming in at 6 am to bring me breakfast, bringing me a change of clothes and lunch if I unexpectedly stayed overnight in lab, and for going to every bakery in DC when I needed a cupcake

Mom
For telling me to stop crying, man up, and get back to work!



Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrome
bNAb	Broad neutralising antibody
Bpool	HIV-1^{+} Serum from clade B infected patients
BSA	Bovine serum albumin
cRMPI	Complete RPMI
DC	Dendritic cell
Env	Envelope
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallisable
FcR	Fc Receptor
G-CSF	Granulocyte colony-stimulating factor
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte macrophage colony-stimulating factor
GrB	Granzyme B
HAART	Highly active antiretroviral therapy
hESC	Human embryonic stem cell
HESN	HIV exposed seronegative
HIV-1	Human immunodeficiency virus type 1
ICE	Infected cell elimination
Ig	Immunoglobulin
IMC	Infectious molecular clone
KIR	Killer cell immunoglobulin-like receptor
LTNP	Long-term nonprogressor
mAb	monoclonal antibody
MC	Monocyte
MFI	Median fluorescent intensity
MP	Macrophage
NAb	Neutralising antibody
NET	Neutrophil extracellular trap
NK	Natural killer cell
PBMC	Peripheral blood mononuclear cell
PBS	PBS
PMN	Polymorphonuclear cell, neutrophil
RT	Room temperature

Chapter 1

Introduction

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1.1 HIV-1 Pandemic

In 1959 a Bantu male living in Kinshasa, DRC died of a mysterious illness (Zhu et al., 1998). Twenty-two years later on June 5, 1981 the CDC MMWR reported 5 cases of *Pneumocystis* pneumonia (PCP) in 5 homosexual men living in Los Angeles. Additional cases of PCP and Kaposi's sarcoma in homosexual men living in NYC and California were reported less than a month later in the July 4, 1981 CDC MMWR report (Masur et al., 1981). These cases were striking because these diseases typically manifested in immunosuppressed transplant patients, not young previously healthy individuals (Fig. 1.1.1).

A. B.

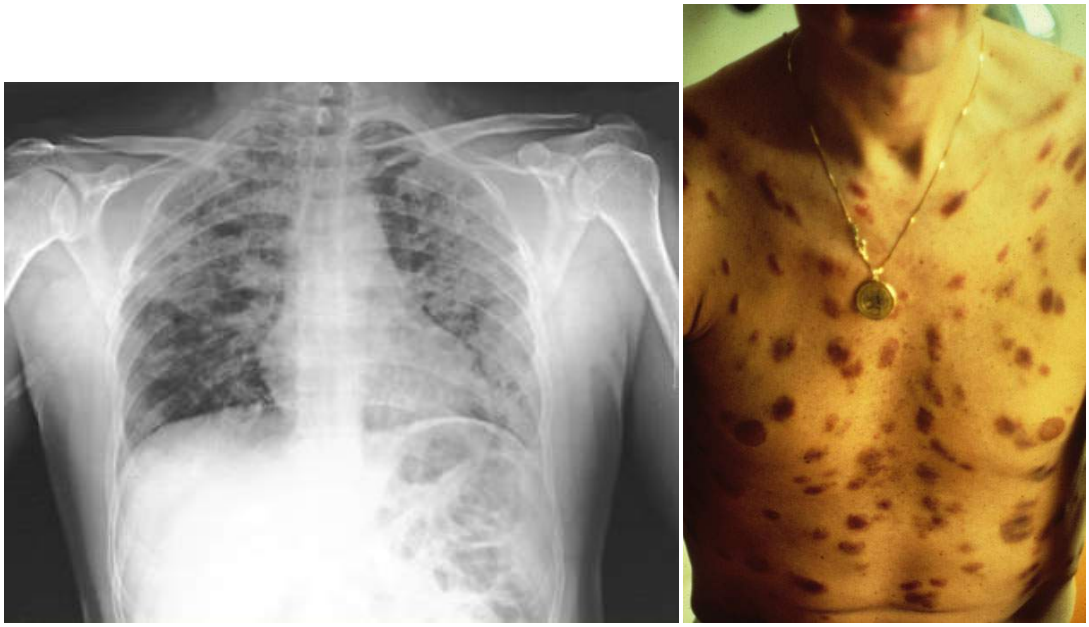


Figure 1.1.1. *Pneumocystic* pneumonia and Kaposi's sarcoma, opportunistic infections characteristic of the early HIV/AIDS epidemic. (A) X-ray of a lung of a patient with *Pneumocystic jiroveci* pneumonia (PCP, formerly called Pneumocystis carinii) (Thomas and Limper, 2004). Image adapted from www.medscape.com. (B) Patient suffering from Kaposi's sarcoma, a cancer caused by human herpes virus 8 (Antman and Chang, 2000). Photo by Paul A. Volberding, MD, UCSF; image adapted from www.hiv.va.gov.

In 1983 Drs. Françoise Barré-Sinoussi and Luc Montagnier at the Pasteur Institute identified lymphadenopathy-associated virus (LAV) as the virus responsible for T cell loss in homosexual and haemophiliac patients (Barre-Sinoussi et al., 1983). They were awarded the 2008 Nobel Prize in Physiology or Medicine for this discovery. In 1984 Dr. Robert Gallo at the National Cancer Institute identified human T-cell lymphotrophyic virus III (HTLV III) as a possible cause of AIDS (Gallo et al., 1984). LAV and HTLV III were identified as the same virus and termed human immunodeficiency virus (HIV). There are two subtypes of HIV, 1 and 2. HIV-1 is more virulent and accounts for most of the infections worldwide (Delves, 2011). HIV-2 was discovered in 1986, is less pathogenic, and largely confined to West Africa such as Senegal, Ghana, Nigeria, and the Ivory Coast (Clavel et al., 1986; Nyamweya et al., 2013). HIV is believed to be simian immunodeficiency virus (SIV), which crossed from non-human primates to humans in Africa during the harvesting of bush meat in the early to mid 20^{th} century. HIV-1 is related to SIV_{cpz} which infects chimpanzees and HIV-2 is closely related to SIV_{smm} which's natural host is the sooty mangabey (Hirsch et al., 1989; Huet et al., 1990).

Routine screening of blood products for HIV did not begin in the United States until March 1985 even though HIV was known to be transmitted through blood as early as 1982. Some countries did not begin screening blood until the 1990s so as a result, most cases of HIV early in the epidemic were due to blood transfusions.

Currently 2 million people are infected and die from AIDS and AIDS-related conditions each year. The UNAIDS organization estimates that there are

approximately 34 million people living with HIV-1. Seven million deaths have occurred since the start of the epidemic with most deaths occurring in sub-Saharan Africa but the epidemic has recently burgeoned in India (Fig. 1.1.2).



Figure 1.1.2. Deaths due to HIV-1. Adapted from http://betterhealthcareforafrica.org.

Note: Henceforth this report will discuss HIV-1.

1.2 HIV-1 Infection

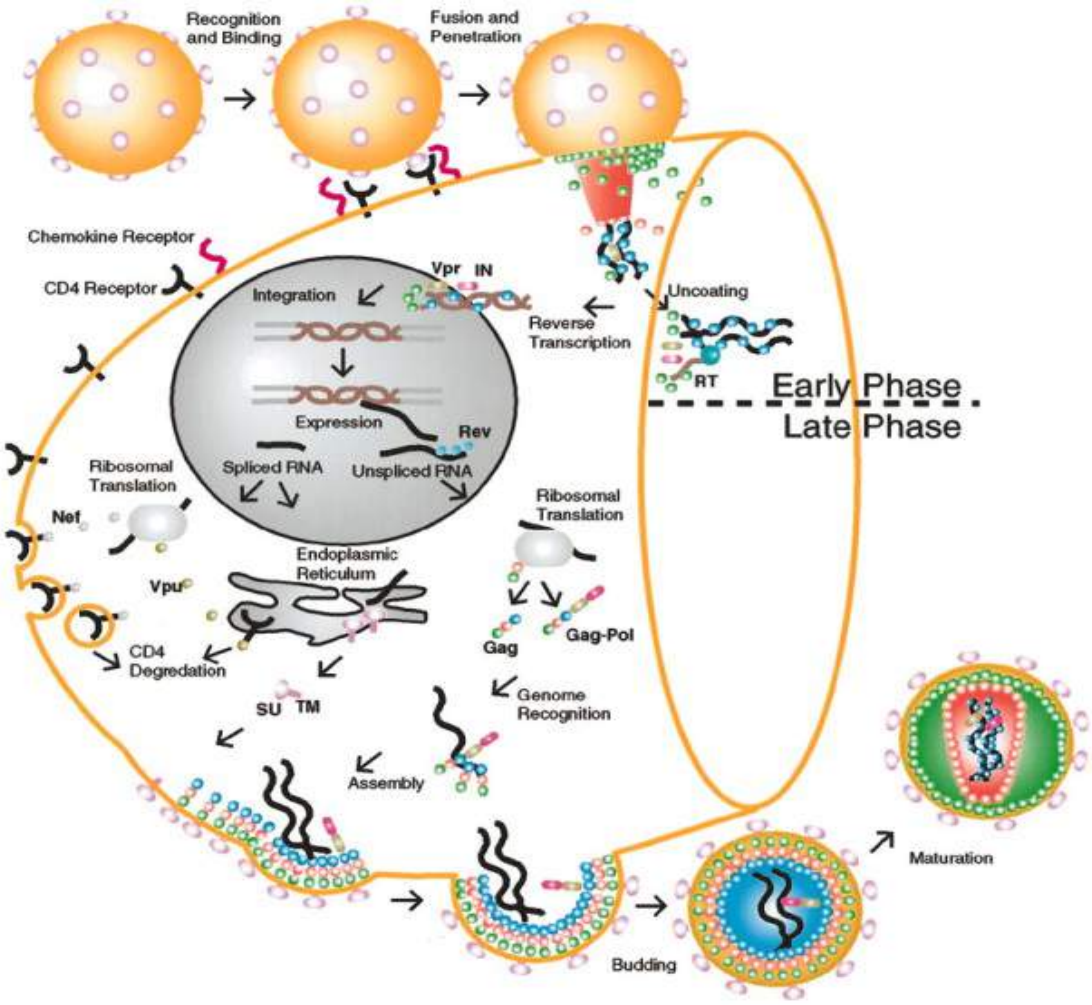


Figure 1.2.1. General features of the HIV-1 replication cycle. The early phase (upper portion of the diagram) begins with CD4 recognition and involves events up to and including integration of the proviral DNA, and the late phase includes all events from transcription of the integrated DNA to virus budding and maturation. Adapted from (Turner and Summers, 1999).

HIV-1 is a retrovirus that infects predominantly $CD4^+$ T cells but also macrophages (MPs) and dendritic cells (DCs). Retroviruses reverse-transcribe their RNA into cDNA which is integrated into the genome of the host cell. $CD4^+$ T helper cells recognize antigen presented by antigen presenting cells via MHC-class II, and secrete cytokines to activate effector cells that clear infectious agents (Delves, 2011). Figure 1.2.1 diagrams the HIV-1 replication cycle. To infect a cell HIV-1 first binds to the cell using non-

specific attachments such as cell surface lectins (e.g. DC-SIGN) and heparan sulfate proteoglycans (Geijtenbeek and van Kooyk, 2003; Patel et al., 1993). The surface of the HIV-1 virion is covered by an envelope spike protein (Env) that is a trimer of the gp120 and gp41 heterodimer (Lu et al., 1995). Gp120 binds to CD4 on the T cell, inducing a conformational change and exposing the coreceptor binding site (Dalgleish et al., 1984; Gomez and Hope, 2005; Lu et al., 1995). X4 or R5 viruses use either CXCR4 or CCR5 coreceptor, respectively, and these viruses have specific tropisms for various cell types. Receptor tropism is determined by the V3 loop of gp120 (De Jong et al., 1992; Prosperi et al., 2010). X4 viruses tend to infect naïve $CD4^+$ T cells and mature DCs whereas macrophages (MPs), immature DCs, and activated/memory $CD4^+$ T cells are infected by R5 virus (Bjorndal et al., 1997). Primary infections tend to be dominated by R5 viruses and over the course of infection tropism frequently switches to X4. Following coreceptor binding a conformational change in gp41 exposes its fusion peptide so that the virion and cell membranes fuse and the viral contents (including two copies of single stranded positive-sense RNA, protease, reverse transcriptase, and integrase) are released into the cell (Friedrich et al., 2011). In the cytoplasm viral RNA is reverse transcribed into cDNA and this preintegration complex is transported into the nucleus where it integrates into host DNA. As host DNA is transcribed so is the integrated viral cDNA. Viral RNA transcripts are exported from the nucleus where spliced RNA is translated into proteins. Unspliced RNA associates with newly created proteins at the host cell membrane where it buds from the host cell as a new

virus particle, ready to infect other cells (Delves, 2011; Friedrich et al., 2011; Turner and Summers, 1999).

Cell-free transmission is one method by which HIV-1 spreads within the body but increasing evidence shows cell-to-cell transmission via virological synapses to be more efficient and probably responsible for most cellular infection in vitro and in vivo (Groot et al., 2008; Jolly et al., 2004; Murooka et al., 2012). HIV-1's crippling effect on the immune system is principally due to the destruction of $CD4^+$ T cells. Recently $CD4^+$ T cell death has been attributed to the process of pro-viral cDNA integration into host cell DNA. Cooper et al. found that host DNA double strand breaks triggers the DNA damage response in which DNA-dependent protein kinase (DNA-PK) is activated thus phosphorylating p53 which induces apoptosis (Cooper et al., 2013).

1.3 HIV-1 Pathogenesis

Most HIV-1 transmission is via sexual intercourse but other modes include drug injection, exposure to contaminated blood such as through transfusion, and mother-to-child vertical transmission (Moir et al., 2011). In sexual transmission CD4-bearing langerhans cells (DCs) at the genital mucosa are likely the first cells to be infected followed by $CD4^+$ T cells that are more productive in replicating HIV-1 (Shattock and Moore, 2003). The genital mucosa is lined with squamous epithelial cells that act as a physical barrier to infection but structural breaches or the transcytosis of infectable cells towards the surface facilitates infection by virions in semen or vaginal secretions. Within two weeks the virus has travelled via draining lymph nodes

to the lamina propria of gut-associated lymphoid tissue (GALT) (Haase, 2010; Li et al., 2005; Zhang et al., 1999). Within a month a productive infection is established resulting in expansive replication of HIV-1 in GALT, massive depletion of memory $CD4^+$ T cells, and peak viremia levels of about 1 million copies of RNA/mL (Li et al., 2005). This acute HIV-1 syndrome may be subclinical or may be characterized by flu-like symptoms. During this time anti-HIV-1 antibodies (Abs) are raised and HIV-1 specific $CD8^+$ T cells emerge. Eventually the patient enters an asymptomatic phase in which plasma viral load decreases to a steady state, $CD4^+$ T cell levels restabilize to near normal levels (800 cells/μL compared to normal levels of 1200 cells/μL), and the virus establishes latent reservoirs in tissues throughout the body. These cellular reservoirs include $CD4^+$ T cells of the GALT, follicular DCs of peripheral lymph nodes (LN), and latently infected resting $CD4^+$ T cells. The LN provides an environment for efficient cell-to-cell transfer because it puts uninfected $CD4^+$ T cell target cells in close contact with infected $CD4^+$ T cells and DCs. During latent infection $CD4^+$ T cells are not activated and therefore not recognized by the immune system, which prevents clearance of these cells and allows for the establishment of a chronic infection.

During chronic infection $CD4^+$ T cells are depleted due to several mechanisms including: HIV-induced apoptosis of T cells, T cell exhaustion, reduced thymic function, targeted elimination of infected cells by effector cells, and the “bystander” effect (Cooper et al., 2013; Cummins and Badley, 2010). As previously mentioned, the integration of HIV-1 DNA results in double strand DNA breaks which trigger p53-mediated apoptosis via DNA-PK

phosphorylation (Cooper et al., 2013). In T cell exhaustion, activation-induced cell death results from increased cell susceptibility to FasL-induced apoptosis in virus infected cells undergoing active replication. T cell exhaustion can also occur by activated T cell autonomous death (ACAD) where Tat and Nef proteins donwnregulate expression of the anti-apoptotic Bcl-2 so that cells are primed for apoptosis (Boudet et al., 1996; Gougeon, 2003; Gougeon et al., 1996; Oyaizu et al., 1993). In fact, the therapeutic administration of IL-2 works by upregulating Bcl-2 expression, staying ACAD and thus potentiating survival of $CD4^+$ T cells (Adachi et al., 1996). HIV-1 infected cells are also targeted by NKs due to HIV-1-induced downregulation of MHC class I and recognized by $CD8^+$ T cells by MHC class I-restricted antigen presentation. Despite apoptosis induced by active infection within a cell or targeted lysis of infected cells (discussed in Sections 1.7 and 1.8), it does not account for the massive depletion observed *in vivo.* Rather, a larger number of uninfected $CD4^+$ T cells are targeted by effectors or have apoptosis induced because of exposure and proximity to viral products in what is known as the bystander effect (Finkel et al., 1995). Several HIV proteins have been described to act by various mechanisms (Alimonti et al., 2003). For example, Tat secreted from infected cells can be endocytosed and upregulate caspase 8 which induces apoptosis (Mann and Frankel, 1991; Zagury et al., 1998). Env is expressed on the surface of infected cells and is also shed, thus available in a soluble form. Gp120 interaction with CD4 and CCR5 co-receptor, and partial gp41 mediated membrane fusion is enough to partially activate the cell but without specific TCR engagement

downstream apoptotic pathways are initiated (Garg et al., 2007; Joshi et al., 2011; Marschner et al., 2002).

The plasma viral load set point during this asymptomatic phase is prognostic of disease progression (Fig. 1.3.1) (Mellors et al., 1996; van der Loeff et al., 2010). Lower viral loads are associated with slower progression. The rate of disease progression is also determined by the strain of virus and MHC-class I haplotype (see Section 1.4). During chronic disease the body is in a constant state of inflammation characterized by increased cell turnover of NKs, B cells, $CD4^+$ T cells, and $CD8^+$ T cells; increased secretion of inflammatory cytokines such as TNF-α and IL-1; and cellular senescence. Eventually cells lose their capacity to regenerate causing infected individuals to be more susceptible to opportunistic infections (Moir et al., 2011).

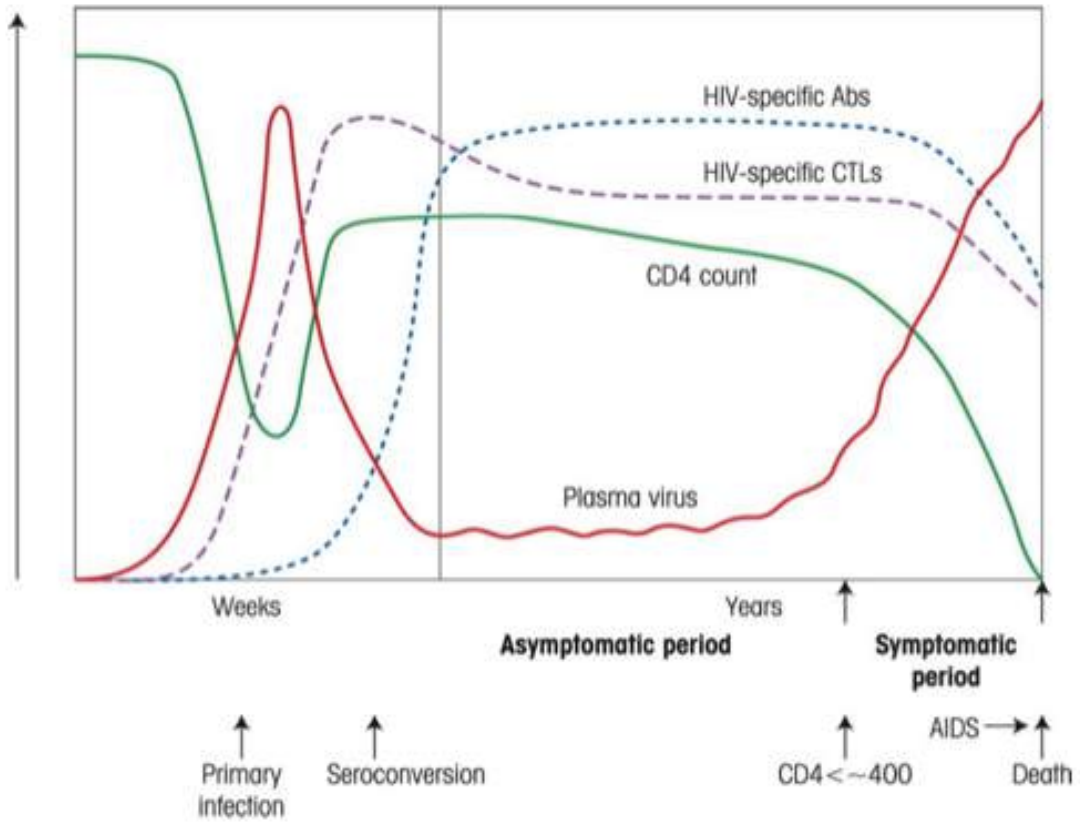


Figure 1.3.1. The typical course of HIV-1 infection. Primary infection is characterized by a rapid rise in plasma virus and a rapid decline in circulating $CD4^+$ T cells. The plasma virus levels peak and decline to a low roughly constant level ("the set point"), which is predictive of the time of progression to disease. The $CD4^+$ T cell count recovers somewhat but to a lower level than prior to infection. The HIV-specific $CD8^+$ T cell response is activated as virus peaks and is probably important in controlling primary infection. The HIV-specific antibody response takes somewhat longer to initiate and results in seroconversion. The neutralising antibody response is yet slower to initiate. Clinical latency follows primary infection for a period of the order of a decade. No symptoms are apparent but depletion of $CD4^+$ T-cells in lymphoid tissues continues. Eventually, $CD4^+$ T-cell depletion is so pronounced that resistance to opportunistic infections begins to wane, leading ultimately to a complete collapse of a functioning immune system and death. Drug intervention can take plasma viral loads below the level of detection and prevent $CD4^+$ T-cell depletion. Adapted from (Delves, 2011).

1.4 Immune control of HIV-1

Once infected with HIV-1 and in the absence of treatment, individuals normally progress to AIDS in about 10 years. AIDS is defined as having a $CD4^+$ T cell count < 200 cells/μL and an opportunistic infection. About 1% of HIV-1-infected people become long-term nonprogressors (LTNP, alternatively elite controllers, EC). These people have been infected for at least 5 years and without treatment maintain stable $CD4^+$ T cell counts and non-detectable viral loads (Casado et al., 2010; Walker and Yu, 2013). Some of the traits contributing to immune control in LTNPs are *HLA-B57* and *B27* haplotypes, and $CD8^+$ T cells with increased capacity for perforin/granzyme B degranulation (Migueles et al., 2008). Rapid progressors comprise about 10% of the population and are those that progress to AIDS within two to three years of infection (Casado et al., 2010; Khanlou et al., 1997). Some of the HLA markers associated with rapid progression are *HLA-B1801* and *HLA-B35*. There are also people repeatedly exposed to HIV-1 but who remain seronegative (HIV-exposed seronegative, HESN) (Kaul et al., 2001a; Rowland-Jones et al., 1995; Rowland-Jones et al., 1998). Data suggests enhanced anti-HIV $CD8^+$ T cell responses, lower levels of certain cytokines (e.g. TNF-α, IFN-γ) at the genital mucosa, antiprotease at mucosal sites, and certain natural killer cell KIR alleles convey protection from infection; but more extensive studies of cohorts of people such as sex workers in Nairobi, Kenya and Contonou, Benin is necessary in order to gain a better

understanding of how they naturally resist infection. (Bienzle et al., 2000; Lajoie et al., 2012; Lajoie et al., 2008; Tomescu et al., 2011).

1.5 Treatment

In 1986 azidothymidine (AZT), a nucleoside reverse-transcriptase inhibitor (NRTI) was first used in clinical trials to treat HIV-1 and by 1987 it had been approved for this use by the FDA (Mitsuya et al., 1985; Nakashima et al., 1986). In 1996 highly active antiretroviral treatment (HAART) was introduced which drastically improved HIV-1 survival rates. HIV-1 was no longer a rapid death sentence but rather, a chronic disease. HAART consisted of using three drugs, two nucleoside reverse transcriptase inhibitors and saquinavir, a novel protease inhibitor (Ho, 1995; Maenza and Flexner, 1998; Palella et al., 1998; Palmisano and Vella, 2011). Since then several classes of drugs that target different phases of the HIV-1 life cycle have been developed including non-nucleoside reverse transcriptase inhibitors, fusion inhibitors, integrase inhibitors, and chemokine receptor agonists (Palmisano and Vella, 2011). Increasing evidence supports the initiation of HAART immediately after diagnosis because the level of $CD4^+$ T cells at the start of treatment is an indicator of the degree of $CD4^+$ T cell restoration (Kitahata et al., 2009; Sterne et al., 2009). As of June 2013, the World Health Organization recommends starting HAART at 500 $CD4^+$ T cells/μL which is elevated from the 2010 recommendation of 350 $CD4^+$ T cells/μL (WHO, 2013). As more people are living longer on these treatments side effects that include immune reconstitution syndrome, drug resistant virus, and cardiovascular disease especially with non-nucleoside reverse transcriptase and protease inhibitors,

is becoming a challenge as significant as managing the disease itself (Moir et al., 2011).

Despite the robust therapies available today, perhaps the greatest challenge in “curing” people of HIV-1 is the persistence of the latent reservoir. Latently infected $CD4^+$ T cells are not activated and therefore not recognized by the immune system, preventing clearance of these cells and driving the establishment of chronic infection (Dolgin, 2013). Dahabieh and colleagues reported that a majority of integrated HIV-1 is latent following $CD4^+$ T cell infection (Dahabieh et al., 2013). Strategies that reactivate dormant HIV-1 in $CD4^+$ T cells, for example with IL-7 or histone deacetylase inhibitor suberoylanilide hydroxamic acid, then clearing these “actively” infected cells for example via $CD8^+$ T cells are of increasing focus in research (Chomont et al., 2009; Shan et al., 2012; Shan and Siliciano, 2013).

Within the past few years there have been reports of people “cured” of HIV-1. In one instance an HIV-1-infected man was “cured” when he received a stem cell bone marrow transplant from a donor whose cells were homozygous for CCR5Δ32 and thus resistant to HIV-1 infection (Allers et al., 2010). A toddler was declared “cured” at 18 months after starting an aggressive course of antiretroviral therapy two days after birth (Cohen, 2013). These reports are encouraging and provide proof of concept, but these treatments are not plausible for the general population.

Perhaps a new paradigm in therapy may be required to eradicate HIV-1. Gavrilyuk et al. propose coupling neutralising antibodies (NAbs) to small molecule inhibitors of viral entry, (Gavrilyuk et al., 2013). The use of RNA aptamers to treat HIV-1 infection is another burgeoning field that is likely to

see more development (Watrin et al., 2009; Wheeler et al., 2013; Yamazaki and Famulok, 2009; Zhou et al., 2009).

1.6 Vaccines

The best treatment for a lethal disease is prevention so efforts to develop a vaccine began immediately after the identification of the causative agent of AIDS. In 1984 Margaret Heckler, the US Health and Human Services Secretary boldly stated that there would be a vaccine for HIV in two years, a claim no doubt based on the success of the polio and small pox vaccine programs at the time, but 29 years later the world still waits.

Upon first exposure to a virus the $CD8^+$ T cell response predominates in clearing the infection. It is believed a $CD8^+$ T cell response will be important in an HIV-1 vaccine because of protective correlates in HESN and LTNPs, although protective $CD8^+$ T cell responses have not been induced by any vaccines to date. The Ab response is secondary and responsible for most of the protective vaccine-induced memory response because memory B cells can be mobilized to secrete Abs within 30 minutes. Passive transfer of Abs is frequently used to treat exposure to viruses such as rabies, hepatitis B, and cytomegalovirus. An attenuated virus vaccine would best generate a memory response because it mimics natural infection at respective host sites, and in the case of HIV, would display Env proteins within the membrane bound from with natural glycosylation patterns or present antigen in the context of MHC class I. Attenuated virus is used for vaccination against small pox, influenza, rotavirus, yellow fever, and varicella zoster (Delves, 2011). In the initial stages of designing a vaccine for HIV-1, a live attenuated SIV with a deletion

to *nef* was used to vaccinate macaques and they were protected from SIV challenge but four years laer he attenuated SIV reverted to a pathogenic form. (Daniel et al., 1992). The potential of reversion of an attenuated HIV-1 vaccine is an ethical barrier against using such a vaccine in people. The primary strategy for HIV-1 vaccination today involves priming with a DNA vector usually containing genes for *gag* and *env* that would evoke a $CD8^+$ T cell response and Abs against Env, and then boosting usually with Env to expand memory B cells, hopefully producing NAbs.

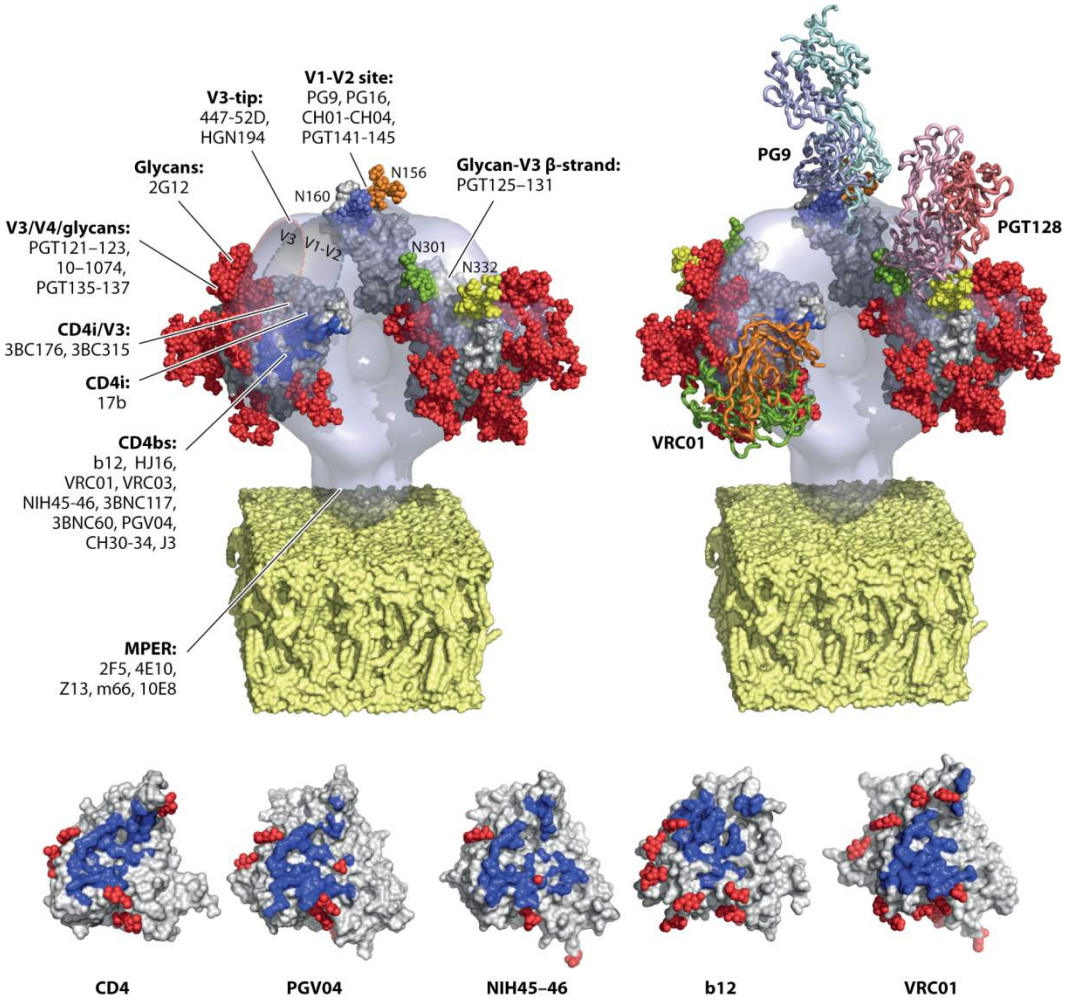
The first HIV-1 vaccine trial, AIDSVAX, was carried out from 1998-2003. It was a gp120-based vaccine designed to induce a NAb response but was halted when it showed no efficacy (Excler et al., 2013). After 2 years, in 2007 the phase IIb STEP Study was suspended after the vaccine, composed of *gag*, *nef*, and *pol* genes in an adenovirus vector (Ad5), failed to reduce viral loads and increased the susceptibility to HIV-1 in vaccinees previously exposed to the vector, Ad5. Most recently in April 2013, a phase IIb trial of HVTN505, which again used an adenovector, was halted due to futility. To date the only successful HIV-1 vaccine trial was the phase III RV144 Thai Trial carried out from 2003-2006. Composed of a prime with a HIV-1 *env*, *gag*, and *pol* genes in a canary pox vector and boosted with VaxGen gp120, it reduced infection by 31.2% although it failed to elicit a strong cellular or NAb humoral response (Rerks-Ngarm et al., 2009). Initially it was speculated that the vaccine's efficacy might have been due to antibody-dependent cellular cytotoxicity (ADCC, discussed further in Section 1.9). Recent analysis shows an inverse correlation between anti-gp120 V2 loop IgG and HIV-1

acquisition but there is a correlation between anti-Env IgA and increased risk (Excler et al., 2013; Karasavvas et al., 2012).

1.7 Preventing and Eliminating Early HIV-1 Infection

1.7.1 Immune response to an infection

There are many immune mechanisms that play a role in the natural defence against HIV-1; virtually every cell is implicated, including $CD4^+$ and $CD8^+$ T cells, natural killer cells (NKs), monocytes (MCs), MPs, DCs, and B cells via Ab production. As in the prevention of many other viral infections one of the primary means is to elicit neutralising Abs (NAbs) that would act to prevent entry of HIV-1 into a cell. Another way to prevent the establishment of fulminant infection would be to kill the initial cells that become infected before multiple rounds of replication occur. In order to do this most vaccine efforts have focused on priming $CD8^+$ T cells for a memory response. Abs and $CD8^+$ T cells are two of the primary ways that body responds to HIV-1 infection (Fig. 1.3.1) however by the time these responses are generated during the natural course of infection HIV-1 has gained traction in the body. Thus far efforts to elicit these protective responses by a vaccine have been mostly ineffective.



Corti D, Lanzavecchia A. 2013.
Annu. Rev. Immunol. 31:705–42

Figure 1.7.1 Model of the HIV-1 Env trimeric glycoprotein bound to broadly neutralising antibodies. The 3-dimensional model of the Env trimer (Electron Microscopy Data Bank code EM-5019) with two of the three gp120 subunits highlighted in gray was positioned on a modelled lipid bilayer. The left panel shows the major sites targeted by broadly neutralising antibodies. The approximate positioning of the V1/V2 and V3 loops is shown, and the CD4 footprint on the gp120 monomer is highlighted in blue. The right panel shows the Fabs of broadly neutralising antibodies VRC01 (3ngb), PG9 (3us2), and PGT128 (3tyg) bound to gp120. Carbohydrates (oligomannose, *red spheres*) were modelled on the unliganded YU2 gp120 core (3tgq) using GlyProt, with the exception of the glycans bound by PGT128 and PG9 (depicted with different colours), which were taken from the structures. The location of PG9 above the trimeric gp120 is approximate; VRC01 and PGT128 Fabs were docked by superposition with the unliganded YU2 gp120 model. The bottom panel shows in blue the footprints of CD4 (1gc1) and the CD4bs-specific antibodies PGV04 (3se9), NIH45-46 (3u7y), b12 (3dnl), and VRC01 (3ngb). Figures were made with PyMOL. Adapted from (Corti and Lanzavecchia, 2013).

1.7.2 Neutralising Abs

NAbs bind to Env, preventing binding of HIV-1 to cellular receptors and/or subsequent membrane fusion. NAbs recognize mature trimers expressed on the surface of virions and infected cells. The envelope glycoprotein (gp) is first synthesized as a gp160 trimer in the endoplasmic reticulum (Checkley et al., 2011). In the Golgi apparatus oligosaccharides are added and gp160 is cleaved by HIV-1 protease into mature gp120 and gp41 subunits that associate by noncovalent interactions. The heterotrimer is secreted from the Golgi to the plasma membrane where incorporation into new virions is directed by matrix protein association with Env C-terminal tail of gp41 (Muranyi et al., 2013). About 7-14 Env are expressed on the surface nascent virion (Chertova et al., 2002; Zhu et al., 2003). This low number is thought to be due to recycling of Env via endocytosis when it reaches the PM or shedding and the paucity of Env helps the virus elude recognition by the immune system. *Env* is highly mutagenic and masking of conserved epitopes by glycans make elicitation of NAbs difficult. Furthermore, immunodominant immune responses are often against immature, misfolded, or decayed Env. Non-neutralising Abs can recognize gp120/gp41 monomers and gp120-gp41 depleted stumps on monomers (Moore et al., 2006). Gp41 stumps on virus and cell membranes are generated when gp120 is shed due the instability of the noncovalent interactions. This shedding can be induced by interaction with soluble CD4 (Moore et al., 2006). In addition, shed gp120 is also thought to be generated by aberrant gp120 processing before functional trimers reach the cell membrane surface (Chertova et al., 2002). Immature gp160 monomers with untrimmed high-mannose glycans included in virus-like particles are likely result from a breakdown in ER processing from HIV-1

induced stress on the cell and so that pg160 is incorporated into budding virus (Crooks et al., 2011).

NAbs start to be generated about 12 weeks after initial infection but the response lags behind the evolution of the virus. In the natural course of infection a patient's Abs are usually not effective in neutralising their current predominant viral variant (Richman et al., 2003; Wei et al., 2003). Naturally raised NAbs have not been shown to play a role in slowing progression to disease in patients such as LTNPs (Bailey et al., 2006; Doria-Rose et al., 2010). Only 10-30% of infected individuals develop broad NAbs (bNAbs) which can neutralise multiple strains of virus. These require several rounds of somatic mutations and are more prevalent in patients with higher viral loads (Liao et al., 2013; Walker et al., 2011). Irrespective of this fact, the search for bNAbs has been an immense part of the HIV-1 vaccine effort because numerous animal studies have demonstrated that NAbs protect against or limit SHIV (simian human immunodeficiency virus)/HIV-1 infection in animals. Fig. 1.7.1 shows most of the bNAbs that have been identified for HIV-1. NAbs elicited by vaccination with HIV-1 Env protected macaques from SHIV challenge (Bogers et al., 2008). Passive transfer of the bNAb b12 protected macaques from challenge with SHIV (Hessell et al., 2009). Klein and colleagues demonstrated that a cocktail of 5 bNAbs could suppress viral loads in humanized mice infected with HIV-1 (Klein et al., 2012). To date no human vaccine has induced NAbs but perhaps stabilizing Env antigen as Schiffner and colleagues did with glutaraldehyde so that constitutively exposed, conserved surfaces are better presented will prove to be an effective strategy in eliciting NAbs (Schiffner et al., 2013). In two studies

Hessell and colleagues demonstrated that effector functions mediated by the Fc portion of NAbs may have played a role in protecting macaques which highlighted in a different manner the importance of Abs in controlling HIV-1 infection (see Section 1.9.3) (Hessell et al., 2007; Hessell et al., 2009).

1.7.3 Elimination of early infected cells

The most robust evidence for preventing and controlling HIV-1 infection shows $CD8^+$ T cells to be essential. Heightened $CD8^+$ T cell responses have been correlated with lack of infection in HESN sex workers, injection drug users, infants of HIV-1^+ mothers, and control of infection in LTNPs (Clerici et al., 1993; Dong et al., 2004; John-Stewart et al., 2009; Kaul et al., 2001b; Migueles et al., 2008; Pinto et al., 1995). Bearing this in mind, other effector cells of the innate immune response that can eliminate infected cells may play an important, yet currently underappreciated role in controlling HIV-1 infection. These cells may work alone sensing infected cells or in concert with Abs of the adaptive immune response that could be generated during the course of infection or by a vaccine.

1.8 Effector Cell Activity

Innate effector cells are so named because they do not need to rearrange their receptor genes in order to recognize antigenic targets, in this case, HIV-1-infected cells. Such effectors are PMNs, MCs and NKs. Their advantage lies in that they can be quickly mobilized in early infection and/or important in prolonged infection when the adaptive immune response is less effective due to viral escape. NKs only kill infected or tumour cells whereas MCs and PMNs can also target free virus. These cells are further discussed.

1.8.1 NK Cells

NK cells (NKs) are large granular lymphocytes. Discovered in 1975 these cells distinguish between normal and abnormal cells, targeting transformed cancer cells, virus-infected cells, or cells having undergone physical or chemical damage (Kiessling et al., 1975a; Kiessling et al., 1975b). NKs comprise 1-6% of leukocytes in peripheral blood (StemCellTechnologies, 2008). NKs recognize viral/tumour cells by two modalities. Normally inhibitory receptors on NKs (including inhibitory killer-cell immunoglobulin-like receptors, KIRs, e.g. KIR2DL; siglecs) bind to MHC-class I molecules on normal cells and this prevents killing by the NK. However tumour and virally infected cells down regulate MHC-class I molecules, so as a result of this "missing-self" signal they are killed. Damaged cells also up-regulate self molecules or express molecules that trigger NK activation receptors (such as FcγRIII, activating KIRs, or NKG2D) in what is termed "stress-induced self recognition" (Di Santo 2006, Vivier 2012).

NKs in HIV-1 infection

NKs play an important role in controlling HIV-1 infection despite their decreased frequency and function as infection progresses (Alter and Altfeld, 2009; Nuvor et al., 2006; Slyker et al., 2012). Certain KIR alleles such as inhibitory KIR3DL1 and activating KIR3DS1 have been implicated in protecting HESN (Tomescu et al., 2011). KIR3DL1 may act by preferentially recognizing cells devoid of specific HLA alleles through HIV-1 infection or it may be characteristic of cells more primed to release cytotoxic granules (Martin et al., 2007). Recently NKs have been shown to place selective pressure on HIV-1, previously only described in $CD8^+$ T cells and Abs. In individuals with inhibitory receptor KIR2DL2 the virus evolved to escape

killing by KIR2DL2-positive NK cells (Alter et al., 2011). NKs have a stimulatory effect on the immune system in the presence of HIV-1 by releasing IFN-γ which stimulates MPs and enhances antigen presentation by DCs (Delves, 2011). In addition, they have also been reported to have a modulatory effect on antiviral $CD8^+$ T cells (Waggoner et al., 2012).

NK cytotoxic effect

Once NK cells recognize pathogenic cells by “missing self” or “altered self” they induce apoptosis in target cells by either of two well-defined mechanisms (Delves, 2011). In the death receptor pathway TNF-α, Fas, and TRAIL ligands on NKs bind respective receptors on the target cell. Engagement of the receptor causes caspase 8 to be cleaved in the target cell. The cell death-signalling cascade continues either through the Bid (BH3 interacting-domain death agonist)/mitochondria pathway or directly downstream through caspase 3. When these specific NK ligands are not engaged the perforin/granzyme pathway predominates. One method of effector-target cell conjugation is via binding of NK FcγRIIIa to the Fc portion of an IgG Ab on the target cell in a process called ADCC (described in Section 1.9.1). Upon crosslinking of the FcR on the NK, there is an increase in Ca^{2+} signalling and polarization of the microtubule (MT) organization centre. Lytic granules containing perforin and the granzyme proteases move in a + to - direction along the MT until they reach the immunological synapse. At the central supramolecular activation complex (cSMAC) recognition and signalling proteins accumulate and actin is cleared (Brown et al., 2011; Stinchcombe et al., 2011). At the peripheral SMAC (pSMAC) additional effector-target cell adhesions are made by integrins on NKs and their

receptors on target cells such as LFA-1 and ICAM-1, respectively (Bryceson et al., 2005). Lytic granules are released into the secretory cleft. Perforin forms a pore in the target cell membrane through which granzyme is delivered. Granzyme B (GrB) has the most killing potential although other granzymes have been identified. GrB activates caspase 8 and target cell apoptosis occurs along the same trajectory as that of the death receptor pathway.

1.8.2 Monocytes

MCs are leukocytes derived from the same myeloid precursor as PMNs (Figure 4.2.3). They comprise 2-12% of leukocytes in peripheral vasculature (StemCellTechnologies, 2008). MCs have fewer and finer granules than PMNs. In response to chemotactic factors they migrate to tissues where they differentiate into MPs and DCs. MCs have a myriad of functions but the primary ones include: (1) phagocytosis by which pathogens, opsonised agents, and apoptotic bodies are cleared, (2) activation of other cells by inflammatory mediators, and (3), antigen presentation, though they are less effective than MPs and DCs (Auffray et al., 2009). Some of the molecules involved in effector mechanisms include reactive oxygen species (ROS), reactive nitrogen species (RNS), complement factors, defensins, prostaglandins, and cytokines such as TNF-α, VEGF, IL-1, and IL-10 (Auffray et al., 2009).

MCs are identified by expression of CD115 and CX3CR1, although CD14, a co-receptor for detecting bacterial LPS is also routinely used. Several subsets of MCs with varying levels of CD14 and other cellular markers exist (Auffray et al., 2009). $CD14^{hi}CD16^{neg}$ MCs represent 80-90% of circulating

monocytes, tend to be larger, and are more phagocytic (Geissmann et al., 2003). $CD14^{hi}CD16^{hi}$ MCs tend to be smaller, produce more cytokines, and express other FcRs, i.e. CD64 (FcγRI) and CD32 (FcγRII) (see Section 1.7.1). There are also $CD14^{dim}CD16^{hi}$ MCs. Though not as extensively studied, one study shows them to have a limited cytokine expression profile and respond to viruses in a TLR7-TLR8-MYD88-MEK-dependent pathway (Cros et al., 2010).

MCs in HIV-1 Infection

Conflicting reports exist as to whether MCs are susceptible to HIV-1 infection given they express CD4. They are mostly shown to be resistant to infection *ex vivo* prior to MP or DC differentiation because of limiting levels of deoxynucleotide triphosphates (O'Brien et al., 1994; Triques and Stevenson, 2004). MCs have innumerable inhibitory and stimulatory effects on HIV-1 virus and virus-infected cells either directly or by stimulating other cells. For example, they can take up virions in phagocytic compartments and once transported to tissues, can establish productive infection as resident MPs which is reported to take place in HIV-1 associated dementia (Gartner, 2000; Kim et al., 2003). These effects are more extensively reviewed in (Sasse et al., 2012). Section 1.9.1 describes MCs and ADCC.

1.8.3 Neutrophils

Eli Metchnikoff first observed PMNs in starfish larvae, engulfing rose thorns (Kumar and Sharma, 2010). They have multi-lobed nuclei giving them the alternative name of polymorphonuclear cell (PMN). PMNs comprise 30-80% of leukocytes in circulation, making them the most abundant white blood cell (StemCellTechnologies, 2008). Once released from the bone marrow into

circulation, PMNs have a life span of 6-10 hours before undergoing spontaneous apoptosis, characterized by DNA degradation, chromatin condensation, and cell shrinkage (Laskay et al., 2008). This is unlike MCs and DCs, which are also from the myeloid lineage. MCs and DCs are primed to undergo apoptosis but do not do so spontaneously whereas PMNs do, especially in HIV-1/SIV infection (Laforge et al., 2011). This is due in part to the fact that pro-apoptotic proteins Bad, Bak, Bax, Bid are constitutively expressed by PMNs (Laskay et al., 2008). Spontaneous apoptosis is also regulated by FasL, reactive oxygen species (ROS), caspase, and MAPK (Salmen et al., 2007).

Dysplastic PMN morphology and neutropenia is characteristic of chronic HIV-1 infection. The PMNs of HIV-1 patients are described as being more primed to apoptosis through a Fas-dependent mechanism that correlates with viral load and these PMNs also have increased ROS production which accelerates apoptosis in a caspase 3-dependent, caspase 8-independent manner (Salmen et al., 2007; Salmen et al., 2004). Neutropenia can also result from HAART or other viral infections, e.g. Epstein-Barr virus.

Pro-inflammatory products are known to enhance the survival of PMNs. GM-CSF, IL-15, and LPS can maintain/increase the expression of anti-apoptotic protein Mcl-1 (Laskay et al., 2008). HIV-1 infection has been shown to stimulate GM-CSF production (Fu et al., 2011) which enhances PMN survival. GM-CSF is routinely used to restore PMN levels in neutropenic infected patients (Barbaro et al., 1997) but because it also poses a risk by possibly stimulating HIV-1 replication, G-CSF is preferentially used (Barbaro et al., 1997; Koyanagi et al., 1988; Levine et al., 1991; Wang et al., 1998).

PMNs have three primary functions:

1. Activation of MCs, MP, NKs, and immature DCs (Kumar and Sharma, 2010; Silva, 2009; Soehnlein, 2009)

2. Antigen presentation via expression of MHC class II molecules in response to GM-CSF, IL-3, and IFN-γ (Kumar and Sharma, 2010).

3. Killing by phagocytosis, degranulation of antimicrobial molecules, and neutrophil extracellular traps (NETs) (Fig. 1.8.1) (Papayannopoulos and Zychlinsky, 2009).

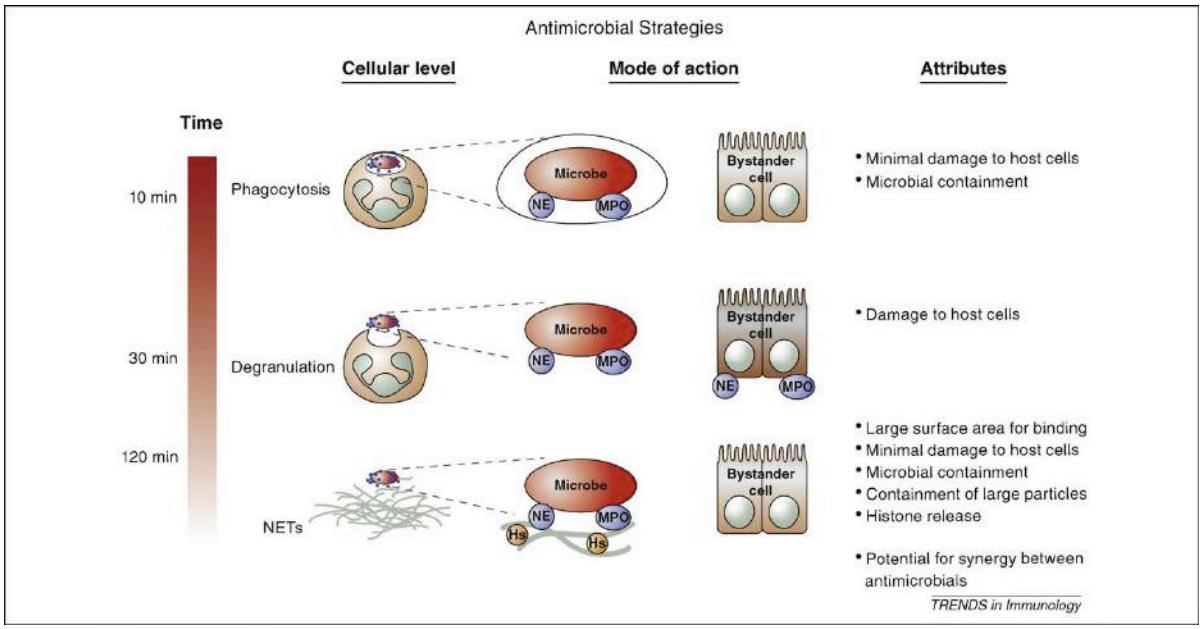


Figure 1.8.1 The antimicrobial strategies of neutrophils. Neutrophils use three major strategies to combat and clear microbes: phagocytosis, degranulation, and NET formation. These operate over different timescales (indicated on the left), and have varying effects on bystander cells (i.e. collateral damage), and have different attributes. Ingestion of microbes into a phagolysosome minimizes damage to host cells during phagocytosis whereas degranulation disseminates granule proteases and can cause widespread damage. The incorporation of granule proteases into NETs limits their diffusion and hence the potential for host cell damage, increases their effective local concentration, and entraps microbes. Red: Microbe, Blue: granular proteins in the phagolysosome, extracellular space, or tethered within the NET, Gray: decondensed DNA of the NET. Abbreviations: Hs, histones; MPO, myeloperoxidase; NE, neutrophil elastase. Adapted from (Papayannopoulos and Zychlinsky, 2009).

The primary role of PMNs is phagocytosis. It is well characterized in bacteria and yeast but there is also evidence of phagocytosis of apoptotic cells, free

virus particles, and virus infected cells (Hashimoto et al., 2007; van Zandbergen et al., 2004). PMNs must be activated in order to perform phagocytosis whereas MPs and DCs do not require activation. In addition to LPS, IFN-γ, and GM-CSF, heat-labile factors (most likely complement) may also be required for phagocytosis of apoptotic cells (van Zandbergen et al., 2004). Apoptotic, but not non-apoptotic, cells are phagocytised by PMNs although PMNs seem to prefer smaller apoptotic bodies as opposed to whole cells (van Zandbergen et al., 2004). They recognize apoptotic cells via of phosphatidylserine (PS) (Hashimoto et al., 2007). Uncoupling proteins have been cited to enhance phagocytosis by lowering the mitochondrial membrane potential and via crosstalk with TIM-4, a receptor that binds PS on apoptotic cells (Park et al., 2011). The ability of PMNs to phagocytise apoptotic cells in HIV-1 infection is important as many of the infected $CD4^+$ T cells undergo apoptosis. The phagocytic capacity of PMNs, MCs and MPs in HIV-1 infection is impaired due to the inhibitory action of Nef or Fas stimulation but it can be restored by HAART (Pugliese et al., 2005).

NETs were first identified by Brinkmann in 2004 (Brinkmann et al., 2004). They are composed of DNA and histone proteins that capture microbial and viral pathogens. To date, two mechanisms of formation have been identified which is extensively reviewed in (Papayannopoulos and Zychlinsky, 2009). In a model described by Papayanopoulos, neutrophil elastase (NE) is released from azurphilic granules upon stimulation by ROS where it translocates to the nucleus and cleaves histones and promotes chromatin decondensation. Myeloperoxidase (MPO) is later recruited from azurphilic granules and also translocates to the nucleus where it binds histones, further enhancing

chromatin decondensation (Remijsen et al., 2011). Eventually the cell ruptures, releasing NETs into the extracellular milieu where they ensnare bacteria, fungus, and parasites (Papayannopoulos et al., 2010). More recently NETs have been shown to have anti-viral activity against influenza, feline leukaemia virus, HIV-1 and poxviruses (Fujisawa, 2008; Jenne et al., 2013; Saitoh et al., 2012; Wardini et al., 2010). Whether they are formed in response to HIV-1-infected cells is unknown.

PMN antiviral effects

PMNs are duplicitous, having both enhancing and inhibitory effects on HIV-1. The anti-viral effects of PMNs are of interest because (1) these cells are not productively infected by HIV-1 unlike other phagocytes, MPs and DCs as they lack CD4 and CCR5 (Johanna Fergusson, unpublished data)(Moriuchi et al., 2000); and (2) they comprise a majority of the leukocytes compared to strictly anti-viral NKs which are only about 5% of the population. A number of studies have described the cytotoxic effect PMNs can have on virus-infected cells. ADCC and antibody-dependent cellular virus inhibition (ADCVI) are of interest because they do not require NAbs which have been hard to elicit in human vaccines although binding Abs have been elicited.

Duval and colleagues created a bispecific Ab composed of cross-linked F(ab) fragments of a non-neutralising anti-gp41 Ab (F240) that recognizes highly conserved epitopes and an anti-IgA FcR (CD89) Ab (14A8). By binding PMN IgA FcR and gp41 on free virus particles or infected cells, it brought them into close proximity thus facilitating PMN-mediated reduction in virus production as measured by a standard ADCVI assay (Duval et al., 2008). PMNs have also been described as mediating ADCC against varicella-zoster virus-

infected firbrobroblasts and HIV-1-infected MOLT-3A lymphoid cell line (Baldwin et al., 1989; Clark et al., 2010; Ihara et al., 1984).

PMNs target free virus particles. Yamamoto and colleagues observed that influenza particles were phagocytised by PMNs and disappeared within 30 minutes (Yamamoto et al., 1989). HIV-1 has also been observed in the phagosome of neutrophils (Orenstein, 2000). Lactoferrin, IL-8, α-defensins, and MIP-1β produced by PMNs have also been noted to have anti-viral effects (Flamand et al., 2004; Fu et al., 2011). Saitoh and colleagues described PMNs detecting HIV-1 in endosomes via TLR7 and TLR8, which triggers NET formation. However HIV-1 counteracts this effect by inducing IL-10 in a CD209-dependent manner, which suppresses ROS generation and thus NET induction (Saitoh et al., 2012).

PMN proviral effects

Stimulated and unstimulated PMNs secrete factors that stimulate HIV-1 replication in PBMCs, Jurkat T cell lines, and MPs. Some of the factors identified include TNF-α, IL-6, Il-8, ROS, GRO-α, cathepsin G, myeloid related proteins, CCL-2, and IL-10 (Fu et al., 2011; Ho et al., 1995; Klebanoff and Headley, 1999; Lane et al., 2001; Moriuchi et al., 2000; Ryckman et al., 2002; Yoshida et al., 2007; Yoshida et al., 2009).

PMNs can also serve as transport vehicles, Trojan horses if you will, for intracellular pathogens, including HIV-1. Intracellular pathogens such as *Chlamydia pneumoniae* and *Leishmania major* can inhibit the release of antimicrobial factors and reduce apoptosis, thus prolonging the survival of PMNs. Infected PMNs can then secrete MIP-1β which recruits MPs that ingest the PMN and parasite that remains intact within its phagosome

(Laskay et al., 2008). Influenza virus has been found within the phagosome and nucleus of PMNs (Yamamoto et al., 1989; Zhao et al., 2008). Although PMNs cannot become infected by traditional CD4 and CCR5 co-receptor pathways, HIV-1 can be taken up within PMNs via phagocytosis mediated by cellular adhesion factors. It has been reported that HIV-1 gp120 can bind to PMNs via glycosaminoglycans and galactosyl ceramides, but not C-type lectins (Gabali et al., 2004). HIV-1 may also bind to PMNs via ICAM-1 and LFA-1 interactions (Silva, 2009). LFA-1 is found on PMNs, but also T cells, B cells, and MPs. ICAM-1 is traditionally found on APCs, MPs, and endothelial cells, including CD4+ T cells. When virions bud from T cells they can carry ICAM-1, making them more infectious to LFA-1 bearing targets such as PMNs (Capobianchi et al., 1994). This leads to a model for infection where if HIV-1 remains intact within the phagosome, when that PMN is phagocytised by MPs, the virus can then propagate. Though described in bacteria and other viruses, what remains to be determined is whether HIV-1 can survive in the phagosome and how it would elude phagosome-lysosome fusion and other anti-microbial molecules.

Another mechanism by which PMNs can spread HIV-1 is by transporting virus extracellularly. Resting, activated, and apoptotic PMNs can bind HIV-1 and transfer it to uninfected CD4s (Gabali et al., 2004; Olinger et al., 2000). Gabali and colleagues reported that binding of HIV-1 to PMNs is enhanced by TNF-α and fMLP but not LPS and is independent of CD4, gp120, and C-type lectins. Activation of PMNs by TNF-α accelerated infection of PHA-activated PBMCs. In a propagation model proposed by Fu and colleagues PMNs bind intact HIV-1 and carry it from peripheral tissue to the mucosa and

LN where the PMNs become activated by various inflammatory mediators such as TNF-α and HIV-1 and are stimulated to produce GM-CSF (Brenchley and Douek, 2008; Fu et al., 2011; Maletto et al., 2006). GM-CSF promotes survival of PMNs and induces HLA-DR expression on PMNs which in turn supports CD4+ T cell activation and thus transfer of HIV-1 from the PMNs to $CD4^+$ T cells.

Taking into account the neutropenic state of an HIV-1 patient, the dysplastic changes to PMNs, and the ability of PMNs to both kill infected cells/virus but also transfer virus, PMNs represent a challenge for the field. A better understanding of these factors will perhaps indicate how recruiting, augmenting, or limiting the activity of these cells can influence the course of HIV infection.

1.8.4 Induction of cellular apoptosis

As described in Section 1.8.1 the mechanism of NK killing of infected cells through death receptor and perforin/GrB pathways is well characterized. However, it is unclear how MCs and PMNs kill infected cells. These cells can recognize HIV-1 infected cells by a variety of mechanisms that include LFA-1/ICAM-1 interactions, or PS expressed by cells that are already apoptotic due to infection, or by engagement of their FcR by antibody coated pathogens or downregulation of MHC class I (Cooper et al., 2013; Gougeon, 2003). In regard to cell killing induced by the engagement of MC and PMN FcRs there are two facets to be considered; what happens in the effector cell upon FcR crosslinking and how does that signalling induce apoptosis in the target cell. Many studies have probed this question but no conclusive mechanism has been revealed. Each FcR and has a different pathway and

different outcome (Sanchez-Mejorada and Rosales, 1998). For example, when FcγRIIIa is crosslinked the MAPK pathway is triggered and downstream signalling occurs via arachidonic acid. In NK cells degranulation of perforin/GrB ultimately occurs. The process could be similar in MCs and PMNs but as they lack perforin and granzyme, the downstream molecules that are activated and essential in an ADCC response remain unclear.

Most studies to address this have used typical inhibitors of secretory products like brefeldin A, concanamycin A, ionomycin; or superoxide dismutase (SOD) or catalase to prevent ROS. In several studies ROS has been shown to be generated during target cell killing but not essential for lysis (Kleinerman et al., 1985; Seim and Espevik, 1983; Takamuku et al., 1996). Inhibiting ROS production in MCs with SOD and catalase, and using MCs from patients with chronic granulomatous disease (who don't produce the superoxide anion) did not affect tumour cell lysis (Kleinerman et al., 1985). The same group also observed that IFN-γ was not essential. Takamuku and colleagues did not observe any reduction in ADCC by MCs of human colorectal cancer cells when MCs were treated with SOD, but they did find that IFN-γ enhanced apoptosis significantly and that TNF-α was essential because an anti-TNF-α Ab inhibited apoptosis (Takamuku et al., 1996). ADCC by PMNs has also been shown to be an oxidative-independent Ca-dependent process (Baldwin et al., 1989; Clark et al., 2010). The MC or PMN molecules involved in ADCC would determine whether the intrinsic or extrinsic apoptotic pathway in the target cell is activated. The intrinsic pathway involves mitochondrial signalling and caspase 9 whereas the extrinsic pathway, triggered by death receptor ligands and perforin/GrB, uses

caspase 8. Both of these pathways eventually signal through caspase 3 (Chen et al., 2013; Gougeon, 2003).

1.9 Antibody-Dependent Cellular Cytotoxicity (ADCC)

1.9.1 Description

Effectors can work alone or in concert with Abs where they can mediate ADCC, ADCVI, or antibody-dependent cellular phagocytosis (ADCP). During ADCC anti-HIV-1 Abs bind to viral epitopes, generally Env, expressed on the surface of the infected cell. Then FcRs on the surface of the effector cell bind the Fc portion of the Ab, bringing the effector and infected target, usually $CD4^+$ T cells, into close proximity. Crosslinking of FcRs on the effector cell by immune complexed Ig and antigen causes the effector cell to induce apoptosis in the target cell either by the release of perforin/GrB by NKs or some other yet to be determined mechanism in MCs and PMNs.

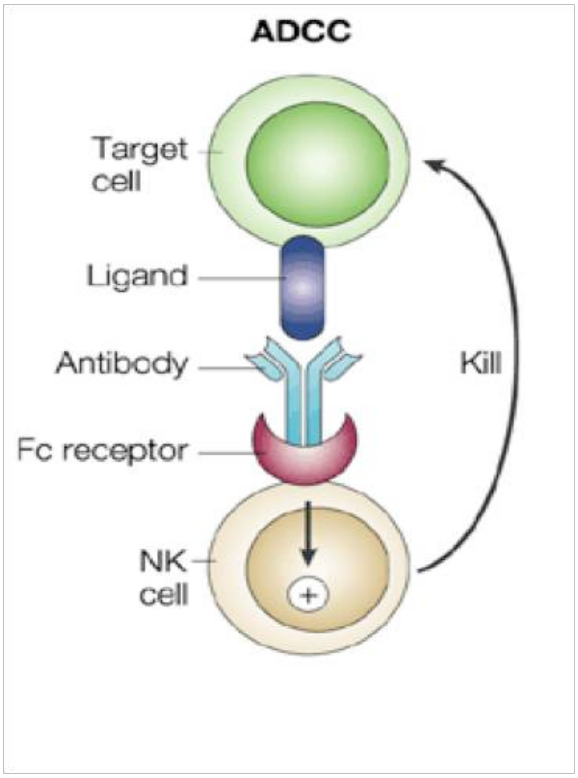


Figure 1.9.1 ADCC by NK cells. Abs bind HIV-1 viral epitopes presented on the surface of infected $CD4^+$ T cells called the target cell. Then an FcγR-bearing effector cell such as an NK binds the Fc region of the antibody. Binding to several Abs results in cross-linking of FcγRs which causes the NK cell to release the pore forming protein perforin which permits entry of GrB into the target cell cytosol inducing apoptosis. Adapted from (Yokoyama and Plougastel, 2003).

There are several FcRs on the surface of cells with the ability to bind different classes of Ig (Nimmerjahn and Ravetch, 2008; Woof and Burton, 2004). Low and medium affinity receptors bind preferentially to immune complexes and play more of a role in ADCC compared to high affinity receptors, which bind monomeric Ig. Table 1.9.1 outlines the various classes of FcRs and their cellular expression. Of note: FcαRI (CD89) binds IgA, is present on PMNs and MCs, and crosslinking causes ADCC. FcεRI and FcεRII (CD23) are present on MCs but these are not believed to trigger ADCC. NK cells only express the medium affinity FcγRIIIa (CD16a) but MCs and PMNs also express this and FcγRI (CD64), FcγRIIa (CD32a), and FcγRIIc (CD32c) which mediates ADCC and ADCP. PMNs exclusively express FcγRIIIb (CD16b) and crosslinking is responsible for superoxide generation.

MW (kDa)	FcγRI (CD64)	FcγRII (CD32)				FcγRIII (CD16)		FcεRI	FcεRII (CD23)		FcαRI (CD89)
	50–70	40				50–80		45–65	45–50		50–70
Major isoforms expressed	FcγRIa	FcγRIIa		FcγRIIb	FcγRIIc	FcγRIIIa	FcγRIIIb	FcεRI	FcεRIIa	FcεRIIb	FcαRIa
Allotypes		LR	HR					NA1 and NA2			
Specificity for human Ig*	IgG1 = 3 > 4 IgG2 doesn't bind	IgG3 ≥ 1 = 2 IgG4 doesn't bind	IgG3 ≥ 1 >>> 2 IgG4 doesn't bind	IgG3 ≥ 1 >> 2 > 4	ND	ND	IgG1 = 3 >>> 2 = 4	IgE	IgE		Serum IgA1 = 2, SIgA1 = SIgA2
Affinity for monomer Ig (M^{-1})	High (10^8–10^9)	Low (<10^7)	Low (<10^7)	Low (<10^7)	Low (<10^7)	Medium (10^7)	Low (<10^7)	Very high (10^{10})	Low (<10^7)		Medium (10^7)
Signaling motif	γ chain ITAM	α chain ITAM		α chain ITIM	α chain ITAM	γ chain ITAM	No signaling motif. Anchored in membrane via glycan phosphatidylinositol (GPI) linkage	γ chain ITAM β chain also present but role unclear	C-type lectin	C-type lectin	γ chain ITAM
Cellular distribution	Monocytes, macrophages, DC, neutrophils (IFNγstim), eosinophils (IFNγstim)	Monocytes, macrophages, neutrophils, platelets, Langerhans cells		Monocytes, macrophages, B-cells	Monocytes, macrophages, neutrophils, B-cells	Macrophages, NK cells, γδ T-cells, some monocytes	Neutrophils, eosinophils (IFNγstim)	Mast cells, basophils, Langerhans cells, activated monocytes	B-cells	B-cells, T-cells, monocytes, eosinophils, macrophages	Neutrophils, monocytes, some macrophages, eosinophils, Kupffer cells, some DC

*Relative affinities of various ligands for each receptor are indicated in decreasing order starting with the isotype with highest affinity. Arrowheads and equal signs are used to show the differences in affinity.

Table 1.9.1 Human leukocyte Fc receptors. Adapted from (Woof and Burton, 2004).

1.9.2 IgA and FcαRI

CD89 is the IgA FcR (FcαRI). Two FcαR are bound by one IgA in a 2:1 stoichiometry compared to IgG which binds FcγR in a 1:1 stoichiometry. IgA is present in several forms. The mucosa contains primarily the dimeric form whereas the serum contains the monomeric (IgA1), dimeric (IgA2), and secretory (SIgA) forms. Mucosal MPs and DCs (Langerhan cells) lack FcαRI. FcαRI is present on PMNs, MCs, and interstitial DCs. Binding of dimeric, immune complexed IgA to FcαRI evokes a proinflammatory response in which phagocytosis, antigen presentation, ADCC, ROS generation, and cytokine release (Fig. 1.9.2). Often microbes are opsonised by dimeric IgA and cleared by PMNs. Serum monomeric IgA moderates the immune response because binding to FcαRI transduces inhibitory signals and one of the sequelae is downregulation of IgE- or IgG-Fc mediated effector functions

(Kanamaru et al., 2007a; Kanamaru et al., 2007b; Wilton, 1978). It does so by recruiting “inhibisomes” which block phosphorylation and thus the function of these activating FcRs.

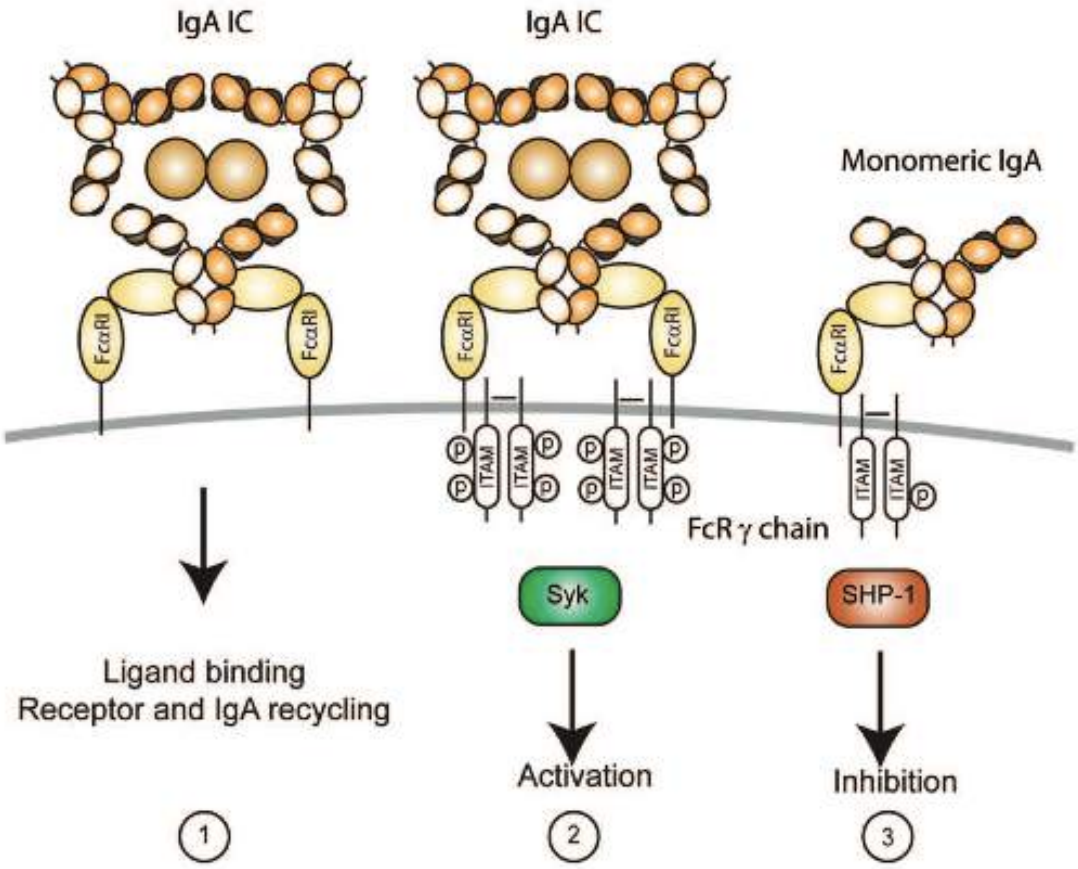


Figure 1.9.2 FcαRI initiates different functional outcome depending on the mode of interaction with its ligand IgA or FcR γ-chain. (1) Functionality of FcαRI “γ-less” receptors is limited to ligand binding and receptor internalisation (although Gly^{248}-FcαRI can trigger IL-6 production as well). (2) Cross-linking of FcαRI via IgA immune complexes (IC) leads to heavily phosphorylated (p) FcR γ-chain ITAMS, which engage Syk. This results in activation and pro-inflammatory responses. (3) Binding of monomeric IgA to FcαRI mediates weaker phosphorylation of FcR γ-chain ITAM and recruitment of SHP-2. This elicits inhibitory signal, which can suppress activation through other Fc receptors. Adapted from (Bakema and van Egmond, 2011a).

While PMNs express FcγRs, the FcαRI is the most effective for stimulating its cytotoxicity. In several studies involving mAbs used to treat cancer, the IgA mAb is superior to IgG in eliciting tumouricidal function by PMNs (Bakema

and van Egmond, 2011a; van Egmond and Bakema, 2013). IgA has the advantage over IgG in being able to recruit the more plentiful PMN effector population by crosslinking FcαRI and stimulating release of leukotriene B4, a potent PMN cheomattractant (van der Steen et al., 2009). However several challenges exist in the study and implementation of IgA for immunotherapy: it is inhibitory in the monomer form, it is susceptible to degradation, and it has a short half life of one week compared to that of IgG which is 3 weeks. Furthermore, studying the effects IgA and FcαRI *in vivo* is more difficult because mice do not have FcαRI so transgenic humanized mice must be used. IgG's advantage in conjunction with NKs is that IgG is more bioavailable and a single NK is able to kill several cells sequentially.

1.9.3 ADCC and HIV-1

ADCC has been studied since early in the HIV-1 pandemic but renewed interest was generated by two studies reported in 2007 and 2009. In a passive transfer study, various forms of NAb b12 was administered to macaques prior to challenge with SHIV. The study groups were (1) control – no Ab, (2) WT b12, (3) b12 KA – a variant of b12 with abrogated complement C1q binding, and (4) b12 LALA – a variant with low FcγR binding, particularly to FcγRIIa and FcγRIIb, and low binding to C1q (Hessell et al., 2007). In the animals treated with b12 WT and b12 KA, 8/9 were protected from SHIV, however, in the group treated with b12 LALA, only 5/9 animals were protected. While neutralization and complement alone are enough to provide sterilizing immunity as evidenced in only 5/9 LALA animals being protected when these functions were intact, the diminished protection from loss of FcγR-binding capacity suggests that FcγR-mediated functions play a role in

sterilizing immunity. These effector functions could be phagocytosis of b12-opsonized virions, ADCC, or ADCVI. The LALA mutation was noted to significantly lessen FcγRIIa (and FcγRIIb) binding so it is plausible that MCs, PMNs, and MPs bearing this receptor acted by phagocytising virions. FcγR-mediated function alone does not account for the protective effects since in over half of the b12 LALA animals neutralization was robust enough to provide protection when complement fixation and FcR binding was ablated. Because ADCC mediated effects are on virus infected cells and not free virions, this study suggested that ADCC or ADCVI could eliminate HIV-1-infected cells *in vivo*.

A study that using a non-neutralising Ab that binds to a similar epitope as a NAb with similar heavy chain modifications, e.g. b6 and b12, would better define the contribution of FcγR functions to Ab protection. In such a study by Burton and colleagues macaques administered b6 were not protected from challenge with SHIV compared to animals receiving b12, highlighting the importance of neutralization in Ab protection (Burton et al., 2011).

It was initially thought that protection by the RV144 vaccine might have been through ADCC because initial analysis did not show NAb and antigen-specific $CD8^+$ T cells to be responsible. An inverse correlation between anti-gp120 V1 and V2 loop plasma IgG and HIV-1 acquisition was observed and anti-Env plasma IgA was associated with increased risk. ADCC was also associated with decreased risk but only when anti-Env plasma IgA levels were low. (Haynes et al., 2012; Rerks-Ngarm et al., 2009; Tomaras et al., 2013).

IgG is the most abundant IgG found in the blood, has a half life of 3 weeks, and is especially good at complement binding and recruiting NK cells for ADCC so it is plausible that some of the protective effect associated with IgG might be via ADCC. IgA is the most abundant Ig in the entire body, found primarily at mucosal sites in its dimeric form but also in the plasma as a monomer or dimer. It cannot fix complement or be bound by NKs to mediate ADCC, but PMNs, MCs, and MPs have FcαRs which allow these cells to phagocytise or kill IgA opsonised targets. Tomaras et al. showed that binding of monomeric IgA isolated from plasma competes with IgG binding for Env, and thus reduces NK-IgG mediated ADCC. A recent report describing enhanced 2F5 affinity for gp41 by switching the heavy chain from IgG to IgA gives further evidence of how Ig isotype affects affinity (Tudor et al., 2012). Given the fact that monomeric serum IgA is known to downregulate IgG mediated effector functions it is not surprising that vaccinees with a higher ratio of serum IgA:IgG were more susceptible to infection and this further suggests that the IgG-Fc mediated ADCC may play a role in preventing HIV-1 infection (Bakema and van Egmond, 2011b; Wilton, 1978).

The RV144 vaccine study was limited in that mucosal samples were not collected therefore the role of mucosal IgA could not be characterized although vaccine trials using the same dosing regimen and designed to study mucosal responses are underway. If IgA were to opsonise HIV-1 or an infected cell and it was bound by FcαRI on PMNs or MCs those cells could kill via ADCC. Since mucosal MPs do not contain FcαRI it is unlikely that IgA opsonisation of virus or infected cell and subsequent phagocytosis by a MP

would lead to infection of the cell but since FcγR are present on MPs IgG could hypothetically facilitate infection.

1.9.4 Macaque and human HIV-1 ADCC studies

Many other macaque challenge studies have implied that ADCC may be important for limiting infection (Alpert et al., 2012; Banks et al., 2002; Gomez-Roman et al., 2005). In a double prime-double boost-challenge model of SIV infection, intranasal/oral priming evoked a better anamnestic humoral and mucosal response than oral/oral priming (Hidajat et al., 2009). While no NAbs were elicited, probably due to the small amount of Env immunogen, Abs mediating ADCC and ADCVI were elicited and only their binding titers after the first boost correlated with reduced viral load 2 weeks post challenge. HIV-1 gene products Env, Nef, and Tat have been shown to be epitope targets for ADCC-mediating Abs (Florese et al., 2009; Johansson et al., 2011). Env is probably the most important as it covers the surface of actively infected cells from which virus is budding. V1/V2 loop-, V3 loop-, coreceptor binding site-, and CD4-induced binding site-recognizing Abs have been shown to be particularly effective in mediating ADCC (Ferrari et al., 2011; Guan et al., 2012; Johansson et al., 2011). It has been suggested that sera from LTNPs exhibit greater ADCC activity, which correlates with the $CD4^+$ T cell count (Ahmad et al., 2001; Baum et al., 1996; Lambotte et al., 2009), but others have observed no correlation between ADCC and disease progression (Dalgleish et al., 1990; Koup et al., 1989; Wren et al., 2013).

1.9.5 ADCC and other viral infections

ADCC has been noted against several other virus infections including influenza, respiratory syncytial virus, and herpes simplex virus (HSV) (Gupta et al., 2013. Kent et al. demonstrated that Abs induced to seasonal flu

facilitate NK-mediated ADCC in macaques against divergent strains of influenza. HSV secretes glycoprotein E which acts as an FcγR, blocking IgG-Fc mediated effector functions such as ADCC and complement binding, enhancing the spread of infection {Lubinski, 2011 #326; Jegaskanda et al., 2013; Kohl, 1991). The extent of ADCC's role in preventing the establishment of primary infection is still unclear for these and several other viral infections. Protection by vaccination in most viral infections such as influenza, human papilloma virus, tick-born encephalitis, small pox, measles, has been correlated with NAbs but this does not exclude the involvement of ADCC (Plotkin, 2010).

1.10 Research Objectives

Elimination of HIV-1-infected cells via ADCC and ADCC's role in vaccine-induced immunity is of increasing interest. Several basic questions regarding the nature of an effective ADCC response have not been addressed or remain poorly understood, including:

- the role of innate effector cells, NKs, MCs, and PMNs, in killing HIV-1-infected cells
- how PMNs and MCs recognize and kill HIV-1-infected cells
- how to enhance the proliferation and recruitment of effector cells to sites of infection
- the ADCC-mediating Ab response in the course of natural HIV-1 infection, and
- the number, affinity, and specificities of Abs necessary to mediate ADCC.

The goals of this study were to:

1. Attempt to generate PMNs from stem cells to provide an inexhaustible supply of identical human PMNs for further study.
2. Compare the relative efficacy of NKs, MCs, and PMNs in killing HIV-1-infected cells and investigate how Ab enhances killing.
3. Image the effector-target synapse to gain insight into mechanisms of killing.

4. Screen clinical serum samples for ADCC and determine whether any correlation with clinical markers of progressive HIV-1 infection (status, $CD4^+$ T cell count, plasma RNA levels) and neutralization exists.
5. Probe the specificities of Abs that evoke potent ADCC.

I managed to generate limited numbers of PMN-like cells from stem cells but the yield was too low to carry out subsequent functional studies. I found that primary NKs and MCs killed virally infected cells to a similar degree and at a level greater than PMNs, however HIV-1-specific Abs significantly enhanced killing of infected cells by all effectors. While I was able to image effector-target cell conjugates and observed ingestion of apoptotic bodies by MCs, I was not able to make any strong conclusions about the process or possible mechanisms involved in ADCC based on these observations. Having determined that NKs mediated the greatest level of ADCC they were used as effector cells in the studies with patient serum. I did not detect any differences in the level of ADCC mediated by LTNPs or progressors nor a correlation with ADCC activity and $CD4^+$ T cell count, viral RNA levels, or NAb titers. ADCC was found to correlate with the level of IgG, specifically IgG1 binding to the surface of infected cells. MAbs were used to explore specificity for ADCC but they did not have levels of ADCC comparable to patient sera.

Chapter 2

Materials and Methods

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2.1 General Notes

Similar techniques were used in labs at NIH and Oxford with minor modifications as indicated. Dulbecco's phosphate buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ was used where PBS is indicated (PAA, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Complete RPMI (cRPMI) medium used to culture primary cells was composed of RPMI 1640 (PAA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA), 100 μg/mL penicillin, 100 μg/mL streptomycin (PAA). Complete DMEM used to culture 293T and TZM-bl cell lines was high glucose DMEM (Gibco, Life Technologies, Carlsbad, CA, USA or PAA) supplemented with 10% FBS and penicillin/streptomycin. Cells were incubated at 37^{o}C with a CO_2 level of 5%. Cells were typically pelleted at 500 x g for 6 minutes, except where otherwise noted.

2.2 Cell Isolation

2.2.1 Purification of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were purified from blood using ficoll density centrifugation. Blood from healthy donors was obtained either from leukapheresis packs (NIH Blood Bank, Bethesda, MD), 50 mL Buffy coat packs (National Blood Service, Midlands and South West, Bristol, UK), or donors from the Sattentau lab using BD Vacutainer® sodium heparin tubes (BD Diagnostics, New Jersey, USA). Blood was diluted 2 fold with PBS. 25 mL of diluted blood was layered on 15 mL of room temperature (RT) Lymphocyte Separation Medium (at NIH; MP Biomedicals, Solon, Ohio, USA) or Hisopaque® 1077 (at Oxford; Sigma-Aldrich, St. Louis, MO, USA). Layers were centrifuged for 25 min at

25^{o}C and 500 x g with gentle deceleration. PBMCs were collected from the interface of the serum and ficoll. PBMCs were washed twice with PBS before isolating specific cell types or freezing.

2.2.2 Cell Isolation

Cells were selected from freshly isolated or frozen PBMCs thawed in complete RPMI with 25 U/mL Benzonase® Nuclease (Millipore, Billerica, MA, USA). CD4^{+} T cells were positively selected using magnetic automated cell sorting according to manufacturer's instructions (CD4 MicroBeads, Miltenyi, Bergisch Gladbach, Germany). Monocytes (MCs) and NKs were negatively isolated from PBMCs and neutrophils (PMNs) from granulocytes using EasySep® Human Monocyte, NK, and Neutrophil Enrichment Kit according to manufacturer's instructions (StemCell, Vancouver, BC, Canada). To isolate PMNs granulocytes were first enriched for using dextran sedimentation and hypotonic lysis as follows: after ficoll density centrifugation granulocytes were collected from the layer below the ficoll and mixed with equal parts of 3% dextran/0.9% NaCl and left at RT for 40 min (Dextran-500, Sigma-Aldrich). The top layer containing granulocytes was removed and pelleted at 250 x g, 4^{o}C, for 10 min. The granulocyte pellet was resuspended in 10 mL ice-cold 0.2% NaCl to hypotonically lyse RBCs followed 30 seconds later by 1.6% ice-cold NaCl to attain a 0.9% NaCl isotonic solution. Cells were pelleted at 250 x g, 4^{o}C for 10 min and then PMNs isolated using the Neutrophil Enrichment Kit.

2.2.3 Storage of Cells

PBMCs were frozen in Recovery Cell Culture Freezing Medium (Gibco, Carlsbad, CA, USA) using a CryoMed controlled-rate freezer (ThermoForma, Waltham, MA, USA) and stored at -140^{o}C at NIH; or in 10% DMSO/45%

FBS/RPMI medium using Nalgene® Mr. Frosty Freezing Container (Sigma-Aldrich) and stored at -80^{o}C at Oxford.

2.3 Serum and Ab Samples

2.3.1 Serum Samples

HIV-infected subjects were recruited from the Clinical Research Center, National Institutes of Health (Bethesda, MD) under protocol 02-I-0086 and signed National Institute of Allergy and Infectious Diseases Investigational Review Board-approved informed-consent documents. Normal serum was obtained from the NIH Blood Bank. Pooled HIV-positive (Bpool) serum was assembled by mixing equal parts of sera from 20 clade B broadly neutralising HIV-infected patients. Normal sera was assembled by mixing equal parts of sera from 10 HIV-negative subjects. All sera were heat-inactivated at 56^{o}C for 1 hour and stored at -80^{o}C.

2.4 HIV-1 Virus Stocks

2.4.1 Preparation of High Titre HIV-1$_{BaL}$

PBMCs were isolated from 2 Buffy coat packs as described in 2.2.1. Two vials of 2 x 10^{7} PBMCs from each donor were frozen for later use. PBMCs from both donors totalling about 2 x 10^{9} were stimulated for 3 days in 100 mL cRPMI supplemented with 1μg/mL PHA and 10 IU/mL IL-2 (PHA/IL-2 media) in a T-175 flask laid flat. Non-adherent cells were removed, leaving approximately 20 mL in the flask to maintain viability of the adherent cells, pelleted, and resuspended in 80mL cRPMI with 10 IU/mL IL-2, returned to the same flask and laid flat for another 2 days. 80 mL non-adherent cells were removed pelleted, and mixed with at least 5.62 x 10^{6} $TCID_{50}$ HIV-1$_{BaL}$

obtained through the NIH AIDS Reagent Program from Dr. Suzanne Gartner, Dr. Mikulas Popovic, and Dr. Robert Gallo (Gartner et al., 1986). 20 mL of media was left in the flask to maintain viability of adherent cells and these too were mixed with HIV_{BaL}. Cells and virus were incubated at 37^{o}C for 4 h then returned to the flasks with adherent cells in 80 mL cRPMI/IL-2 media. At 2 and 9 days post-infection, 20 x 10^{7} PBMCs from each donor were thawed in cRPMI and added to the culture flask. After 5 days 80 mL of supernatant was harvested, cells pelleted, and the cell-free supernatant concentrated 60-fold in 300 kDa Vivaspin® 20 centrifugal concentrators (Vivaproducts, Littleton, MA, USA). 80 mL of cRPMI/IL-2 was added to the pelleted cells which were returned to the flask with adherent cells and left at 37^{o}C. From post-infection Day 5 to Day 15 virus was harvested in the same manner. Virus was stored in 50 μL aliquots and stored at -170^{o}C.

2.4.2 Preparation of other HIV-1 infectious molecular clones (IMC) and pseudovirus

2.4.2.1 Acquisition and Amplification of Plasmids

Plasmids were acquired from various sources as described in the appropriate chapter. Plasmids were propagated in Stbl2™ cells (Invitrogen, Life Technologies, Carlsbad, CA, USA) and purified using EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. For the production of NL4-3 eGFP virus, pNLC4-3 and pNLC4-3^{EGFP} was obtained from Clare Jolly (University College London, London, UK) (Muller et al., 2004).

2.4.2.2 Transfection of 293T cells

Plasmids were propagated in Stbl2™ cells (Invitrogen, Life Technologies, Carlsbad, CA, USA) and purified using EndoFree Plasmid Maxi Kit (Qiagen,

Valencia, CA, USA) according to manufacturer's instructions. Each IMC was subsequently transfected into 293T LentiPhos cells using FUGENE HD (Roche, Indianapolis, IN, USA) according to manufacturer's instructions. MLV *env* pseudovirus was generated by cotransfecting 293T cells with pSG3$^{\Delta env}$ and SC-A-MLV-*env* plasmids using Fugene 6 (Roche). NL4-3-eGFP was generated by cotransfecting 293T with pNLC4-3 and pNLC4-3^{EGFP} plasmids using PEI.

Transfection of IMCs and MLV pseudovirus were performed as follows: 293T cells were seeded in T-75 flasks in complete DMEM to obtain 50-80% confluency on the day of transfection. In a 15 mL conical 18 μg of DNA was mixed with 774 μL DMEM then 108 μL Fugene HD or Fugene 6 added and the tube gently tapped. The transfection medium was left at RT for 15 min. Complete DMEM was removed from 293T/LentiPhos cells and replaced with 7 mL fresh complete DMEM and the transfection medium gently pipetted onto the cells. Cells were left for 3 days at 37^{o}C.

Transfection for NL4-3-eGFP was performed as follows: 293T cells were seeded in 10 cm culture dishes in complete DMEM to obtain 50-80% confluency on the day of transfection. In tube A, 12 μg each of pNLC4-3 and pNLC4-3^{EGFP} was mixed with 2.5 mL DMEM. In tube B, 2.4 mL DMEM + 100 μM PEI was combined and the contents of Tube B added dropwise to a gently vortexing tube A. The transfection medium was left at RT for 20 min. Complete DMEM was removed from 293T cells and the transfection medium was added to cells and left at 37^{o}C. After 4 hours the transfection medium was removed and replaced with 5 mL complete DMEM for 2 days at 37^{o}C.

2.4.2.3 Concentration and Storage of Virus

Cell-free supernatant was harvested after 3 days and concentrated 60-fold in Amicon 100kDa centrifugal filtration conicals (Millipore). Virus stock was stored in 50 μL aliquots at either -80^{o}C (at NIH) or -170^{o}C (at Oxford).

2.4.3 Titration of Virus Stock

In order to determine the working amount of virus to use for infection of 500 x 10^5 CD4$^+$T cells, virus was diluted 1-, 2-, 4-, and 8-fold with cRPMI/IL-2 starting at 1x = 50 μL virus. Virus stocks were then mixed with 10 μL Viromag beads (Oz Biosciences, Marseille, France) and magnetofection carried out as described in 2.5. After 48 h cells were fixed in 4% formaldehyde (FA) for 15 minutes, permealbilized, and stained with KC57-RD1 or KC57-FITC (Beckman Coulter, Miami, FL, USA) at a 1:100 dilution in BD Perm/Wash™ Buffer (BD Bioscience, San Jose, CA, USA). p24 expression was assessed by FACS using either FACS Aria IIu (NIH) or FACS Calibur Cytometer (Oxford) (BD Biosciences, Franklin Lakes, NJ, USA).

2.5 Magnetofection of CD4$^+$ T Cells

CD4$^+$ T cells were selected as described in 2.2.2 and resuspended in blasting media at 1 x 10^6 for 3 to 4 days. Blasting media consisted of cRPMI supplemented with 180 ng/mL OKT3, 180 ng/mL anti-CD28, and 10 IU/mL IL-2. Cells were pelleted and resuspended at 1 x 10^7 in cRPMI/IL-2 and 5 x 10^5 (50 μL) added to wells of 96-well flat bottom tissue cultured plate. 50 μL of virus (diluted in cRPMI/IL-2 if necessary) was mixed with 10 μL Viromag beads (Oz Biosciences) for 15 minutes. 60 μL of magnetized virus was added to each well of cells, leaving one well with no virus for an uninfected

control. The plate was centrifuged in a pre-warmed incubator at 500 x g, 2 min, 37^{o}C. The plate was then placed on a pre-warmed magnet at 37^{o}C for 15 min. 100 μL of cRPMI/IL-2 was added drop-wise to each well so as not to disturb the cell layer and returned to the incubator. Cells were used 48 h post magnetofection. This typically results in 30 – 60% infection of cells.

2.6 Fluorometric Cytotoxicity Assay

One of the initial goals of this project was to adapt a CD8^{+} T cell killing assay to provide a more robust assay for HIV-specific ADCC (Migueles et al., 2008). Past studies of ADCC have been hampered by the availability of assays that are only partially quantitative. There are four primary methods that people use to measure ADCC (Chung et al., 2008). The most prevalent is the chromium release assays (CRA), which is highly variable, does not specifically account for the mechanism of killing, and is insufficiently quantitative (Kohl et al., 1977). In an example of an ADCVI assay, targets, serum, and effectors are incubated for 24 hours and the supernatant is applied to the B95-8 cell line for 5-7 days. The endpoint is reduction of viral infection of the B95-8 cells (Forthal and Landucci, 1998). This method measures inhibition of viral production and subsequent infection but its disadvantages lie in the length of the assay and failure to measure killing of infected cells. Another assay is the intracellular cytokine staining ADCC (ICS-ADCC) assay measures IFN-γ, the primary cytokine responsible for the antiviral activity of many cells including NKs, CD8^{+} T cells, Th1, and DCs; and also CD107a, a marker of degranulation in CD8^{+} T cells and NKs (Stratov et al., 2008). The advantages of this assay are that multiple effector

functions can be measured and all cells and Abs are from the same patient, but it is still a proxy that does not measure HIV-infected cell killing. The rapid and fluorometric antibody-dependent cellular cytotoxicity (RFADCC) assay stains the cytosol with CFSE and cell membrane separately, directly measuring cell killing but requires whole protein (Gomez-Roman et al., 2006). While use of protein pulsed targets is a sensitive method, it likely places artificially high amounts of protein on the surface of infected cells, potentially over-estimating the efficacy of ADCC.

2.6.1 Fluorometric Cytotoxicity Assay at Oxford

Infected $CD4^+$ T cell targets were washed with 1% bovine-serum albumin (BSA)/PBS (Sigma-Aldrich) and consecutively labelled for 30 min at RT with 1 μL LIVE/DEAD® Fixable Far Red stain (Invitrogen) in 100 μL PBS and 7.5 anti-CD3 PerCP/Cy5.5 (BioLegend, San Diego, CA) in 100 μL PBS. Autologous effectors isolated from fresh whole blood were labelled for 30 min at RT in 100 μL PBS: MCs, 1:10 anti-CD14 FITC (BD Biosciences); NKs, 3:10 anti-CD56 FITC (BioLegend); PMNs, 2:10 anti-CD66b FITC (BioLegend). Cells were combined at an effector:target (E:T) ratio of 10:1 (300×10^5: 30×10^5) in a 96-well round-bottom plate (RBP) and pelleted for 2 min before the addition of serum diluted in cRPMI. Cells were again pelleted for 30 s and incubated at 37°C for 1 hour at 37°C, fixed at 4°C with 4% FA, permeabilized 30 min in 0.1% saponin/3%FBS/PBS buffer (PermWash Buffer, PWB), and stained for intracellular viral capsid p24 using 1:100 KC57-RD1 (Beckman Coulter, Miami, FL) in PWB for 30 min at 4°C. Samples were analyzed on a BD FACS Calibur™ (BD Biosciences) to determine infected cell elimination. Data were analyzed using FlowJo software (TreeStar, San Carlos, CA). HIV-1 infected $CD4^+$ T cell elimination (ICE) was calculated as

[(percent p24 expression of infected targets – percent p24 expression of infected targets mixed with effectors and sera)/percent p24 expression of infected targets]×100. Experiments were performed in duplicate for all donors.

2.6.2 Fluorometric Granzyme Cytotoxicity Assay at NIH

Infected $CD4^+$ T cell targets were labelled with 0.8 μL LIVE/DEAD® Fixable Violet (Invitrogen) in 100 μL PBS. Autologous PBMCs were rested in cRPMI overnight and NK effectors isolated on the day of assay. Effectors were labelled with 1 μL LIVE/DEAD® Fixable Far Red stain in 100 μL PBS. Both targets and effectors were resuspended in a 3-fold dilution of Granzyme B (GrB) substrate (GranToxiLux, OncoImmunin, Gaithersburg, MD), combined at an E:T of 10:1, pelleted, and incubated in the presence of HIV^+ serum or MAb in a 96-well RBP at 37^{o}C for 1 h. Cells were washed and resuspended in 1%BSA/PBS, harvested into tubes, and placed on ice prior to analysis. Samples were analyzed on a FACSAria IIu cytometer (BD Biosciences). From the lymphocyte population LIVE/DEAD® Far Red stain-positive effectors were excluded and LIVE/DEAD® Violet stain-positive targets were selected. Intracellular GrB content was assessed in the 2,000 LIVE/DEAD® Violet stain positive events collected. The remaining cells were subsequently fixed with Cytofix/Cytoperm™ (BD Biosciences) at 4^{o}C for 30 min and stained for intracellular viral capsid p24 using 1:50 KC57-RD1 and 1:50 anti-CD4 phycorythrin-Cy7 (BD Biosciences) in Perm/Wash™ Buffer (BD Biosciences) to confirm infected cell elimination by FACS. Data were analyzed using FlowJo software. ICE was calculated as [(percent p24 expression of infected targets with effectors – percent p24 expression infected targets mixed with

effectors and sera)/percent p24 expression of infected targets with effectors] x 100. Results are reported by subtracting the background of targets-effectors-normal serum for patient serum samples and targets-effectors for mAbs to determine killing attributable solely to the presence of IgG. Experiments were performed in duplicate on 3 separate days with similar results. In preliminary experiments the coefficient of variation for ICE was 15.2% across 3 days.

2.7 Cell Count, Viability, and Size Measurements

For stem cell experiments in chapter 4 cells were measured by Nucleoview®. 19 µL of culture was added to 1 µL of Solution 13 AO DAPI (ChemomMetic A/S, AllerØd, Denmark), and 10 µL loaded onto NC-Slide A8™. Slides were loaded into NucleoCounter® NC-3000™, and readings analyzed using NucleoView® software. At NIH cells and viability were counted by Guava ViaCount Assay (Millipore) usually at a 1:40 dilution. Otherwise, cells were counted my standard trypan blue exclusion staining.

2.8 Statistical Analysis

Statistics were performed as described in subsequent chapters.

Chapter 3

Comparative Efficiency of HIV-1-Infected T Cell Killing by NK Cells, Monocytes, and Neutrophils

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3.1 Abstract

HIV-1 infected cells are eliminated in infected individuals by a variety of cellular mechanisms, the best characterized of which are cytotoxic T cell and NK cell-mediated killing. An additional antiviral mechanism is antibody-dependent cellular cytotoxicity. Here I use primary $CD4^+$ T cells infected with the BaL clone of HIV-1 as target cells and autologous NK cells, monocytes, and neutrophils as effector cells, to quantify the cytotoxicity mediated by the different effectors. This was carried out in the presence or absence of HIV-1-specific antisera to assess antibody-dependent or intrinsic mechanisms of cellular cytotoxicity. I have shown that at the same effector to target ratio, NK cells and monocytes mediate similar levels of both antibody-dependent and antibody-independent killing of HIV-1-infected T cells. Neutrophils mediated significant antibody-dependent killing of targets, but were less effective than monocytes or NK cells. Laser-scanning confocal microscopy confirmed effector cell induced apoptosis and revealed that MCs rapidly ingest apoptotic bodies of killed cells. These data have implications for acquisition and control of HIV-1 in natural infection and in the context of vaccination.

3.2 Introduction

The primary target of HIV-1 infection in vivo are $CD4^+$ T cells. Not only are these thought to be the first cells infected after sexual transmission, but they

also account for up to 90% of the infected cell burden *in vivo* (Zhang et al., 1999). Control of established HIV-1 infection is mediated predominantly by $CD8^+$ T cells (Reynolds et al., 2008), but there is also evidence that NK cells (NKs) play a role (Martin et al., 2002; Martin et al., 2007; Sun et al., 2011). NKs can effect HIV-1 infected cell killing either directly, or via antibody-dependent cellular cytotoxicity (ADCC (Berger and Alter, 2011; Jost and Altfeld, 2013)). Although the role of ADCC in the control of HIV-1 infection is unclear, it does appear to contribute to the protective effect of Abs *in vivo* (Hessell et al., 2007). The role of monocytes (MCs) is less well established. Recent reports have demonstrated monocyte killing of an HIV-1-infected T cell line *in vitro* via an ADCC mechanism (Kramski et al., 2012a; Kramski et al., 2012b). However, the target cells used in these assays were a recombinant antigen-coated immortalized cell line rather than infected autologous primary cells, and the relative efficiency of killing compared to NK cells was not revealed. Neutrophils (PMNs) are innate immune cells with properties that include rapid infiltration into sites of inflammation, intrinsic phagocytic and other antibacterial activity, and expression of Fc receptors (FcRs) allowing mediation of Ab-mediated effector mechanisms (Mantovani et al., 2011). Although PMNs are classically described as cells with activity against extracellular pathogens (Borregaard, 2010; Nathan, 2006), they have antiviral activity and can reduce influenza virus infection in cell culture by ADCC-type effector mechanisms (Fujisawa, 2008; Hashimoto et al., 1983). In addition, their function against HIV-1-infected cells has been proposed in the presence and absence of HIV-1-specific antibodies (Baldwin et al., 1989; Jenkins et al., 1993). The rapid recruitment of PMNs, and with somewhat

delayed kinetics, MCs, to sites of inflammation mean that if these cells have antiviral activity then they may influence HIV-1 transmission and/or established viral replication (Demirkhanyan et al., 2012).

Chapter Hypotheses

Given the role NKs, MCs, and PMNs have in eliminating infected cells, understanding to what extent and how they do so against HIV-1-infected cells is important. The cytotoxic mechanism of NKs is well characterized but that of MCs and PMNs is not. A number of studies have shown that mediators essential to the antimicrobial activity of these cells, such as ROS and IFN-γ, are generated but not essential for a cellular response (Baldwin et al., 1989; Clark et al., 2010; Kleinerman et al., 1985; Takamuku et al., 1996). Laser-scanning confocal microscopy would be a useful tool to examine cytotoxicity by these cells because it would show gross morphological changes associated with killing mechanisms exclusive to these cells. There are very few reports using microscopy to describe MC and PMN target cell ADCC but phagocytosis by MCs and trogocytosis by PMNs have been described (Horner et al., 2007b; Kramski et al., 2012b). Neutrophil extracelluar traps (NETS) may be another mechanism. I show that MCs have killing activity equivalent to NKs both in the absence and presence of HIV-1 specific antibodies, and that PMNs can also kill HIV-1-infected T cells via an antibody-dependent mechanism. Microscopy confirmed effector cell induced apoptosis and revealed that MCs rapidly ingest apoptotic bodies. NET formation by PMNs in response to infected cells was not observed nor the ingestion of apoptotic bodies as would be expected by these phagocytes.

My specific aims were to:

1. Compare HIV-1-infected cell killing by NKs, MCs, and PMNs.
2. Compare how NK, MC, and PMN HIV-1-infected cell killing is enhanced by HIV-1 antiserum.
3. Use confocal microscopy to image the effector-target cell synapse to gain insight as to how HIV-1-infected cells were eliminated, either by phagocytosis, trogocytosis, or in the case of PMNs, NETs.

3.3 Materials and Methods

3.3.1 Sample collection

Bpool serum was collected as described in Section 2.3.1. HIV^- serum and blood were collected from uninfected donors in accordance with the University of Oxford Occupational Health Service guidelines; written and verbal consent was obtained from all donors.

3.3.2 Cell isolation and characterization

Heparinised blood was collected from seven HIV^- donors and leukocytes separated using Ficoll density centrifugation as described in Section 2.2.1 and 2. Briefly, NKs and MCs were negatively isolated from PBMCs using EasySep® Human NK and Monocyte Kits. Granulocytes were enriched for using dextran sedimentation and hypotonic lysis and PMNs isolated from granulocytes using the Neutrophil EasySep Enrichment Kit. $CD4^+$ T cells were positively selected for using MACS CD4 MicroBeads. The purity of cells was accessed for each experiment by indirect immunofluorescence and flow cytometry using the following mAbs: CD3 (UCHT1 clone), CD56 (MEM-188 clone), CD14 (M5E2 clone), CD66b (G10F5 clone). The MC fraction did not

stain for CD3, CD8, or CD66 but had a small percentage of CD56 labelled cells, which may represent non-classical $CD56^+$ MCs (Sconocchia et al., 2005).

3.3.3 Killing assay

Killing assays were performed as described in Section 2.6.1. In brief, $CD4^+$ T cells were magnetofected with HIV-1_{BaL} for 48 h, reaching an average infection across experiments of 54.8%. On the day of the assay infected $CD4^+$ T cell targets were consecutively labelled with LIVE/DEAD® Fixable Far Red and anti-CD3 PerCP/Cy5.5. Autologous NK, MC, and PMN effectors were isolated and labelled with the aforementioned FITC-labelled antibodies. Cells were combined at an E:T ratio of 10:1 in the presence or absence of heat-inactivated serum for 1 h at 37^{o}C, fixed, permeabilized, and stained for intracellular viral capsid p24. Samples were analyzed by flow cytometry. The readout for the killing assays was HIV-1 infected $CD4^+$ T cell elimination (ICE).

3.3.4 Statistics

Statistical analysis was performed with Prism® (GraphPad Software, LaJolla, CA, USA). Comparisons of independent groups were made by one-way or two-way ANOVA with Bonferroni post-test.

3.3.5. Confocal Microscopy

NK, MC, and PMN effector cells were isolated as described in Section 2.2.2. $CD4^+$ T cells were magnetofected 2 days prior with $HIV_{NL4\text{-}3\text{-}eGFP}$ as described in Section 2.5. NK, MC, and PMN effectors were stained with Cell Tracker Orange CMRA (CTO) (Invitrogen). A 10 mM stock of CTO was prepared by mixing a 50 µg vial at RT with 9 µL of DMSO. CTO was diluted to a working dilution of 2 µM by mixing 1 µL in 5 mL of RPMI without phenol red and

warmed to 37^{o}C. Cells were resuspended in 2 µM CTO at 1 x 10^{6} cells/mL and incubated at 37^{o}C for 30 min. Cells were washed and rested at 37^{o}C in cRPMI for 30 min. In a 1.5 mL microcentrifuge tube 500 x 10^{5} effectors were combined with 250 x 10^{5} infected T cells and 100-fold diluted Bpool serum in a total volume of 50 µL cRPMI and incubated at 37^{o}C for 20 min, as determined to be the optimal length of time to observe conjugates of still viable CD4^{+} T cell targets and effectors. Cells were washed in 1% BSA/PBS and fixed in 4% FA. Cells were resuspended 7 µL 1% BSA/PBS/2mM EDTA and combined with 10 µL VECTASHIELD® Hard Set Mounting Medium with DAPI (Vector Labs, Burlingame, CA, USA) on an X-tra® adherent slides (Leica Biosystems, Newcastle Upon Tyne, UK). Images were acquired on an Olympus Flouoview 1000 Confocal using sequential capture at 100X and analyzed using Olympus Fluoview Software. 3D reconstructions of Z stacks were prepared using Imaris software (Bitplane, Zurich, Switzerland).

3.4 Results

3.4.1 Purity of isolated effector and target cell populations

Primary target and effector cells were isolated from PBMCs freshly obtained from seven normal healthy subjects and immediately prepared for the killing assay. Different cell subsets were isolated using magnetic bead-based negative selection, and purity assessed using flow cytometry and markers specific for the individual cell types: CD4^{+} T cell (CD3); NK (CD56); MC (CD14) and PMN (CD66b). Mean purity across seven experiments was as follows: CD3^{+}CD4^{+} T cells = 99.4%, NK cells = 98.7%, MCs = 92.5% and PMNs = 100% (Fig. 3.4.1).

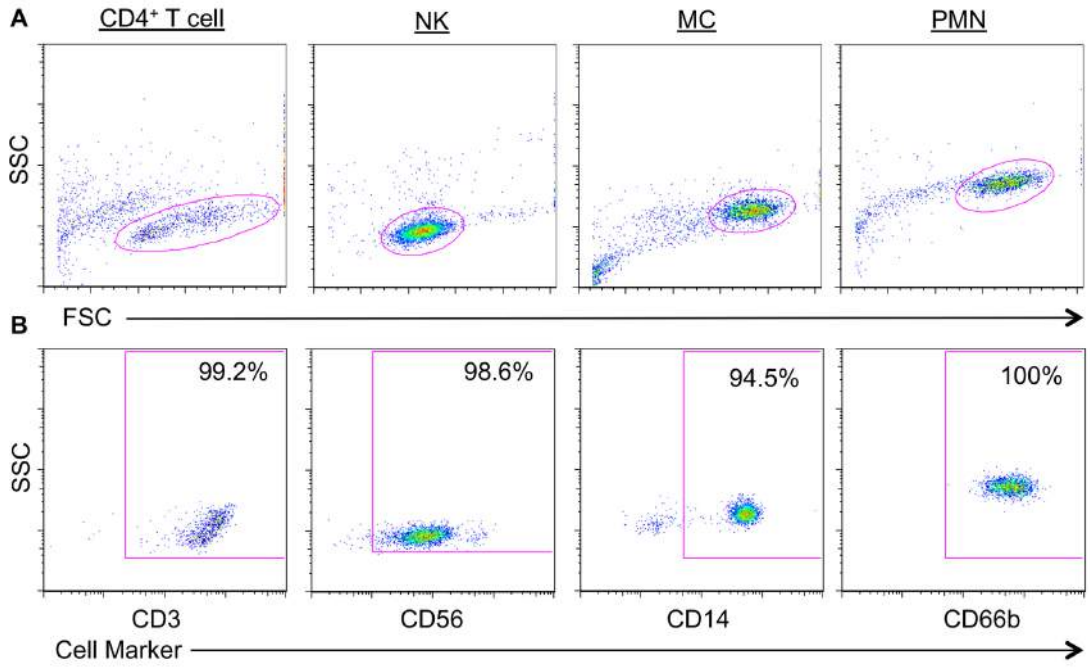


Figure 3.4.1. Purity of isolated target and effector cells. Isolated cells were stained with an appropriate single cell marker for identification. The percent positive populations (B) were assessed on live-gated cells (A): $CD4^+$ T cell = $CD3^{hi}$, NK = $CD56^{dim}$, MC = $CD14^{hi}$, PMN = $CD66b^{hi}$.

3.4.2 Infected cell elimination (ICE) assay

The killing assay was based upon that previously described in (Smalls-Mantey et al., 2012) and detailed in Section 2.6.1. Briefly, $CD4^+$ T cells were stimulated for 72 h and subsequently magnetofected™ with HIV-1_{BaL} for 48 h, yielding an average infection across seven experiments of 54.8%. Non-viable infected $CD4^+$ T cell targets were excluded from analysis by selective labeling with a dead cell label. Autologous effectors were isolated and combined at an effector:target ratio of 10:1 in the presence or absence of heat-inactivated human serum for 1 h at 37^{o}C, fixed, permeabilized, and stained for intracellular viral Gag. Target cell killing (HIV-1 infected $CD4^+$ T cell elimination - ICE), was evaluated by quantifying the loss of Gag^+ $CD3^+$ T cells from the live cell gate compared to Gag^+ T cell loss in the absence of

effector cells (Fig. 3.4.2).

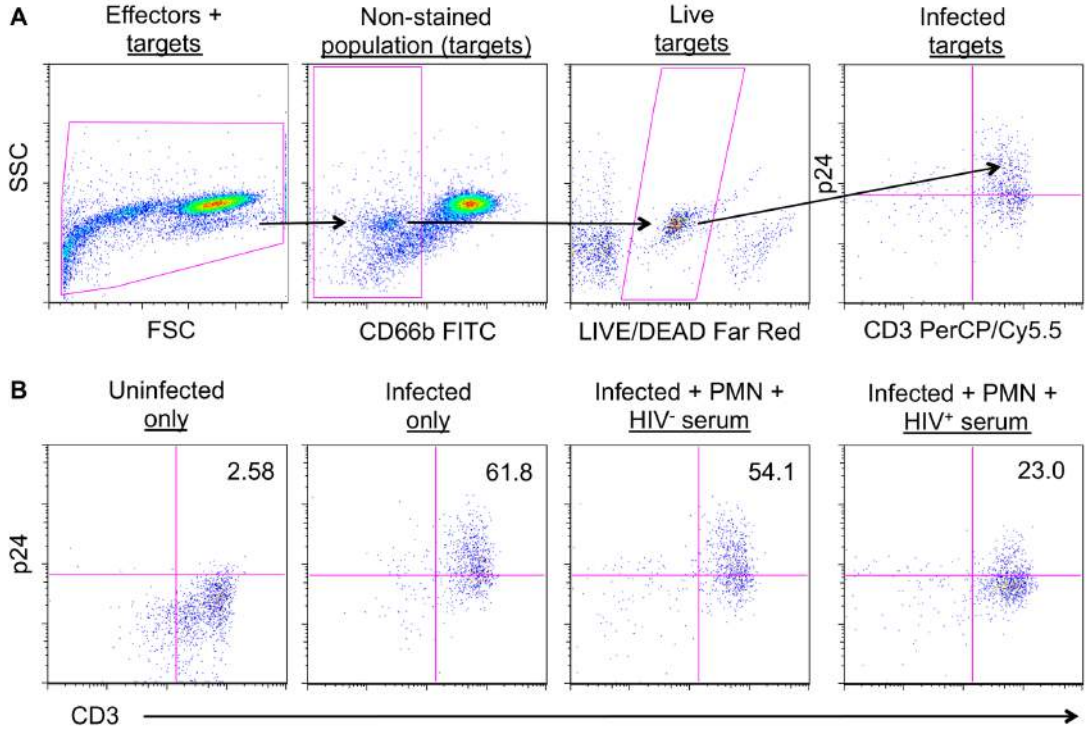


Figure 3.4.2. Gating strategy for ICE Assay. (A) Effector cells were labelled with the phenotypic marker mAbs shown and targets labelled with LIVE/DEAD stain and mixed. Live $CD4^+$ T cells (targets) were identified by gating on live cells negative for the effector label (NK, MC, or PMN-FITC). Cells were fixed and stained for p24 to determine the frequency of infected targets. (B) Elimination of HIV-infected cells. Representative data show % p24 positive cells for uninfected $CD4^+$ T cells, infected $CD4^+$ T cells, infected $CD4^+$ T cells + PMN + HIV^- (control) serum, and infected $CD4^+$ T cells + PMN + HIV^+ (Bpool) serum. A reduction in the number of p24-stained cells is represented as % ICE and indicates cell death. In the example described above, $\%\ ICE_{Infected\ CD4+PMN+Bpool} = [(61.8-23.0)/61.8] \times 100 = 62.8\%$.

3.4.3 NKs, MCs, and PMNs mediate ICE

Killing was evaluated in seven subjects in the absence of human serum or using serum from an $HIV\text{-}1^-$ donor (control serum) to assess non-ADCC-mediated killing, or in the presence of pooled $HIV\text{-}1^+$ (Bpool) serum to assess ADCC. Serum was diluted serially 5-fold from 1:20 to 1:62,500 (Fig. 3.4.3). There were inherent differences in the level of cytotoxicity mediated by subjects' effector cells against HIV-1 infected $CD4^+$ T cells (Fig. 3.4.3, no

serum) that was not significantly modulated by control serum. Killing efficiency varied substantially between donors. In some donors killing by all effector cell types was negligible in the absence of immune serum, whereas in others NK and MC killing varied from 20-40%. In general PMN killing in the absence of antiserum was absent or weak. Pooling the data from all donors revealed a clear increase in killing efficiency with increasing concentration of antiserum, with NK and MC yielding approximately equivalent killing of ~65% and PMNs mediating ~47% ICE at a serum dilution of 1/20.

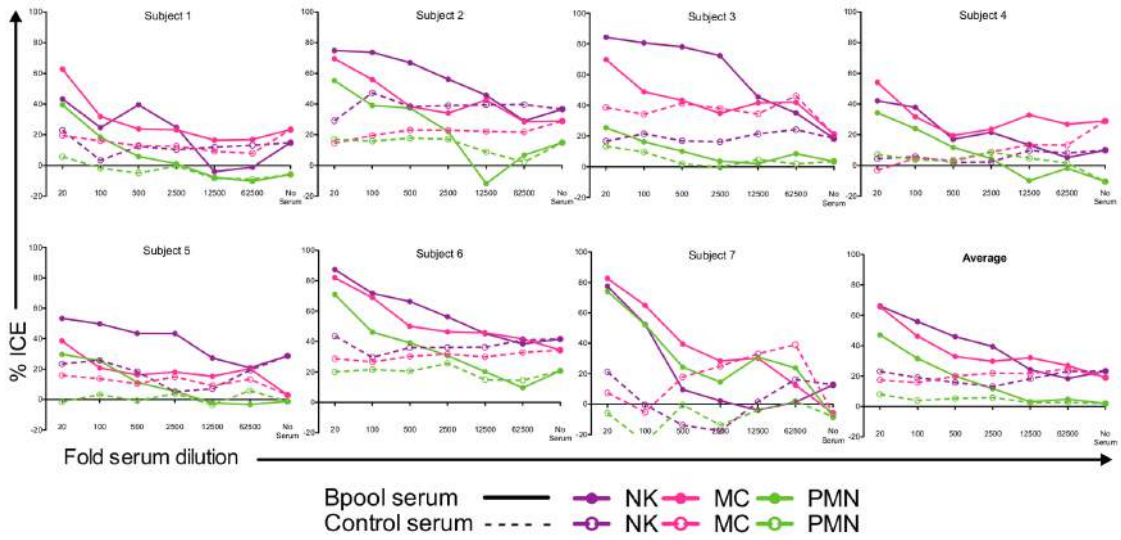


Figure 3.4.3. NKs, MCs, and PMNs mediate ICE. Percentage ICE mediated by NKs, MCs, and PMNs in seven subjects, at an effector:target ratio of 10:1 without or with Bpool (HIV-1^{+}) or control (HIV^{-}) serum at serial 5-fold dilutions starting at 1:20. Each datum point represents the mean of duplicate samples.

3.4.4 NKs, MCs, and PMNs differ in killing activity

These trends were confirmed by carrying out an area under the curve analysis of log-transformed x-axis curves of killing at all dilutions of antiserum or in the absence of antiserum in the combined data set. NKs and MCs alone had a highly significant intrinsic ability to kill infected cells (Fig. 3.4.4A, no serum/effectors compared to effectors + control serum, $P < 0.001$) whereas

PMNs showed a modest but insignificant trend to intrinsic killing (no serum/effectors compared to effectors + control serum $P > 0.05$). ADCC due to HIV-1-specific Abs titrated out at higher serum dilutions to yield values similar to killing of effectors with or without uninfected control serum (Fig. 3.4.3). HIV-1 specific Abs in Bpool serum significantly enhanced killing by NKs ($P < 0.001$), MCs ($P < 0.01$), and PMNs ($P < 0.05$) (Fig. 3.4.4A) when compared to control serum. NKs mediated statistically equivalent levels of killing to MCs, whereas PMNs mediated a significantly lower level of ADCC compared to NKs ($P < 0.05$) (Fig. 3.4.4B).

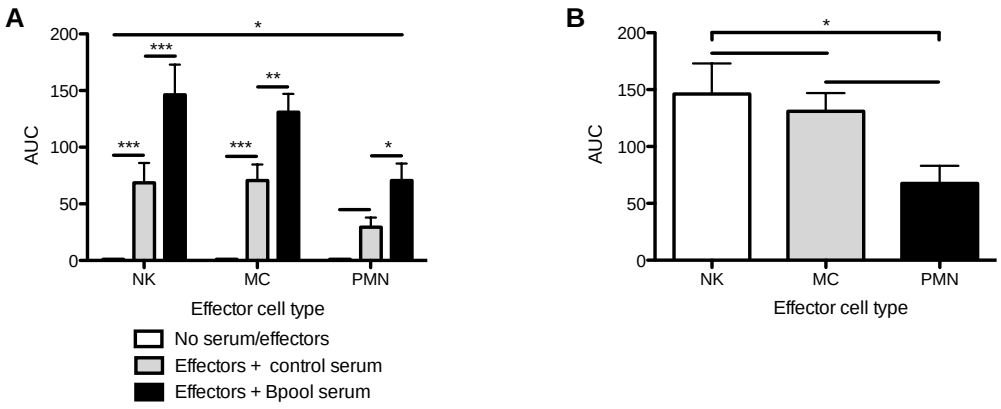


Figure 3.4.4. NKs, MCs, and PMNs differ in killing activity. Total area under the curve (AUC) of % ICE curves presented in Figure 3 for log_{10}-transformed serum dilutions from 1:20 to 1:62,500 was calculated for all 7 subjects and means calculated. Comparisons of independent groups were made by two-way or one-way ANOVA (A and B, respectively) with Bonferroni post-test. (A) Comparison of AUC for infected cell elimination in the absence of serum or effectors (AUC set at 1), effector cells with control serum, and effector cells with Bpool serum. HIV-1-specific Abs enhance killing by NKs ($P < 0.001$), MCs ($P < 0.01$), PMNs ($P < 0.05$). (B) Comparison of AUC for effector cells with Bpool serum. NKs and MCs mediate similar levels of ADCC; PMNs mediate significantly less ADCC than NKs ($P < 0.05$).

3.4.5 Imaging the ADCC synapse

Infected $CD4^+$ T cell-effector conjugates treated with Bpool sera were imaged using laser-scanning confocal microscopy (LSCM). Effector cells were

prelabeled with CTO and $CD4^+$ T cells were infected with $HIV_{NL4\text{-}3\text{-}eGFP}$ that secretes soluble GFP so the entire cell fluoresces green when infected. Nuclei were stained with DAPI when mounting so changes in nuclear morphology associated with apoptosis could be observed. Images are from one experiment and are representative of four independent experiments. Fig. 3.4.5 shows staining for individual target and effector cell populations. The infected cells appear larger than uninfected cells and represent syncytia formation, characteristic of HIV-1 infection. Fig. 3.4.6 shows single slice views of infected T cells and effectors mixed with Bpool serum at an E:T of 2:1. Images in Fig. 3.4.7. are a 3D reconstruction of *z*-stack slices. At least 2 effector cells are in apposition with infected cells whereas uninfected cells in the field are largely ignored by effectors (Fig. 3.4.6 and 3.4.7). In Fig 3.4.7 infected cells with fragmented cellular membranes in contact with effector cells represents cells having undergone apoptosis presumably as a result of interaction with the effector cell. Of note, MCs are seen to have phagocytised small infected cell fragments of the apoptotic cell they are contact with, confirming similar observations made by Kramski et al. (Kramski et al., 2012b).

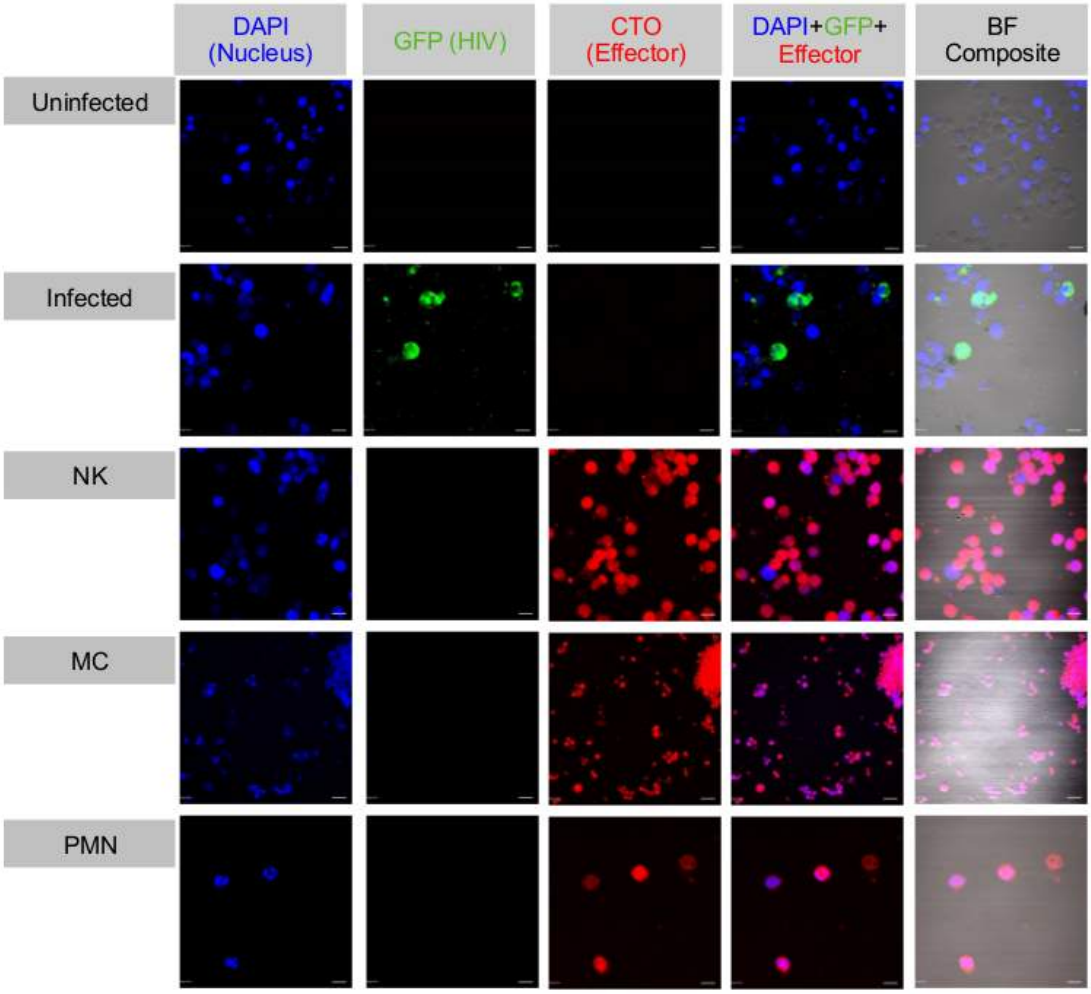


Figure 3.4.5. Single cell staining. Single optical sections taken through the centre of individual effector and target cell populations with LSCM at 100X. Targets and Effectors were stained as follows: Uninfected (none); NL4-3-eGFP infected T cells (GFP, green); NK, MC, PMN (CTO, CellTracker™ Orange, red). All cell nuclei were stained with DAPI (blue). Scale bar (white)= 10 μm.

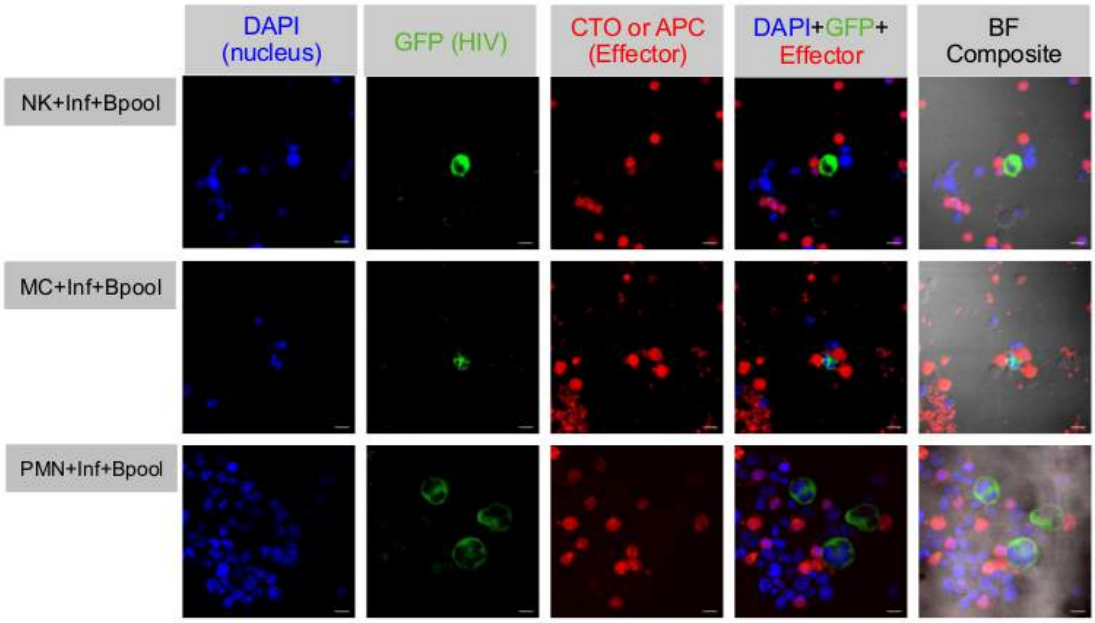


Figure 3.4.6. Detection of HIV-1-infected T cell and effector cell conjugates. NL4-3-eGFP infected T cells (GFP, green) combined with 1:100 Bpool serum and effectors (CTO, red) for 20 min were fixed and single optical sections taken through the centre with LSCM at 100X. Cell nuclei were stained with DAPI (blue). Single colours, merged, and merged with brightfield overlay (BF composite) are shown for each conjugate. Scale bar (white) = 10 μm.

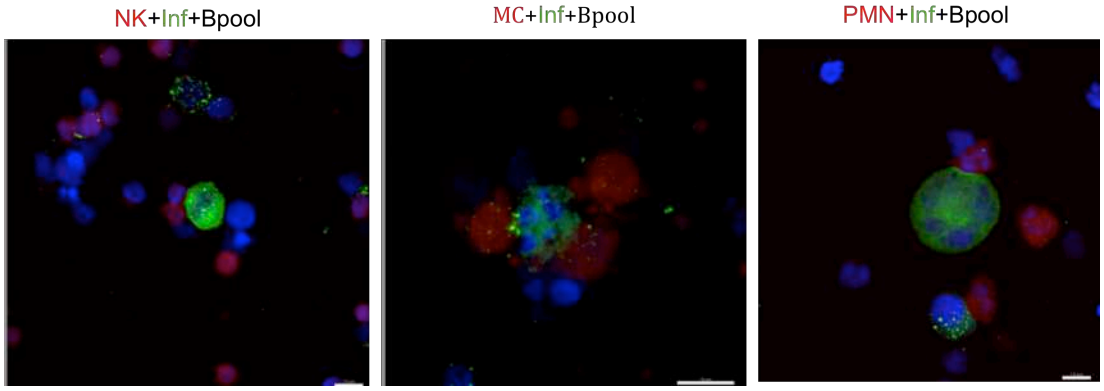


Figure 3.4.7. 3D reconstruction of target-effector-serum conjugates. A 3D reconstruction of Z-stack *x-y* sections (step size = 0.3 μm). Conjugates imaged at 100X, E:T = 1:1. Scale bar (white) = 10 μm.

3.5 Discussion

Others have reported intrinsic killing and ADCC mediated by NKs, MCs, and PMNs in several different assays (Baldwin et al., 1989; Jenkins et al., 1993; Kramski et al., 2012b) but in this study I quantitatively compare the relative efficiency of these three effector cell types using a highly biologically relevant assay system. Either contaminating $CD8^+$ T cells were in the NK-lymphocyte population (Jenkins et al., 1993); or where NKs, MCs, and PMNs were compared it was in a cancer system (Ashraf et al., 2009). I show that NKs and MCs mediate similar levels of ADCC whereas PMNs mediate significantly less ADCC than NKs and MCs. These differences may be attributable to differing levels of FcγR expression, divergent mechanisms with different efficiencies of killing, or both. NKs only express activating FcγRIIIA (CD16a) (Nimmerjahn and Ravetch, 2008) while MCs and PMNs in addition express both activating and inhibitory FcγRs, FcγRI (CD64) and FcγIIA/B (CD32a/b) (Kramski et al., 2012a; Metelitsa et al., 2002; Nimmerjahn and Ravetch, 2008). Kramski et al. (Kramski et al., 2012a) observed more FcγR-IgG binding to PMNs and MCs than NKs. Although they observed greater IgG binding to PMNs, the mechanism was unclear since the relative expression of FcγRs and level of IgG binding to specific FcγRs was not shown. NK cytotoxicity mediated by perforin/granzyme and Fas/FasL has been well characterized (Smalls-Mantey et al., 2012; Smyth et al., 2005). However the mechanism by which PMNs and MCs mediate ADCC is still undefined. Both MCs and PMNs use reactive oxygen species to kill bacteria and while this is generated in response to HIV-1 and during ADCC, it is not essential for killing (Horner et al., 2007a; Koller and LoBuglio, 1981; Saitoh et

al., 2012). MCs and PMNS also contain similar secretory granules such as $CD63^+$ azurphilic granules (Metelitsa et al., 2002), although arguably not perforin and granzyme (Metkar and Froelich, 2004), which may have a role in ADCC. Neutrophil extracellular traps (NETs) have recently been identified as a mechanism by which PMNs kill fungi, bacteria, influenza-infected cells, and most recently, HIV-1 virions (Saitoh et al., 2012). It could be that PMNs exhibit less intrinsic killing and ADCC than NKs or MCs because they use NETs resulting from lysis of PMNs, which may be a less cell-specific and efficient mode of killing.

In the absence of Ab, infected cells are recognized and targeted for killing by different mechanisms in effectors. NKs recognize virus infected cells that have downregulated MHC class I. Early apoptotic cells expressing phosphatidylserine (PS) are recognized by TIM-4 on MPs and phagocytised (Fadok et al., 1992; Miyanishi et al., 2007). TIM-4 has been described in antigen presenting cells such as B cells, DCs, and MPs, and in PMNs, but not in MCs (Kobayashi et al., 2007). HIV-1 infected cells expressing PS may be recognized by other receptors on MCs and targeted for killing.

PMNs and MCs need no priming to mediate their effector functions, and are the first cells recruited to sites of inflammation. PMNs in particular are recruited very rapidly and in high numbers, and so may make up for their reduced killing efficiency by increased effector:target ratios. The lamina propria of mucosal tissues are the first sites of viral replication after HIV-1 transmission, following this, the virus migrates to gut-associated lymphoid tissue (GALT) in which uncontrolled viral replication is associated with massive $CD4^+$ T cell death and local inflammation (Haase, 2010). Such an

inflammatory response is likely to recruit PMNs and MCs (Hunt, 2010). Whether these cell types can modulate early HIV-1 infection by establishing an antiviral environment is an important question that remains to be addressed. In a prophylactic vaccine setting, in which HIV-1-specific Abs were raised, PMNs and MCs could participate in ADCC of HIV-1-infected cells during early infection, potentially increasing Ab effector functions. Thus understanding how innate effector cells kill HIV-1 infected cells will provide important insights into pathogenesis and may inform strategic vaccine designs that would allow us to harness their cytotoxic capacity in acute HIV-1 infection.

There have been several studies that used laser scanning confocal microscopy (LSCM), live cell LSCM, electron microscopy (EM), and Förster resonant energy transfer (FRET), to study the NK immunological synapse that describe the F-actin network and lytic granules or the sequential killing of target cells by NKs. (Bhat and Watzl, 2007; Brown et al., 2012; Mace and Orange, 2013). Fewer studies have looked at MCs and even fewer at PMNs. One study uses transmission EM and LSCM to describe target-effector membrane exchange at the PMN-cancer target cell ADCC synapse (Horner et al., 2007b). Others have used imaging flow cytometry with MCs and reported uptake of target apoptotic bodies (Helguera et al., 2011; Kramski et al., 2012b). It appears that the Kramski study is the only one using HIV-1 (antigen coated) target cells. By fixed-cell LSCM I too observed the uptake of apoptotic bodies by MCs but was not able to make any conclusion about

events surrounding PMN-mediated apoptosis. Several attempts to observe ADCC using live-cell microscopy were unsuccessful but further optimization of the assay with regards to cell number, timing, vessel container, and E:T ratio may prove effective. In conjunction with the fixed-cell LSCM method I have established, one would be poised to do more specific studies of the HIV-1-infected target-effector cell synapse.

Chapter 4

Neutrophil Differentiation from Human Embryonic Stem Cells

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4.1 Abstract

A majority of research into HIV-1-infected cell killing has focused on $CD8^+$ T cells, NK cells, and monocytes as effectors. Neutrophils (PMNs) also have anti-viral and cytotoxic capabilities but are less studied perhaps due to technical limitations. They are short-lived *ex vivo* and are not viable after cryopreservation so they must be isolated from fresh blood immediately prior to use. I sought to generate a steady, homogenous source of PMNs from human embryonic stem cells (hESCs) to use in experiments. Three different methods were used to produce PMNs from hESCs based on similar studies that had used hESCs and induced pluripotent stem cells. hESC line HUES-2 cells were first formed into embryoid bodies for 4 days and then differentiated for 21 days in primary differentiation medium (PDM) containing the following growth factors: insulin-like growth factor II (IGF II), vascular endothelial growth factor (VEGF), stem cell factor (SCF), Fms-like tyrosine kinase 3-ligand (FLT3-L), thrombopoieten (TPO), and granulocyte colony-stimulating factor (G-CSF). The 3 conditions differed in the medium used and supplementation with IL-3: (1) X-Vivo 10™, IMDM, and IMDM + IL-3. After 25 days cells were maintained until Day 67 in terminal differentiation medium (TDM) without growth factors except for G-CSF in half of the samples. Floating cells were collected and productivity of differentiation assessed by cumulative cell harvest, viability, cell size, CD66b expression, morphology, and leukocyte alkaline phosphatase staining. All conditions produced cells with features of PMNs. IMDM supplemented with G-CSF in the TDM was determined to be the best condition. This study exhibits three ways to derive

PMNs from hESCs in a feeder-free system, which could prove useful for further research applications.

4.2 Introduction

NK cells (NKs) and monocytes (MCs) have been extensively used in studies of HIV-1-infected cell killing. Few studies have focused on the antiviral effect of neutrophils (PMNs) or their capacity to kill HIV-1-infected cells. One of the challenges of using PMNs in research is that they must be isolated immediately prior to use because once isolated they die rapidly, they are not viable after cryopreservation, and few PMN cell lines are available. PMNs do not survive long in circulation or culture without stimulation. They have a life span of 6-10 hours in circulation, and 30 minutes after activation and engulfing bacteria (Laskay et al., 2008). It is generally accepted that PMNS survive for about 6 hours in culture. GM-CSF is known to extend the life span of PMNS (Brach et al., 1992). In my hands, PMNs remained nearly 100% for at least 24 hours after which viability decreased (Fig. 4.2.1). Viability was enhanced by the addition of 10 ng/mL GM-CSF.

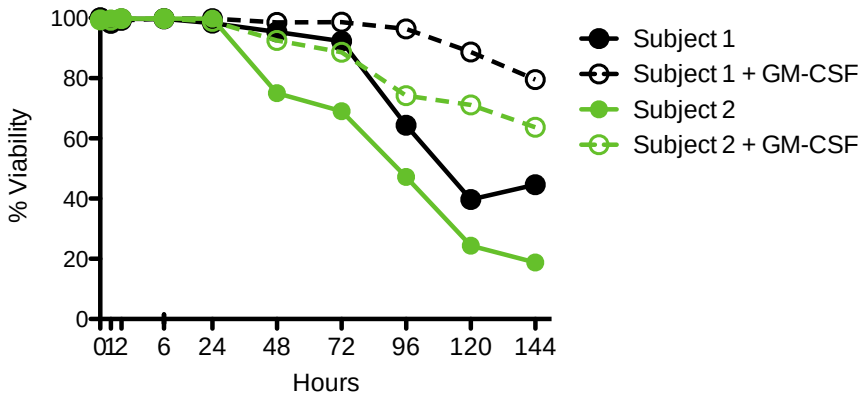


Figure 4.2.1. PMN viability. Viability of primary PMNs ± GM-CSF was monitored over 6 days. The NucleoView® cell counter system, which uses the DAPI to identify intact nuclei, was used to measure viability as described in Section 2.7.

While buffy coats could be used to obtain PMNs (leukocyte cones did not yield many PMNs), the cells have already been *ex-vivo* for 24 hours when received in the late afternoon so it would be logistically difficult to use these suboptimal cells. PMNs do not survive a freeze-thaw cycle as virtually all died within 1 hour of being thawed (Figure 4.2.2). Nishimural *et al.* also reported the death of all PMNs within 2 hours of a thawing from frozen (Nishimura, Mitsunaga, and Juji, 2001). The human promyelocytic cell line HL-60 can be induced to differentiate into cells with PMN-like qualities by all-trans retinoic acid but it is preferable to use primary cells as opposed to artificial systems (Breitman et al., 1980; Martin et al., 1990).

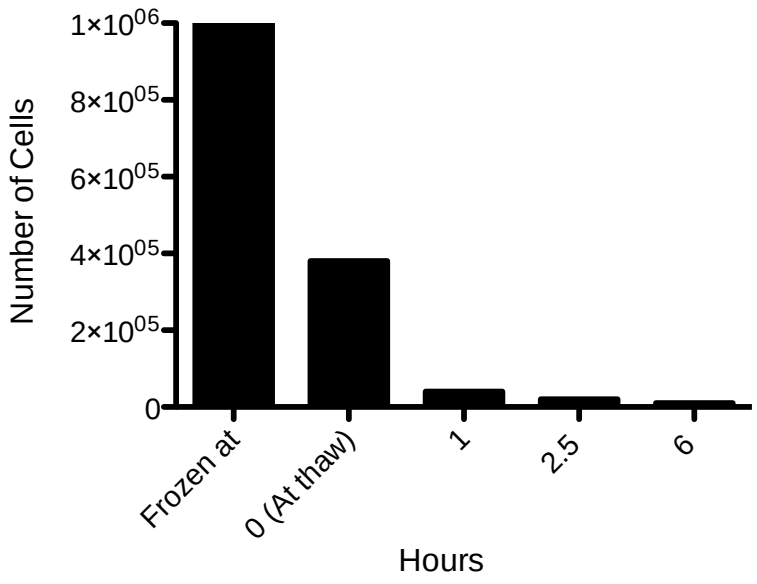


Figure 4.2.2. PMN survival. Survival of cryopreserved primary PMNs after thawing was monitored over 6 hours using the NucleoView® cell counter system.

The differentiation of human embryonic stem cells (hESCs) into PMNs was undertaken in order to generate a steady, homogenous source PMNs that

could be used in the study PMN-anti-cellular/antiviral activity. At the time of this study four other labs had been able to derive PMNs from hESCs or induced pluripotent stem cells (iPS). Figure 4.2.3 illustrates the growth factors that drive hematopoesis from multipluripotent stem cells. Saeki *et al.* generated PMNs from KhES-3 cell line without the use of feeder cells (Saeki et al., 2009). Yokoyama et al*.* used KhES-3 cell line with mouse embryonic fibroblasts as feeder cells (Yokoyama et al., 2009). Morishima et al*.* used iPS cell lines derived from human dermal fibroblasts with mouse bone marrow stromal cell line OP9 (Gao et al., 2010; Morishima et al., 2011), and Zou et al. used iPS cells generated from mesenchymal stem cells from a bone marrow aspirate of a patient with X-linked chronic granulomatous disease and OP9 feeder cells (Zou et al., 2011). The use of irradiated feeder cells is to provide nutrients for stem cells, however they potentially introduce unknown xenotropic factors therefore we did not use them in our system. I used the HUES-2 hESC line developed from cleavage-and blastocyst-stage embryos (Cowan et al., 2004). We used the growth factors as described by Saeki *et al.* because of their use of a feeder-free system but we also added IL-3 which induces differentiation and proliferation of the myeloid lineage to one sample as done by Morishima et al. to see if it would further drive PMN differentiation (Morishima, 2011; Saeki et al., 2009). The growth factors used by Saeki et al. were insulin-like growth factor-II (IGF-II), vascular endothelial growth factor-A (VEGF), stem cell factor (SCF), Fms-like tyrosine kinase 3-ligand (Flt3-L), thrombopoietin (TPO), and granulocyte colony stimulating factor (G-CSF). IGF-II is a fetal growth factor (MeSH D12.644.276.937.420). VEGF plays a role in vascular growth and permeability (MeSH

D12.644.276.100.800.200). SCF is a general hematopoietic growth factor (MeSH D12.644.276.374.410.800). Flt3-L stimulates production of hematopoietic cells (GenBank AF282985-6 (bovine)). TPO stimulates the production of platelets and proliferation of megakaryocytes (MeSH D12.644.276.374.410.240.750). G-CSF stimulates differentiation, proliferation, and survival of neutrophil granulocytes and activates mature blood neutrophils (MeSH D12.644.276.374.410.240.350).

Chapter Hypotheses

Given the success of four other groups in deriving PMNs from hESC I sought to do the same in a feeder-free system.

My specific aims were to:

1. Compare three different formulations of media, growth factors, and further supplementation with G-CSF in their efficiency of generating PMNs.
2. Characterize these cells based on size, CD66b expression, morphology, LAP staining, chemotaxis, and phagocytosis.

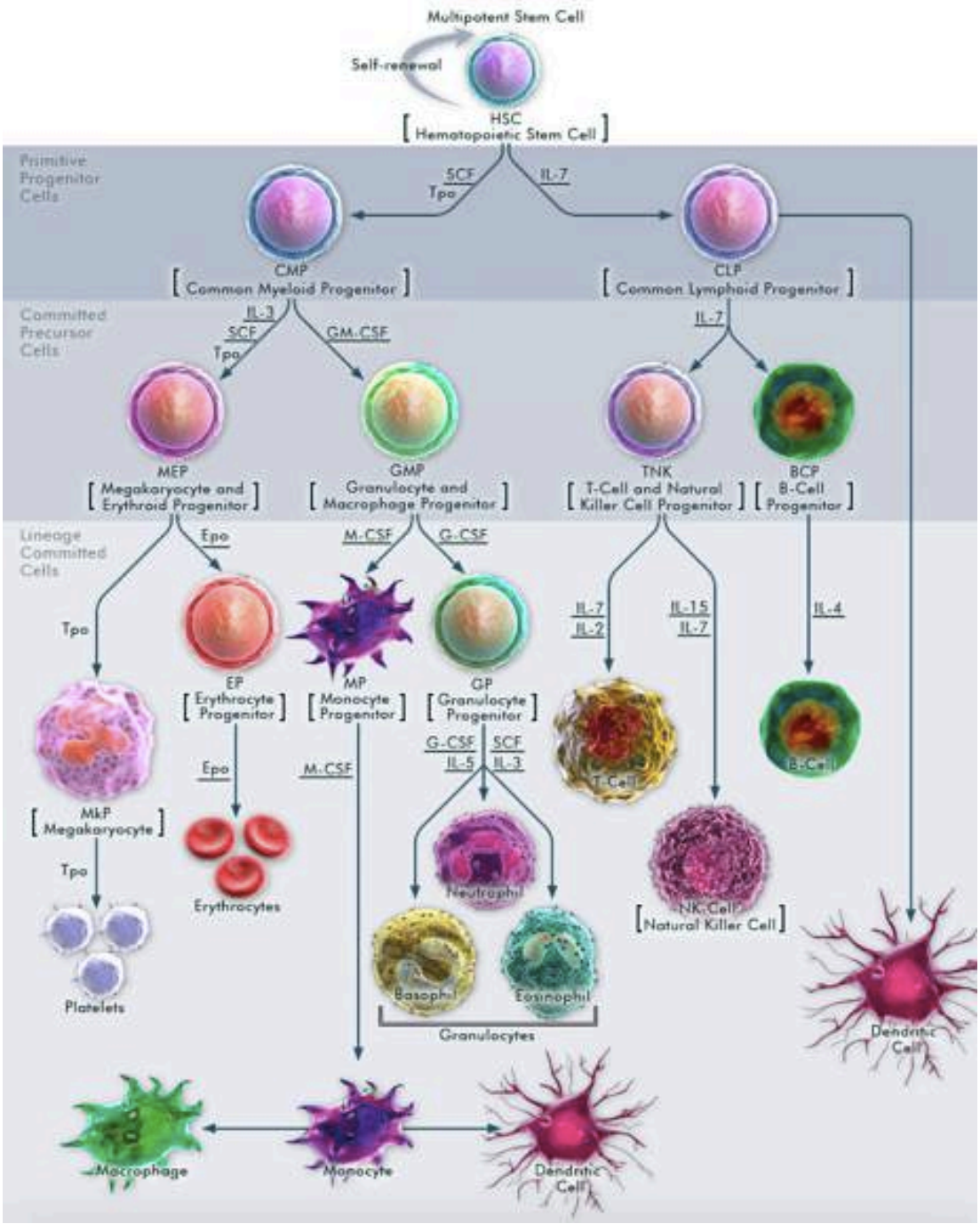


Figure 4.2.3. Hematopoesis from multipotent stem cells. Taken from www.eBioscience.com ©2012 eBioscience.

4.3 Materials and Methods

4.3.1 Culture of hESC

Human ESC line HUES-2 was obtained from the University of Harvard and cultured by members of the James Martin Stem Cell Facility (Oxford) as described in Karlsson *et al.* (Karlsson et al., 2008). Briefly, Cells were maintained on mitomycin C-inactivated mouse embryo fibroblasts (MEF) in 0.1% gelatin-coated 6-well TC plates with 2 mL of hESC medium. hESC medium contained KO-DMEM, 2% KO-Serum Replacement, 0.5 % albumin, 2 mM GlutaMAX™-I (L-glutamine), 1 % nonessential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin (pen/strep), 55 µM β-mercaptoethanol (β-ME), and 10 ng/mL basic fibroblast growth factor. Media was changed daily and cells passaged every 5 days to prevent differentiation.

4.3.2 Formation of Embryoid Bodies (EB)

hESC medium was removed from HUES-2 cells and replaced with 2 mL of EB medium which consisted of DMEM (PAA), 10 % FBS, 2 mM GlutaMAX™ (Gibco®, Invitrogen), 100 µM β-ME, 100 U/mL penicillin and 100 µg/mL streptomycin (PAA), and growth factors (all from Miltenyi): 20 ng/mL IGF-II, 20 ng/mL VEGF, 100 ng/mL SCF, 100 ng/mL Flt3-L, 50 ng/mL TPO, and 100 ng/mL G-CSF. Cells were not immediately transferred to X-Vivo or IMDM media because James Lab observations show that hESC do not fare well when transferred to specialized medium with growth factors immediately. Wells containing HUES-2 were scored 100 times (10x10) with a needle and cells agitated with a P-1000 pipette tip. Patches of cells and media were transferred to ultra-low adherence 6-well plates (Corning, Tewksbury, MA, USA) and cultured for 4 days at 37^{o}C. After 1 day cells had defined edges which confirmed EB formation.

4.3.3 Differentiation of hESC into PMNs

After 4 days EBs were transferred to Primary Differentiation Medium (PDM). Three different conditions were set up:

a) X-Vivo: This medium is used for MC differentiation so it was used to see if it would also generate PMNs. X-Vivo Medium composition: X-Vivo 10™ (Lonza, Walkersville, MD, USA), GlutaMAX™, β-ME, pen/strep, and growth factors as described above. No FBS was added.

b) IMDM: This condition was set up as described by Saeki et al. (Saeki et al., 2009). IMDM medium composition: Iscove's Modified Dulbeco's Medium (IMDM, Lonza), 15% FBS, GlutaMAX™, β-ME, pen/strep, and growth factors.

c) IMDM/IL-3: IL-3 induces differentiation and proliferation of the myeloid lineage. It was used by Morishima et al. (Morishima, 2011). IMDM/IL-3 medium composition: IMDM, 15% FBS, GlutaMAX™, β-ME, pen/strep, and growth factors, and 25 ng/mL IL-3 (R&D Systems, Minneapolis, MN, USA).

d) FM MC: Factory Medium MC – This condition was set up a positive control for ESC differentiation into MC using the standard protocol from the James Lab (Karlsson et al., 2008). FM composition: Advanced DMEM (Gibco), GlutaMax™, β-ME, pen/strep, 25 ng/mL IL-3, and 50 ng/mL M-CSF (R&D).

e) X-Vivo 15 MC: This condition was set up as a positive control for hESC differentiation into MC using a second standard protocol from

the James Lab. X-Vivo 15 medium composition: X-Vivo 15™ (Lonza), GlutaMax™, β-ME, pen/strep, 25 ng/mL IL-3, 50 ng/mL M-CSF (R&D). By Day 4 EBs had round edges. EBs and media were transferred to a 15 mL conical and EBs allowed to settle for a few minutes. EB supernatant containing dead cells was removed. 3 mL of PDM was added to conicals and 15-20 EBs transferred to a well of an adherent 6-well TC treated plates. A minimum of 10 EBs is needed per well to ensure enough paracrine signalling. The plate was gently rocked to ensure equal distribution of cells and left at 37^{o}C. Two wells of each condition were set up. Media was changed every 4-5 days by removing 2 mL and replacing with 2 mL fresh PDM.

4.3.4 Terminal Differentiation

On day 25 cells in PMN-inducing conditions were gradually transitioned to terminal differentiation medium (TDM) by removing 2 mL of culture media and replacing with TDM. MC controls were maintained in the same medium. TDM consisted of either X-Vivo 10™ or IMDM, 15% FBS, GlutaMAX™, β-ME, and pen/step. Growth factors were withdrawn because they were no longer needed and IL-3 removed because it is too general a factor for myeloid differentiation. To half of the wells 100 ng/mL G-CSF was added to drive PMN differentiation. Every 4-5 days 2 mL of media was replaced with fresh TDM.

The process of differentiating neutrophils is illustrated in Fig. 4.3.1.

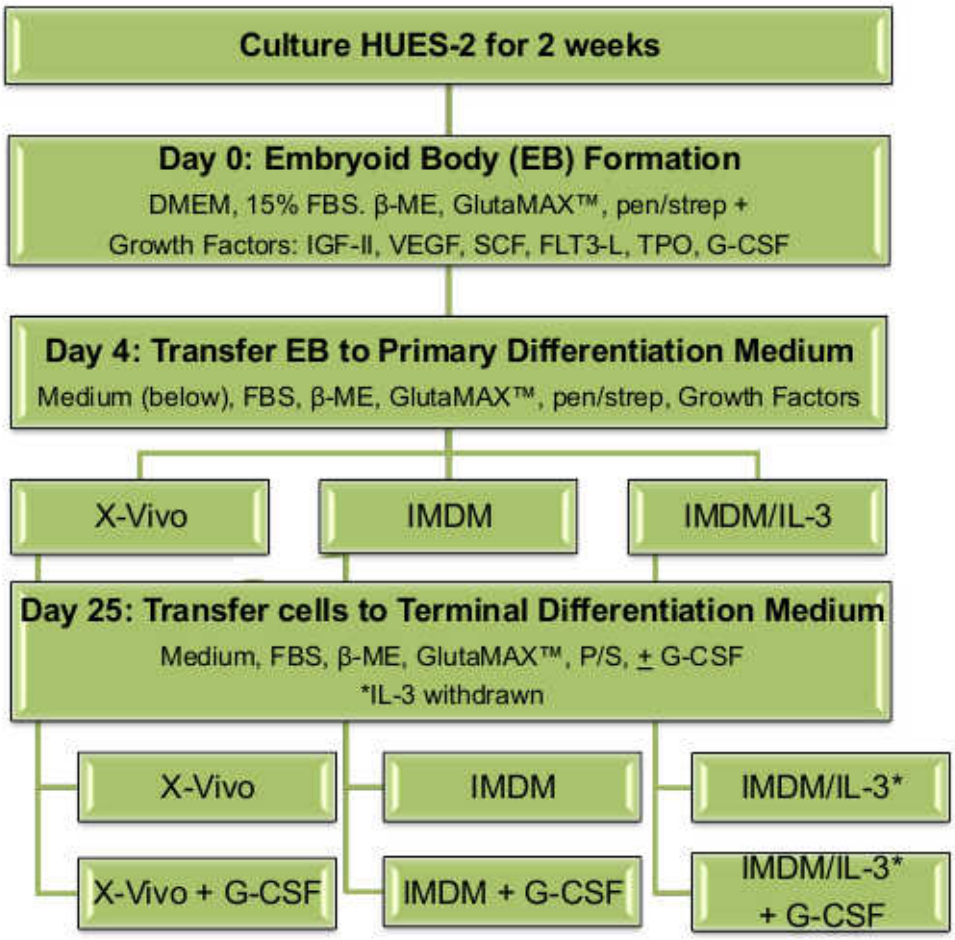


Figure 4.3.1. PMN differentiation from hESC.

4.3.5 CD66b Staining

2 mL of cell culture was removed and pelleted at 450 x g for 6 min. Cells were resuspended in 25 µL of blocking buffer (PBS, 1 % FBS, 10 µg/mL huIgG) for 10 min at 4^{o}C and then the appropriate amount of stain and volume topped up to 50 µL of 1% BSA/PBS. 10 µL of CD66b FITC (BioLegend, San Diego, CA, USA) and 10 µL Mouse IgMκ FITC Isotype Control (BioLegend) was used. Cells were stained for 30 min at 4^{o}C, washed twice with 1%BSA/PBS and then fixed with 2% FA for 30 min at 4^{o}C. Cells were washed and resuspended in 1% BSA/PBS and analyzed by FACS on BD FACS Calibur.

4.3.6 Cytospin

Slides and filter papers were assembled in the cytospin holder units. 20,000 cells were resuspended in 200 μL of PBS and added to slide-holder units. Cells were spun in a Shandon CytoSpin 3 (Thermo Scientific, Waltham, MA, USA) for 4 min at 500 rpm and subsequently stained.

4.3.7 Rapid Blood Stain

Hema Gurr® Rapid Staining Set (VWR International LLC, Radnor, PA, USA) was used to stain cells. Slides were dipped into Solution 1 (methanol) for 1 min, Solution 2 (eosin solution) for 30 s, Solution 3 (methylene blue solution) for 30 s, and rinsed thrice in dH_2O.

4.3.8 Mounting and Imaging Slides

Slides were dried at 37^{o}C for 15-30 min, 1 drop of DPX mountant (Sigma-Aldrich) and 1.0 coverslip applied, and dried at RT overnight before imaging at 40X on EVOS® XL Core Microscope (Electron Microscope Sciences, Hatfield, PA, USA).

4.3.9 Leukocyte Alkaline Phosphatase Staining

Cells were stained with Leukocyte Alkaline Phosphatase (LAP) Kit (Sigma-Aldrich) according to manufacturer's protocol.

4.4 Results

4.4.1 G-CSF increases cumulative cell harvest

Every 4-5 days when culture media was refreshed by removing 2 mL of media and replaced with a fresh 2 mL of media, the total number of cells in 2 mL of culture was counted and summed with previous counts to determine the yield (Fig. 4.4.1). Under all conditions the yield increased until transfer to terminal differentiation medium (TDM) on day 25 after which it took about 2

weeks for cells to acclimatise and again start producing increasing number of cells at day 39. For PMN-inducing conditions IMDM/IL-3 had the greatest yield, then X-Vivo and IMDM, which nearly paralleled each other. The addition of G-CSF increased the yields of all media types. X-Vivo15 MC followed by FM MC controls had the highest yields overall.

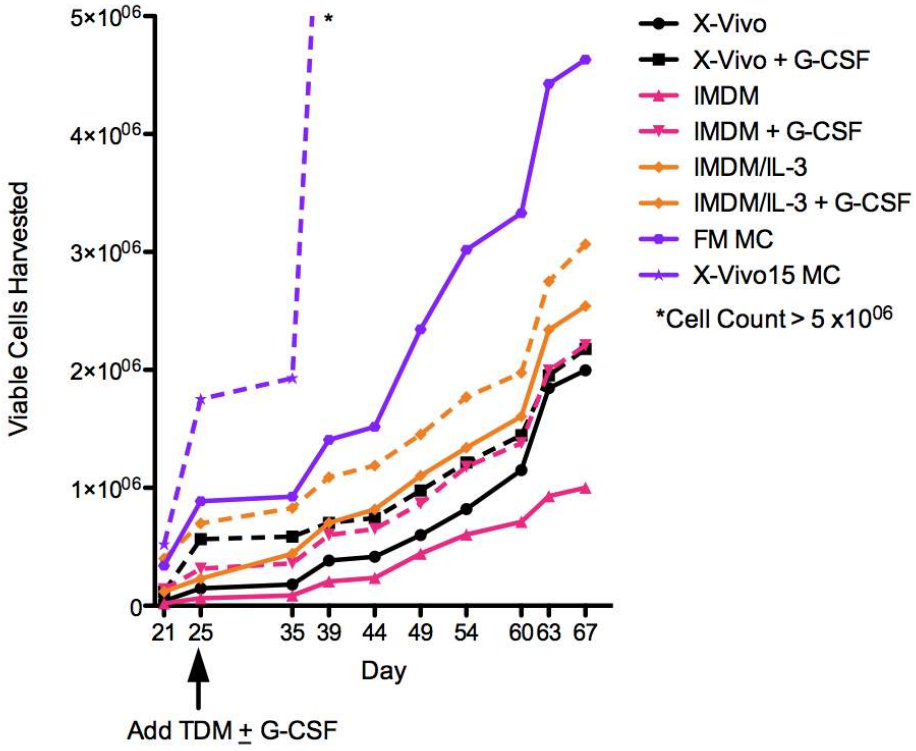


Figure 4.4.1. Cumulative cell harvest. 2 mL of cell culture was counted using NucleoView®, and cumulative cell counts plotted. The second condition in each coloured pair is represented by a dotted line, i.e. G-CSF or X-Vivo15 MC.

4.4.2 G-CSF enhances cell viability

Every 4-5 days when culture medium was refreshed viability was measured (Fig. 4.4.2). PMN-inducing conditions IMDM/IL-3 and IMDM had similar viability. Cells in X-Vivo medium were least viable. The addition of G-CSF increased viability of cells in all types of media. Mean viability over the 42 days was: X-Vivo = 52.57%, X-Vivo + G-CSF = 63.88%, IMDM = 66.94,

IMDM + G-CSF = 85.80%, IMDM/IL-3 = 71.92%, and IMDM/IL-3 + G-CSF = 87.44%. The X-Vivo15 MC and FM MC controls had the greatest viability overall averaging 91.26% and 89.90%, respectively.

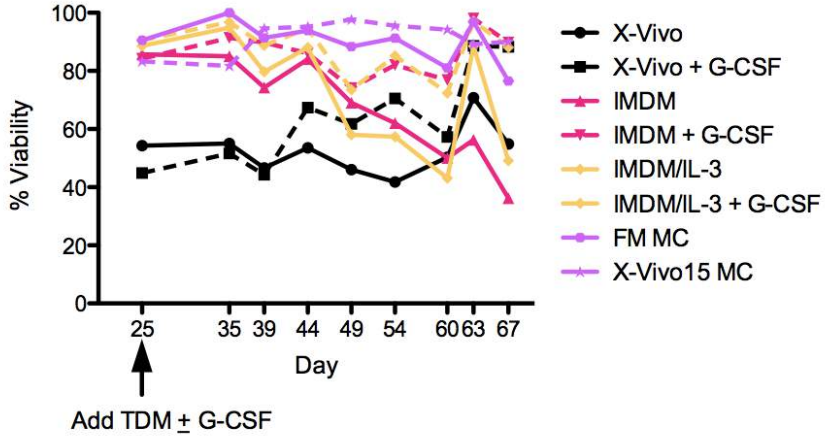


Figure 4.4.2. Cell viability. Cell culture viability was counted using NucleoView®. The second condition in each coloured pair is represented by a dotted line, i.e. G-CSF or X-Vivo15 MC.

4.4.3 Cell diameter

Every 4-5 days when culture medium was refreshed cell size was measured using NucleoView® cell counter (Fig. 4.4.3). The standard size of PMNs isolated from whole blood is 10-15 µm; PMNs isolated from whole blood had a diameter of 10.5 µm. Cells in PMN-inducing conditions had a similar diameter that averaged 12.8 µm. The average diameter of X-Vivo15 MC and FM MC controls were 17.2 µm and 15.5 µm, respectively, which is within the 12-20 µm range of blood-derived MCs.

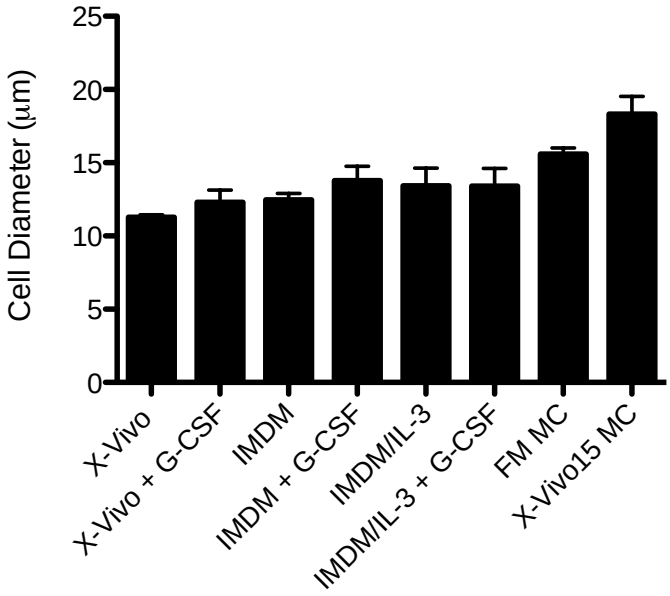


Figure 4.4.3 Cell diameter. Cell size was measured using NucleoView®. Nine measurements from Day 25 to Day 67 were averaged. Mean + SEM is plotted.

4.4.4 IMDM generates a greater proportion of CD66b$^+$ cells than IMDM/IL-3 or X-Vivo

In order to identify PMNs, day 25 cells in PDM and day 35 cells in TDM were stained for granulocyte maker CD66b (Fig. 4.4.4). Staining was carried out as described in Section 4.3.5. Among the PMN-inducing conditions at day 25, IMDM had the most positive staining for CD66b (42.3%), followed by IMDM/IL-3 (37.9%), then X-Vivo (24.5%) (Fig. 4.4.4.A). Similar to day 25 CD66b staining on day 35, IMDM +/- G-CSF had the greatest CD66b staining (71.8% and 50.4%), followed by IMDM/IL-3 +/- G-CSF (31% and 30.8%), and X-Vivo +/- G-CSF 9.3% and 17.8%) (Fig. 4.4.4.B). On day 25 (not shown) and day 35 FM and X-Vivo15 MCs stained intensely for CD66b but this paralleled the isotype control. This staining was probably non-specific but is unexpected given that human IgG was used to block non-specific FcR binding (Section 4.3.5). A High level of binding of the IgM isotype control was

also seen in MCs, despite routine blocking with FCS and human IgG. Perhaps blocking with IgM would have reduced non-specific staining. CD66b staining for freshly isolated human PMNs is also shown (Fig 4.4.4.B).

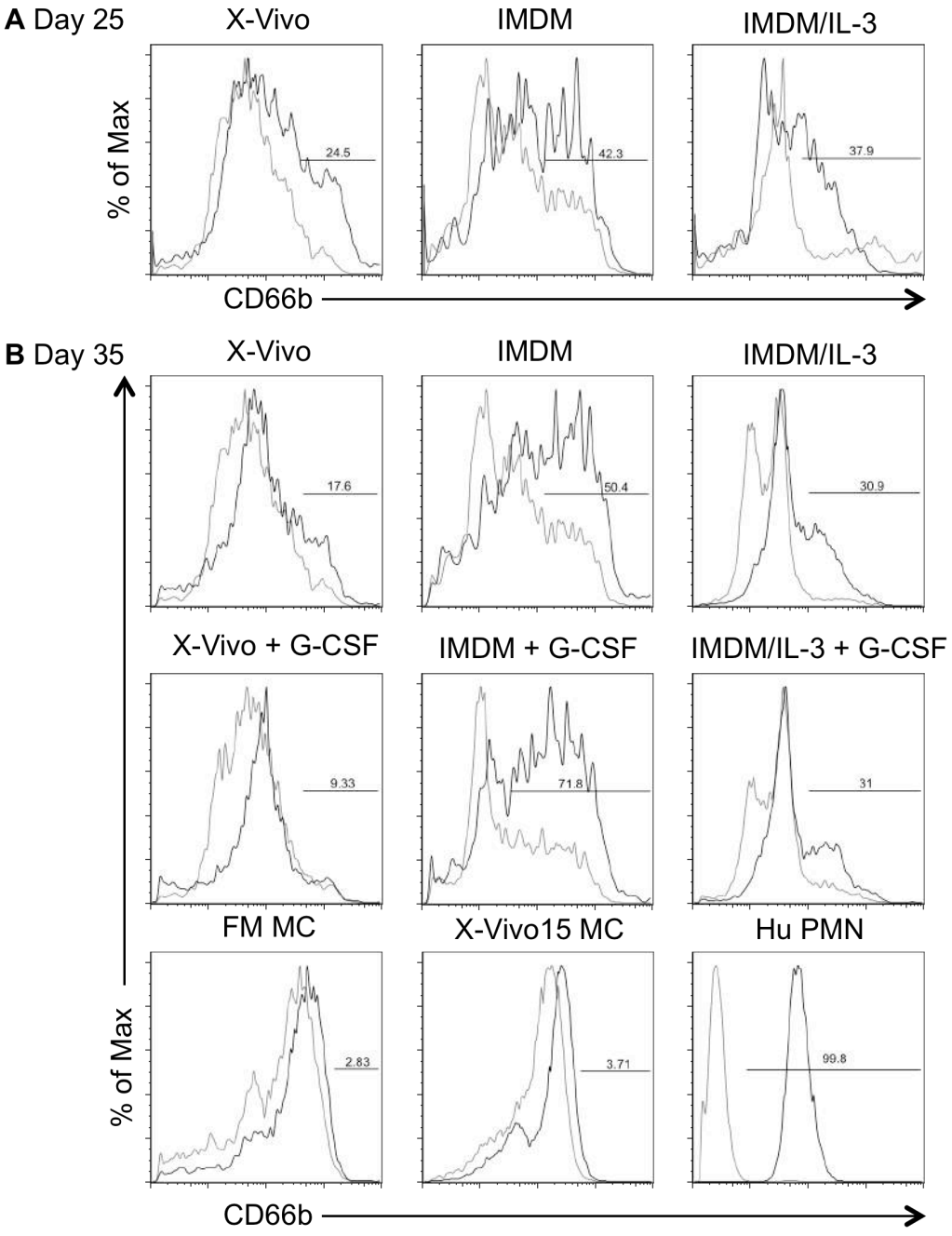


Figure 4.4.4. CD66b staining. (A) Day 25 cells in PDM and (B) day 35 cells in PDM were stained for CD66b (black). (B) Day 35 cells in TDM were stained for CD66b (black). Each cell type was stained with isotype control to which it is compared (gray). Note: In (A) isotype controls from Day 35 were used for X-Vivo and IMDM samples.

4.4.5 IMDM and IMDM/IL-3 generate a greater proportion of PMNs

In order to identify cells by morphology, day 25 cells were stained using Hema Gurr Rapid Blood Stain Kit (see Section 4.3.6-8). It stains in a similar manner to Wright Giemsa stains where neutrophilic granules stain light blue and nuclei red-violet. PMNs were identified as cells with banded/multilobed nuclei. IMDM/IL-3 and IMDM had 12% and 11% PMNs, respectively compared to X-Vivo with only 3%. FM MC and X-Vivo15 MC had 2% and 1% PMNs respectively.

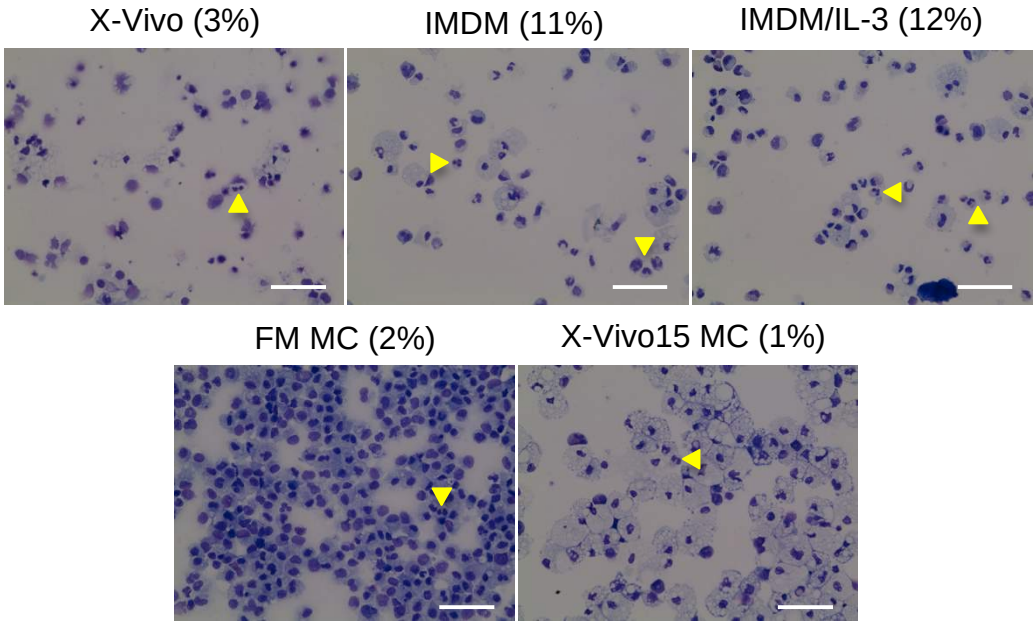


Figure 4.4.5 Rapid blood stain. Day 25 cells were stained using Hema Gurr Rapid Blood Stain Kit, mounted on slides by cytospin, and imaged at 20x. PMN-type cells are indicated by yellow arrowheads. Scale bar = 20 µm. Images are representative of 3 taken for duplicate samples.

4.4.6 IMDM produces the greatest proportion of LAP positive cells

In order to identify functional PMNs beyond simple morphology after day 25, cells were stained for Leukocyte Alkaline Phosphatase (LAP) (see Section 4.3.9). Granules in the cytoplasm stain pink while the nucleus stains blue. LAP is a phosphatase that functions best in basic environments (Rambaldi et al., 1990). It is present only in band and segmented PMNs. It is higher in 3rd

trimester/post-partum women thus PMNs from a woman in her 7th month of pregnancy were used as a positive staining control. Cells were stained on Day 28 and 39. Cells in IMDM +/- G-CSF condition had the most LAP positive cells; at day 28 = 23%/33%, and at day 39 = 25%/16%, respectively. Cells in IMDM/IL-3 +/- G-CSF were 5%/11% LAP positive at day 28, and 5%/11% positive at day 39. Cells in X-Vivo +/- G-CSF had the least amount of LAP activity; at day 28 = 33%(indeterminate)/0% and at Day 39 = 4%/14%. The presence of G-CSF in the media reduced the number of LAP positive cells. Neither FM MC nor X-Vivo 15 MC at Day 28 or Day 39 stained for LAP. Of note was the morphology of non-LAP staining cells. LAP positive cell were compact with multi-segmented nuclei, however all other cells in all conditions were large - nearly twice the size of the others, and highly vacuolar, which is characteristic of hESC-derived MC (personal communication, Sally Cowley).

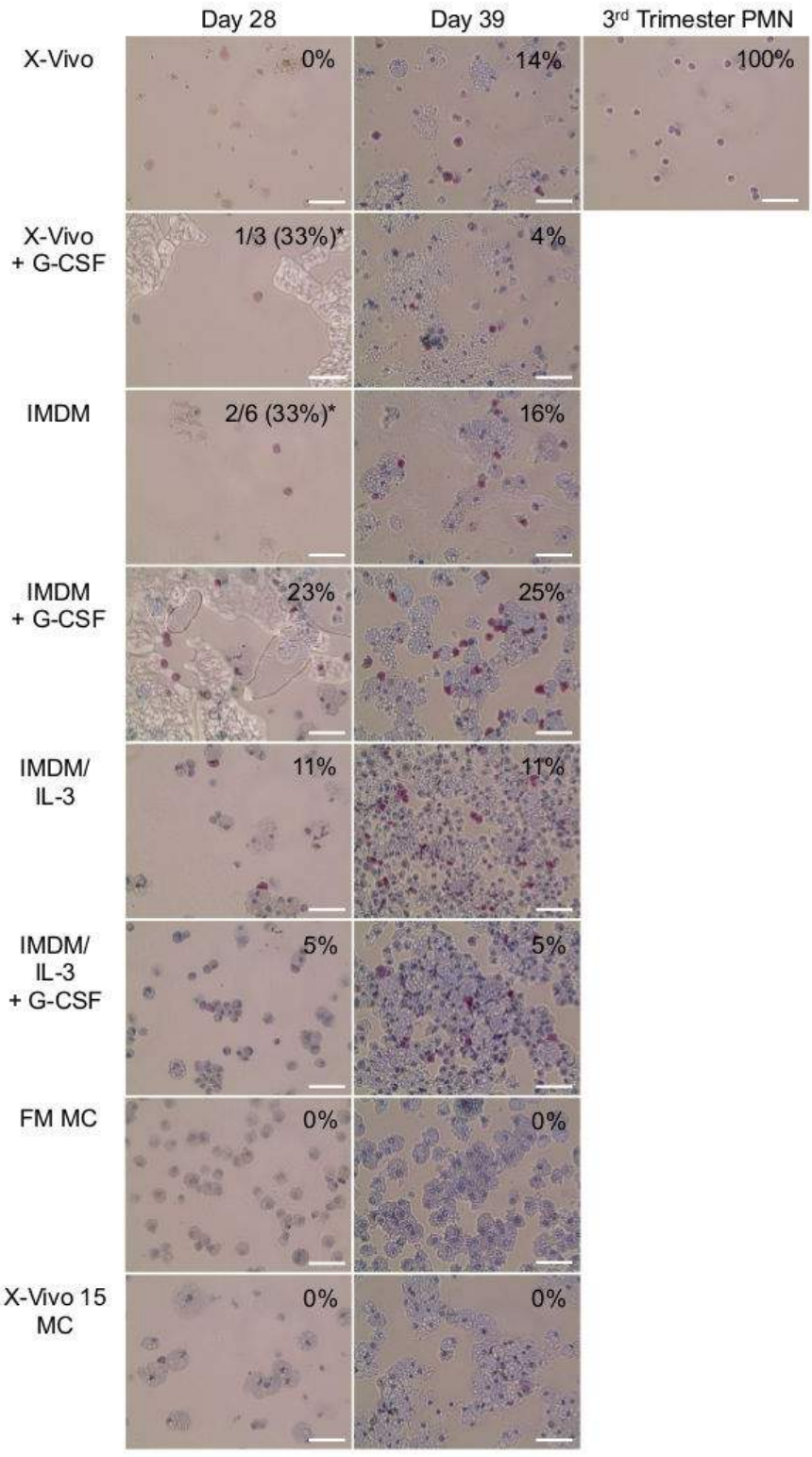


Figure 4.4.6. Leukocyte Alkaline Phosphatase (LAP) staining. Day 28 and day 39 cells stained for LAP. PMNs from a woman in her 7th month of pregnancy were used as a positive control for LAP staining. Cells were mounted to slides using cytospin, stained with Sigma LAP detection kit, and imaged at 20x. The percentage of LAP positive cells is noted in the upper right-hand corner. Scale bar = 20 µm. *Cells were dislodged from the slide so numbers may not represent true values.

4.4.7 Chemotaxis assay

I sought to further characterize the functional capacity of hESC-derived PMNs by chemotaxis. N-formyl-Met-Leu-Phe (fMLP) is a PMN chemoattractant produced by bacteria (MeSH D02.886.030.676.450.440) (Showell et al., 1976). IL-8 is another PMN chemoattractant with 2 primary active isoforms: the 72-aa isoform is produced by macrophages and the 77-aa isoform is produced by endothelial cells. fMLP and either the 72-aa or 77-aa isoform were used to observe the motility of Day 44 IMDM/IL-3 cells or granulocytes. The chemotaxis assay was performed as described by Saeki et al. (Saeki et al., 2009)). A 3 µm FluoroBlok™ Insert (BD Biosciences) was placed in a 24-well culture dish. In the bottom chamber 500 µL 2.5% FBS/HBSS with 100nM fMLP and 10 ng/mL of IL-8. The top chamber was filled with 2 x 10^5 cells in 500 µL 2.5% FBS/HBSS. Cells were left at 37^{o}C for 4 h after which cells in both chambers were counted. Virtually no cells were recovered from the lower (<1500) and the top (<4000) chambers. To verify that cells had not simply adhered to the insert cells were scraped off of the membrane and <1000 cells were present. The few cells recovered from the bottom chamber were harvested, mounted on slides, and LAP staining attempted but the cells were lost due to the use of non-adherent slides. The recovery of few cells in the bottom of the chemotaxis chamber may have been due to not having enough cells to start with, the pores size being too small, or maybe the need for Ca^{2+} or Mg^{2+} in the HBSS. No further attempts were made with this assay.

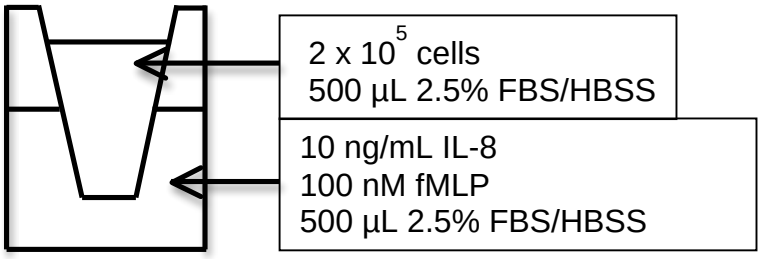


Figure 4.4.7 Chemotaxis assay set-up. FluoroBlok™ inserts were placed in 24 well plates and reagents added as diagrammed.

4.4.8 Phagocytosis assay

Three attempts to develop a phagocytosis assay using PMNs were made. The first two attempts using hESC-derived PMNs was unsuccessful but a third with primary PMNs was successful. The following protocol was successful and could be used for the observation of phagocytic ability of hESC-derived PMNs.

1. Isolate PMNs (either from whole blood, or possibly by chemotaxis. If desired, stimulate PMNs with 10 ng/mL of GM-CSF (determined to be optimal stimulant from titrations of GM-CSF, G-CSF, and PMA, Fig. 4.4.8.B).
2. Resuspend cells at $1x10^6$ cells/mL and place 1 mL in a 24-well plate for 1 h at 37^{o}C. Wash cells with PBS in a 15 mL conical and resuspend at 2 x 10^6 in HBSS/Ca/Mg.

3. To a 1.5 mL microcentrifuge tube add 1 x 10^5 zymosan particles then add 2 x 10^5 cells in 100 µL HBSS/Ca/Mg. Rotate gently at 37^{o}C for 10 min. Add 100 µL of stop solution (10% FBS and 2 mM NaF in HBSS without Ca^{2+} and Mg^{2+}) and centrifuge at 150 x g for 10 min. Resuspend cells in 200 µL PBS,
4. Cytospin, and stain with Rapid Blood Stain Kit if using light microscopy or not at all if using fluorescent microscopy.

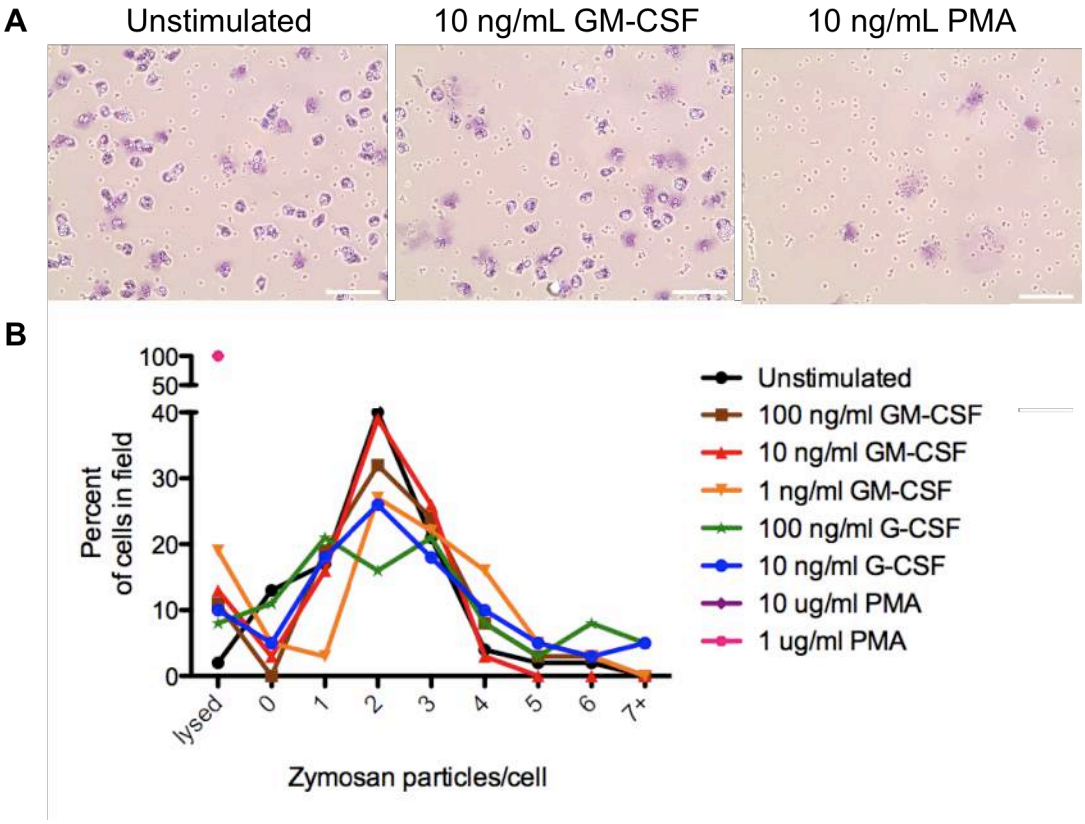


Figure 4.4.8 Phagocytosis assay. Experiments were carried out as described in Section 4.4.8. PMNs must be activated to perform phagocytosis therefore various concentrations of GM-CSF, G-CSF, and PMA were used to stimulate PMNs prior to mixing with zymosan particles (van Zandbergen et al., 2004). It should be noted that zymosan alone should also produce enough stimulus to induce phagocytosis. (A) Light microscopy images of zymosan uptake by PMNs in the presences of stimulants. 40x magnification. Scale bar = 20 µm. A greater proportion of G-CSF-stimulated cells as compared to GM-CSF-stimulated cells were undergoing lysis. PMA induced total lysis of cells. (B) Zymosan uptake peaked at 2 particles/cell. 10 ng/mL GM-CSF was selected as the optimal concentration to stimulate cells because it had the greatest percentage of cells with 2 zymosan particles and fewer phagocytising cells undergoing lysis although unstimulated cells are equally capable of phagocytosis and could be used.

4.5 Discussion

	X-Vivo	IMDM	IMDM/IL-3	Comments
Cell Harvest	+	++	+++	Enhanced by G-CSF
Viability	+	+++	+++	Enhanced by G-CSF
CD66b	+	+++	++	Enhanced by G-CSF
Rapid Blood Stain (morphology)	+	++	++	N/A
LAP	+	+++	++	Diminished by G-CSF

Table 4.5 Summary of assessments of PMNs derived from hESCs.

This present study describes an optimized protocol for deriving PMNs from hESCs without the use of feeder cells. hESCs were cultured in X-Vivo, IMDM, or IMDM/IL-3 mediums supplemented with specific growth factors and monitored for 67 days. Cell number, viability, CD66b staining, morphology, and LAP activity were used to assess the progress of differentiation and the results are summarized in Table 4.5. IMDM + G-CSF and IMDM/IL-3 without G-CSF produced the most PMNs but when considering specific markers like CD66b and LAP, IMDM + G-GSF appears to be the best of the three conditions for growing PMNs.

Should another attempt be made to grow PMNs from hESCs a better chemotaxis assay must be designed as a way to enrich for PMNs for downstream use. It may also be possible to enrich for PMNs using a ficoll gradient then dextran sedimentation. In addition, measuring reactive oxygen

species (for example with a carboxy-DCFDA, Invitrogen) or the generation of NETs in response to stimulants like GM-CSF and PMA could also be done to assess normal cell function. The ultimate goal would be to use these cells in anti-viral and cytotoxic assays as described in the previous chapter. Based on this study recommendations on how to derive PMNs from hESCs were made to other scientists, resulting in a successful attempt to derive PMNs from induced pluripotent stem cells (iPSC)(Walter Hansler) using IMDM + G-CSF + growth factors, thus validating this method. Walther Hansler used iPSC-CGD1.1, a human IPS line that was developed by reprogramming the fibroblasts of a CGD patient with the *p47^{phox}* mutation (Jiang et al., 2012). While these two initial studies resulted in low PMN yields, this method represents a xeno-free system for generating PMNs from hESCs, which could be used in basic research, drug screening, or regenerative medicine.

Chapter 5

Antibody-Dependent Cellular Cytotoxicity Against Primary HIV-1-Infected $CD4^+$ T Cells Is Directly Associated with the Magnitude of Surface IgG Binding

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5.1 Abstract

Antibody-dependent cellular cytotoxicity (ADCC) is thought to potentially play a role in vaccine-induced protection from HIV-1. The characteristics of such antibodies remain incompletely understood. Furthermore, correlates between ADCC and HIV-1 immune status are not clearly defined. The sera of 20 HIV-1^+ patients was screened for ADCC. Normal human peripheral blood mononuclear cells were used to derive HIV-infected $CD4^+$ T cell targets and unstimulated autologous, freshly-isolated natural killer cells in a novel assay that measures granzyme B (GrB) and HIV-1-infected $CD4^+$ T cell elimination (infected-cell elimination [ICE]) by flow cytometry. Polyclonal serum mediated greater levels of ADCC than anti-HIV-1 envelope glycoprotein (Env)-specific monoclonal antibodies and serum-mediated ADCC correlated with the amount of IgG and IgG1 bound to HIV-1-infected $CD4^+$ T cells. No correlation between ADCC and viral load, $CD4^+$ T cell count, or neutralisation of HIV-1_{SF162} or other primary viral isolates was detected. Sera pooled from clade B HIV-1^+ individuals exhibited breadth in killing targets infected with HIV-1 from clades A/E, B, and C. Taken together, these data suggest that the total amount of IgG bound to an HIV-1-infected cell is an important determinant of ADCC and that polyvalent antigen-specific Abs are required for a robust ADCC response. In addition, Abs elicited by a vaccine formulated with immunogens from a single clade may generate a protective ADCC response *in vivo* against a variety of HIV-1 species. Increased understanding of the parameters that dictate ADCC against HIV-1-infected cells will inform efforts to stimulate ADCC activity and improve its potency in vaccinees.

5.2 Introduction

To better understand the characteristics of an effective ADCC-mediating antibody response, some of the qualities of HIV-1-infected patient sera that are associated with high levels of ADCC were compared, including neutralisation and IgG binding to HIV-1-infected $CD4^+$ T cells. Having compared ADCC mediated by NK cells (NKs), monocytes, and neutrophils, and finding that NKs were the most effective (see chapter 3), they were used as effectors in assays to compare serum and mAb-mediated ADCC. In addition, the ability to use NKs after cryopreservation without loss of functionality allowed for the collection and storage of large quantities of PBMCs by leukapheresis, therefore it was possible to use the same donor's cells for all experiments where comparative analysis was done.

In the case of ADCC mediated by NKs, the Fcγ receptor IIIa (FcγRIIIa) on the surface of NKs binds to the Fc of IgG1 or IgG3 (Nimmerjahn and Ravetch, 2008). Upon cross-linking of the Fcγ receptor, NKs release the pore-forming protein perforin, which permits entry of granzymes into the target cell cytoplasm, inducing apoptosis. NK cell-mediated killing of targets has been examined in some prior reports. However, the aim of many of these studies was not to understand the qualities of patient sera that mediate high levels of ADCC. Most prior studies were directed at understanding a specific function of NKs (Brunetta et al., 2009; Chung et al., 2009; Johansson et al., 2011; Liu et al., 2009; Sowrirajan and Barker, 2011; Stratov et al., 2008) or antibody (Ferrari et al., 2011; Klein et al., 2009; Moldt et al., 2011; Trkola et al., 1996; Tudor and Bomsel, 2011). To this end, they have examined NK cell-mediated

ADCC in the context of mAbs or heterologous cell lines or have measured indirect markers of ADCC such as cytokine expression by NKs (Chung et al., 2008; Forthal and Landucci, 1998; Gomez-Roman et al., 2006). In addition, many prior studies have used protein-pulsed, rather than infected target cells (Chung et al., 2009; Johansson et al., 2011). These targets may not closely approximate the situation *in vivo,* in that they have relatively high levels of surface HIV-1 Env, which does not account for variability in conformation or glycosylation, and HIV-1 Env is not presented on the cell surface in a native, receptor-unligated form. Thus, while thought to have a protective role in humans, the characteristics of HIV-1-specific Abs in patient sera that mediate ADCC remain incompletely understood.

Chapter Hypothesis

$CD8^+$ T cell-mediated cytotoxicity has clearly been demonstrated to account for delayed HIV-1 disease progression, for instance in long-term nonprogressors (LTNPs), but discrepancies exist in the literature as to whether NK-mediated ADCC has the same effect. Since neutralisation does not correlate with slower progression it is unlikely another Ab-mediated affect, ADCC, does either. I found that high levels of ADCC are not associated with plasma viral load, peripheral blood $CD4^+$ T cell count, or specificity was observed. Rather, high levels of ADCC were most closely associated with high levels of IgG, especially IgG1 binding to primary HIV-1-infected cells.

My specific aims were to:

1. Determine whether patient serum-mediated ADCC had any correlation with clinical markers of progressive HIV infection (status, $CD4^+$ T cell count, plasma RNA levels) and neutralisation.
2. To probe the specificities of Abs that evoke potent ADCC.

5.3 Materials and Methods

5.3.1 Study Participants

HIV-1-infected subjects were recruited from the Clinical Research Center, National Institutes of Health (Bethesda, MD), and signed National Institute of Allergy and Infectious Diseases (NIAID) Investigational Review Board-approved informed-consent documents. Sera from 9 long-term nonprogressors (LTNPs) and 11 progressors were examined in this study. LTNPs were defined as having set point plasma viral RNA levels of <50 copies/mL, stable $CD4^+$ T cell counts, and no opportunistic infection in the absence of highly active antiretroviral therapy (HAART). Progressors had been off HAART for >5 years at the time of sampling, had been diagnosed with HIV >7 years prior to sampling, and had declining $CD4^+$ T cell counts or $CD4^+$ T cell counts of <400 cells/μL. HIV-seronegative donors were recruited from the National Institutes of Health donor aphaeresis clinic. A single seronegative donor was used for data contained in each figure of this work. These donors were typed for FcγRIIIa polymorphisms (Ahlgrimm et al., 2011). Results in Fig. 7.3.2, 4, 6, 8, and 9 were from studies performed with cells from a single donor homozygous for a valine at position 158. This polymorphism is associated with greater binding of IgG1 and IgG3 on NKs

and greater ADCC compared to cells from individuals homozygous for phenylalanine at this position (Koene et al., 1997; Wu et al., 1997). Results presented in Fig. 7.3.5 were from studies performed with cells from a single donor that was heterozygous at position 158, considered to give intermediate binding to FcγRIIIa.

5.3.2 Storage of Samples

PBMCs were collected as described in Section 2.2.1. Bpool and Normal sera were assembled as described in Section 2.3.1. Samples were stored as described in Section 2.2.3.

5.3.3 Antibodies

A novel human-murine chimeric anti-CD3 antibody was used as a positive control for NK cell-mediated $CD4^+$ T cell lysis and was provided by Hing Wong (AltorBioscience, Miramar, FL, USA). This chimeric antibody was constructed by overlapping PCR amplification of cDNA templates encoding the heavy- and light-chain variable regions of OKT3 antibody from the OKT3 clone (ATCC, Manassas, VA, USA) and the human IgG1 constant region or kappa fragment, respectively, as previously described (Han et al., 2011). The coding sequences of the light- and heavy-chain fusions were cloned into a modified retrovirus expression vector, pMSGV-1 (Hughes et al., 2005), that carries the neomycin or puromycin resistance gene, respectively, to construct the expression vectors. The expression vectors were cotransfected into the CHO cells, and the clone producing the chimeric antibody was isolated by limiting dilution. Antibody was purified by protein A chromatography.

MAbs 2F5 and 2G12 were purchased from Polymun Scientific (Vienna, Austria). B12, b12-LALA, and b12 non-fucosylated variant Abs were provided

by Dennis Burton (The Scripps Research Institute, La Jolla, CA, USA) and produced as previously described (Hezareh et al., 2001).

VRC01, 17b, 447-52D, 2.2C, and 211C were provided by John Mascola (Vaccine Research Center (VRC), NIH, Bethesda, MD) (Wu et al., 2011). The VRC01 DE variant was provided by Gary Nabel (VRC). The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120 mAbs A32 and 48D from James E. Robinson (Moore et al., 1993; Thali et al., 1993; Wyatt et al., 1995).

Expression plasmids encoding the 4-116, 4-554, 4-133, 4-221, and 6-129 Abs isolated from patients 4 and 6 were cloned, expressed, and purified as previously described (Scheid et al., 2009). Briefly, Abs were produced by cotransfecting heavy- and light-chain IgG expression plasmids into 293T cells using Fugene 6 (Roche, Indianapolis, IN, USA). Supernatants were harvested after 3 days and purified on EconoPac columns (Bio-Rad, Hercules, CA, USA) using recombinant protein A Fast Flow Sepharose beads per the manufacturer's instructions (GE Healthcare, Piscataway, NJ, USA). After Ab purification, elution buffer was exchanged 3 times with PBS in Amicon Ultra 30-kDA centrifugal filtration conical (Millipore, Billerica, MA, USA). IgG was purified from Bpool and patient 4 sera using a Montage antibody purification kit with PROSEP-G medium per manufacturer's instructions (Millipore).

Ab yields were often 1 to 5 mg/mL, as determined by enzyme-linked immunosorbent assay.

5.3.4 Viruses

Several replication-competent infectious molecular clones (RC-IMCs) were used in this study. Two of them, pCH162.c and pCH067.c represent the full-length transmitted/founder (T/F) virus sequences inferred from two trial CHAVI 001 subjects infected with clade C HIV-1 (Christina Ochsenbauer and John Kappes, unpublished data) and were recently derived by applying methods essentially as described previously (Ochsenbauer et al., 2012). Other RC-IMCs that were utilized expressed *env* genes of interest derived from clade B, C, or A/E HIV-1 in *cis* in NL4-3-derived proviral backbones (Env IMCs) with or without a reporter gene, using an approach previously described (Edmonds et al., 2010; Ochsenbauer and Kappes, 2009): pNL-YU2.ecto, pNL-THRO.ecto, pNL-LucR.T2A-AEC1081 c03.ecto, pNLENGli-AE.CM235.ecto, and pNL-96ZM.ecto. SF162 IMC was provided by Cecilia Chang-Meyer (Rockefeller University, New York, New York, USA). For production of murine leukaemia virus (MLV) pseudovirus, pSG3$^{\Delta env}$ and SV-A-MLV-*env* were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAIID, NIH (Landau et al., 1991; Wei et al., 2002; Wei et al., 2003).

Plasmids were amplified using Endo-Free Maxi Kits (Qiagen), transfected into 293T LentiPhos with Fugene HD (or 293T with Fugene 6 for MLV *env* pseudovirus), concentrated and titred as described in Sections 2.4.2 and 3.

5.3.5 TZM-bl Neutralisation Assay

Neutralisation of pseudovirus by serum was assessed by measuring luciferase reporter gene expression under the control of an HIV-1 LTR in TZM-bl cells after one round of viral infection. Sera were diluted serially 5 fold

starting at 1:10 and ending at 1:781,250. Duplicate dilutions were prepared in complete DMEM (cDMEM; 10%FBS, pen/strep) in a 96-well RBP. 10 μL diluted Ab was added to a 96-well FBP and 40 μL of HIV-1_{S162} pseudovirus at 100TCID50 added, leaving two rows free of Ab and one free of virus. Virus and Ab were incubated at 37^{o}C for 30 min. Meanwhile, TZM-bl cells were trypsinized, counted, and resuspended at 5x10^5 cells/mL in cDMEM with 70 μg/mL DEAE-dextran (Sigma-Aldrich). 20 μL of cell suspension (10,000 TZM-bl) were added to each well and plates left overnight at 37^{o}C. The next day 130 μL cDMEM was added to each well and the plate returned to the incubator overnight. After a total of 48 hours, media was aspirated, 50 μL Reporter Lysis Buffer (Promega, Madison, WI, USA) was added, the cells lysed on a shaker for 30 min, and 50 μL transferred to an opaque white 96-well FBP. 50 μL Luciferase substrate was added to each well, and relative luciferase units (RLU) immediately measured using Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). To determine 50% inhibitory dose (ID_{50}) that reduced RLU by 50% non-linear regression was fit to serum dilution neutralisation curves. ID_{50} is the reciprocal of the serum dilution that neutralised 50% of the virus. Geometric mean was calculated from serum ID_{50} titers against 20 tier 2 primary HIV-1 isolates.

5.3.6 Fluorometric Granzyme Cytotoxicity Assay

The ADCC assay was performed as described in Section 2.6.2 with the following modifications. $CD4^+$ T cells were selected from cryopreserved PBMCs. Autologous NKs were selected from cryopreserved PBMCs that had been rested overnight at 2 x 10^6 cells/mL in complete RPMI.

5.3.7 IgG Binding Isotype

HIV-1-infected $CD4^+$ T cells or NKs were washed twice with PBS, incubated with serum or mAb diluted in cRPMI at 37^oC for 30 min, washed twice with 1%BSA/PBS and stained for cell-bound IgG using either 1:5 anti-IgG peridinin chlorophyll protein (PerCP; BD Biosciences) or 1:1000 anti-IgG1 biotin (BD Biosciences). 1:100 streptavidin-allophycocyanin (APC; Jackson ImmunoResearch, Westgrove, PA, USA) was used as a tertiary stain for IgG1 biotin. For infected $CD4^+$ T cells, after staining for IgG or IgG1 samples were washed in 1% BSA/PBS, fixed using Cytofix/Cytoperm™, and stained for p24 using KC57-FITC (Beckman Coulter). Samples were analyzed using flow cytometry. The median fluorescent intensity (MFI) in infected targets cells was quantified.

5.3.8 Statistics

Comparisons of independent groups were made by the Wilcoxon two-sample test. Correlations were determined by the Spearman rank method. The Bonferroni method was used to adjust *P* values for multiple testing. Statistics were performed by Claire Hallahan (Biostatistics Research Branch, NIAID, NIH, Bethesda, Maryland, USA).

5.4 Results

5.4.1 Fluorometric granzyme cytotoxicity assay measures ADCC activity of sera against primary HIV-1-infected $CD4^+$ T cells

To provide a quantitative analysis of ADCC against primary HIV-1_{SF162} -infected cells, a novel assay for measuring NK cell-mediated cytotoxicity as previously described was used. SF162 is a HIV-1 clade B, R5-tropic virus originally isolated from CSF. It is weakly resistant to neutralisation (Cheng-Mayer et al., 1989). Primary $CD4^+$ T cell targets were obtained from HIV-1-uninfected controls and infected *in vitro* with HIV-1_{SF162}. These cells were then mixed with autologous negatively isolated NKs in the presence or absence of serum from HIV-infected patients (Fig. 5.4.1). NKs from uninfected controls were used to avoid variations in NK function associated with viral load or clinical status. ADCC activity was measured in two ways: by delivery of granzyme B (GrB) to target cells and by ICE (Migueles et al., 2008). OKT3 with a human IgG1 Fc was constructed for use as a positive control. Titration of this antibody in this assay showed maximal delivery of GrB activity at 64.2% and 50% activity at 0.023 µg/ml (Fig. 5.4.1D). NK effectors mixed with uninfected $CD4^+$ T cells and pooled sera from patients infected with clade B HIV-1 (B-pool serum) served as our control for HIV-1 specificity and the standard by which we gated for p24-positive cells (Fig. 5.4.1C). Infected targets mixed with effectors and normal (HIV-negative) serum served as an additional control for non-HIV-1-specific ADCC. In this example, the uninfected target-serum-effector control had 2.64% GrB delivery. Infected targets had 8.76% GrB and 79.5% infection, as determined

by staining for p24. Infected targets, normal serum, and effectors had 12.1% GrB. Adding HIV-1^+ serum to infected targets-effectors resulted in a 3-fold increase in GrB, 42.9%, and 27.7% ICE. To further characterize ADCC activity against primary HIV-1-infected cells, GrB and ICE were measured in patient sera over a range of dilutions. Sera from 10 HIV-1^+ patients, Bpool serum, or normal sera were diluted 5-fold from 1:100 to 1:62,500 (Fig. 5.4.2). Consistent with some prior work, there was evidence of reduced ADCC activity at the lowest serum dilutions (highest serum concentrations) (Ferrari et al., 2011; Florese et al., 2009). This “prozone-like effect” occurs when too much Ab saturates the antigen so that few high-affinity Abs bind the antigen or IgG aggregates compete with antigen bound IgG for FcRs (Kipps et al., 1985). Peak ICE was observed at a 1:100 dilution for 70% (7/10) of the patient samples. While peak GrB often did not parallel with peak ICE for a given serum sample, GrB and ICE levels followed the same trend. Diluting serum 62,500-fold almost completely ablated ADCC by most sera. ICE and GrB levels were significantly correlated at the 1:500 dilution ($r = 0.75$, $P = 0.04$) (Fig. 5.4.3).

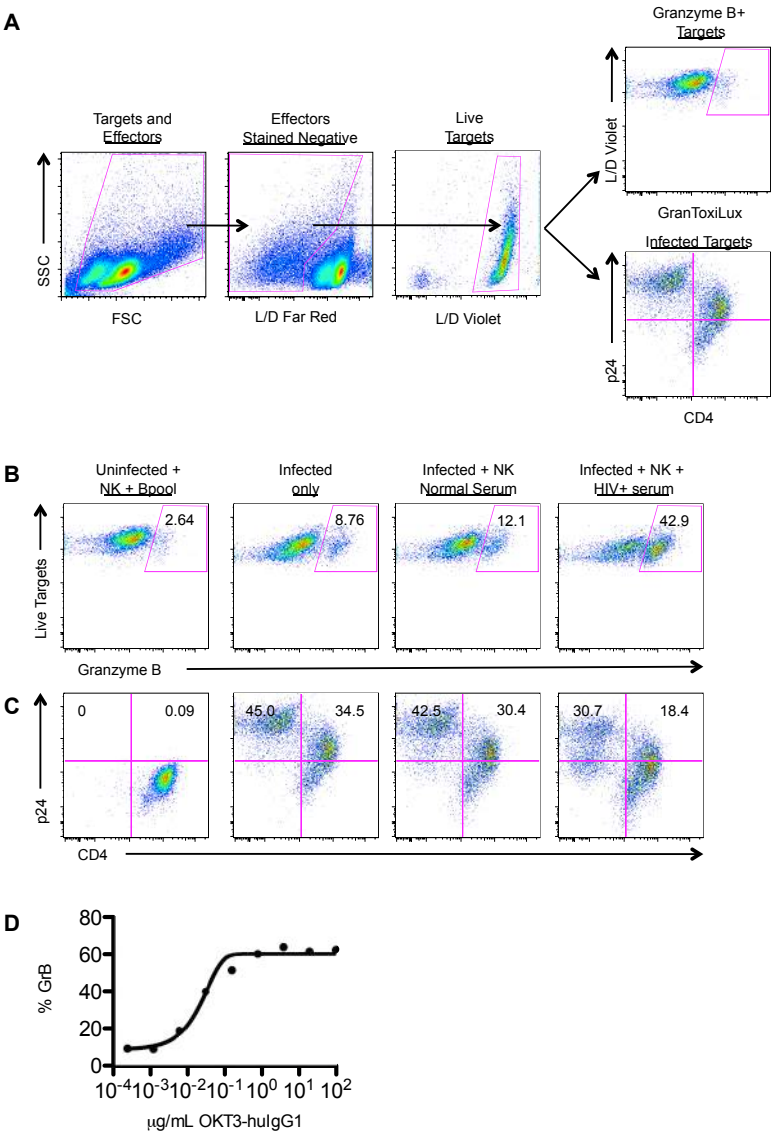


Figure 5.4.1. Fluorometric granzyme cytotoxicity assay effectively measures serum-mediated cytotoxicity against CD4$^+$ T cells. (A) Gating strategy for granzyme B-positive and infected targets. Live CD4$^+$ targets (LIVE/DEAD [L/D] violet stain positive) were selected from the negatively stained NK cell population (LIVE/DEAD far red stain negative). GrB cleaved GranToxiLux substrate, indicating active GrB present within live targets. Live, CD4$^+$ targets were also fixed and stained for p24 to determine the frequency of infected targets. SSC, side scatter; FSC, forward scatter. (B) Cellular GrB content. (C) Elimination of HIV-infected cells. Representative data show active GrB in live targets or p24 in CD4$^+$ T cells. Some infected cells show downregulation of CD4. A reduction in the number of p24-stained cells is represented as ICE and is an indication of cell death. In the example described above, $ICE_{infected\text{-}NK\ cells\text{-}serum}$ = [(42.5 - 30.7)/42.5] × 100 = 27.7%. (D) A novel human anti-CD3 Ab (OKT3-huIgG1) mediates delivery of GrB by NKs to CD4$^+$ T cell-infected targets.

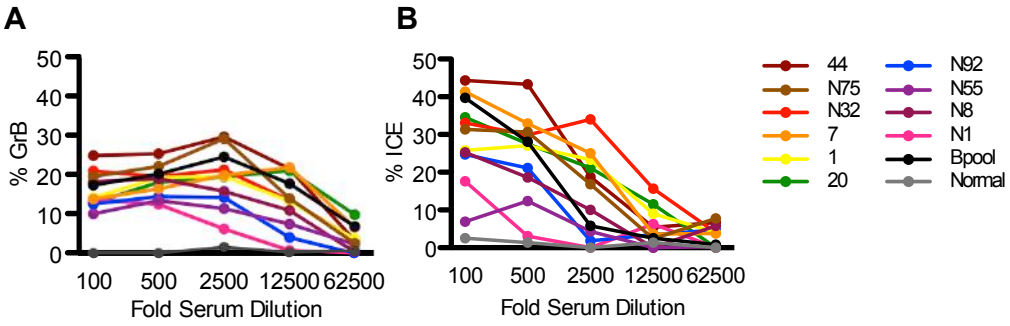


Figure 5.4.2. Antibodies mediate ADCC in a dose-dependent manner. GrB delivery (A) and ICE (B) by serum at serial 5-fold dilutions starting at 1:100 for sera from 10 HIV-1^{+} patients, Bpool serum, and normal serum.

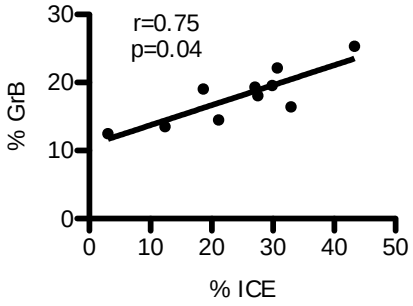


Figure 5.4.3. GrB correlates with ICE at a 500-fold patient serum dilution.

5.4.2 ADCC does not correlate with $CD4^+$ T cell count, viral load, or neutralising antibody (NAb) titers

Some, but not all, prior work has associated the ADCC activity of sera with clinical status, $CD4^+$ T cell count, or viral load. It has been suggested that sera from rare individuals who do not progress to AIDS without antiretroviral therapy (elite controllers [ECs] or LTNPs) exhibit greater ADCC activity, which correlates with the $CD4^+$ T cell count (Ahmad et al., 2001; Baum et al., 1996; Lambotte et al., 2009), while others have observed no correlation between ADCC and disease progression (Dalgleish et al., 1990; Koup et al., 1989). We sought to determine whether greater HIV-1-specific ADCC was associated with clinical status. We compared the sera of 9 LTNPs and 11 progressors (Table 5.4.1; Fig. 5.4.4A) in our assay using HIV_{SF162}-infected primary $CD4^+$ T cells. ICE levels ranged from 19.70% to 40.00% for LTNPs and 16.30% to 52.30% for progressors (Fig. 5.4.4B). The median GrB levels were 19.80% and 20.05% for LTNPs and progressors, respectively ($P > 0.5$). The median ICE levels for LTNPs and progressors were 29.80% and 36.90%, respectively ($P > 0.5$). Neither GrB nor ICE was correlated with the $CD4^+$ T cell count or viral load ($P > 0.5$ for each correlation) (Fig. 5.4.4C and D). If one examines the correlation between viral load and ICE among progressors only, there was also no correlation ($r = 0.33636$, $P = 0.31$). There was also no correlation between ADCC activity and titers of neutralising Abs against HIV_{SF162}, the virus used to infect ADCC targets ($P = 0.47$; Fig. 5.4.4E), or with the geometric mean of neutralising titers against 20 tier 2 primary isolates ($P > 0.5$; Fig. 5.4.4F) (8).

Patient no.	Diagnosis yr	$CD4^+$ T cell count (no. of cells/ml)	Plasma HIV RNA load (no. of copies/ml)	HLA class I allele:		
				A	B	C
LTNP						
7	1985	401	<50–77	1, 2	57	6
30	1990	229	<50–416	31, 74	51, 57	7, 16
47	1994	407	<50–56	1, 74	57, 81	7, 18
72	1998	1, 287	<50	30, 33	42, 58	2, 17
73	1991	801	<50	2, 3	7, 57	6, 7
74	1988	851	<50	11, 32	35, 50	4, 6
79	1998	501	<50	1, 30	42, 57	7, 17
84	1984	776	<50	3, 26	14, 57	7
93	1986	544	<50	3, 68	14, 35	4, 8
Progressors						
1	1985	545	19, 260	3, 66	7, 58	7
20	1985	1, 069	7, 166	1	52, 57	6, 12
21	1984	497	10, 714	2, 10	8, 27	1, 7
44	1986	754	1, 274	1, 24	27, 37	2, 6
59	1986	633	585	23, 30	7, 57	7, 15
63	1986	1, 096	1, 945	30	57, 81	18
69	2000	717	5, 275	2	44, 57	5, 6
70	1984	584	2, 885	1, 24	8, 27	2, 7
131	1989	578	85, 981	2, 11	35, 57	4, 6
265	1991	478	6, 343	23, 30	15, 41	2, 8
266	1992	235	25, 366	24, 33	35, 42	4, 17

Table 5.4.1 Patient characteristics.

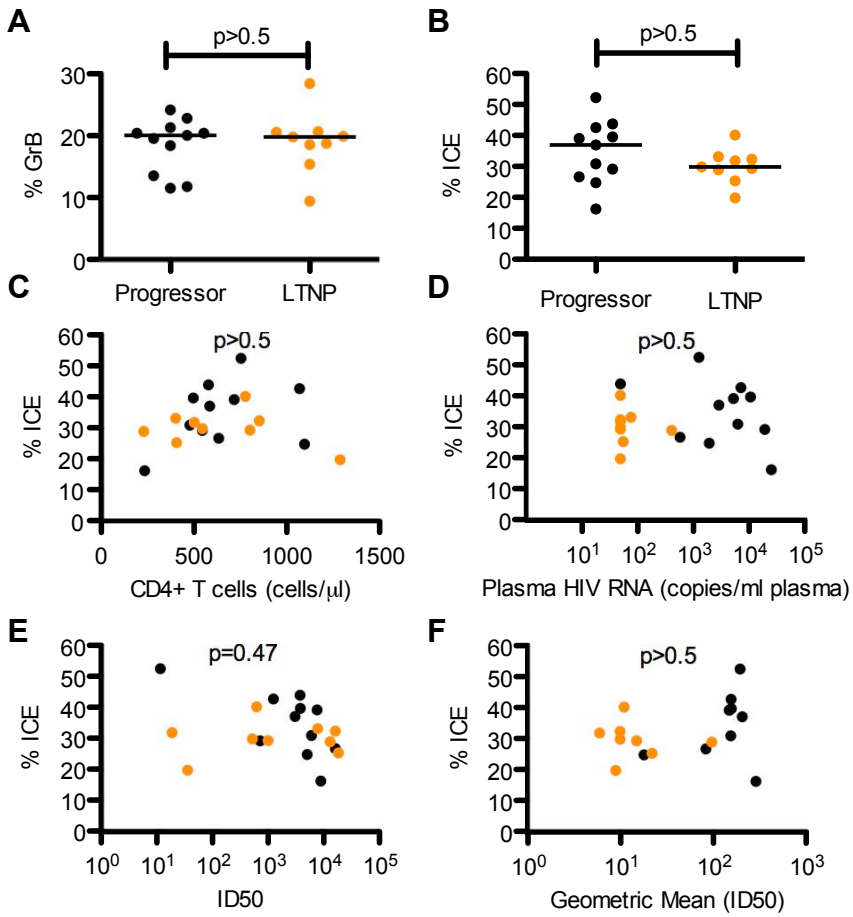


Figure 5.4.4. ADCC activity of sera does not correlate with clinical status, CD4$^+$ T cell count, viral load, or NAb titers. The ADCC activity of sera from 20 HIV-1$^+$ patients diluted 100-fold was compared by patient status for GrB activity (A) and ICE (B). ICE values for individual patients were also compared to CD4$^+$ T cell count (number of cells/μL) (C), viral load (number of copies/mL plasma) (D), NAb titers (50% inhibitory dose [ID50]) against HIV-1_{SF162} (E), and the geometric mean of NAb titers (50% inhibitory dose) against 20 tier 2 primary isolates (F). Orange, LTNPs; black, progressors.

5.4.3 HIV-1 antiserum exhibits breadth in mediating ADCC

We investigated the ability of cells infected with a range of isolates to be killed in this assay. B-pool serum was used to measure ADCC against CD4$^+$ T cells infected with isolates from clade B (SF162, THRO, YU2, 96ZM), C (CH162, CH067), and A/E (C1081, CM235) viruses (Fig. 5.4.5). Clade B

(Bpool) sera were able to kill 35.50% to 73.30% of infected cells from all three clades (ICE for clade B, SF162 = 35.50%, THRO = 60.25%, YU2 = 66.58%, and 96ZM = 41.18%; ICE for clade C, CH162 = 62.8%, and CH067 = 61.65%; ICE for clade A/E, C1081 = 73.3% and CM235 = 49.50%).

Although HIV-1 Env is widely considered the major target for ADCC-mediating Abs, there are prior reports of Abs specific for other HIV-1 gene products mediating ADCC (Rook et al., 1987). To probe whether recognition of HIV Env by serum Abs was essential for ADCC, we examined killing of targets infected with MLV Env-pseudotyped SG3$^{\Delta env}$. There was a 90.14% reduction in ICE against MLV compared to that obtained with SF162, confirming that most ADCC activity within these sera was Env specific (Fig. 5.4.5).

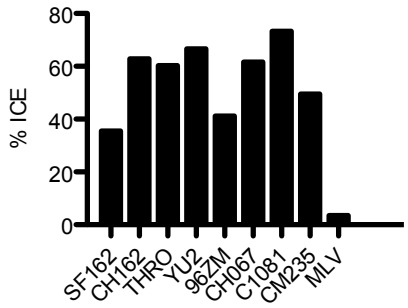


Figure 5.4.5 Serum exhibits breadth in mediating ADCC. Bpool serum mediates ADCC against clade B (SF162, THRO, YU2, 96ZM), clade C (CH162, CH067), and clade A/E (C1081, CM235) viruses. MLV is the pSG3$^{\Delta env}$ backbone with MLV *env.*

5.4.4 Effects of Ab specificity and affinity on ADCC against primary HIV-1-infected targets

In theory, the level of ADCC activity of an HIV-1-specific Ab may be affected by the specificity, affinity, orientation when bound to Env, and/or quantity on the cell surface. Although mAbs with a range of specificities have been used

in ADCC assays against Env-pulsed cell lines, they have not been widely used against primary HIV-1-infected $CD4^+$ T cell targets. Primary cell targets are likely to have less Env antigen on the cell surface and are less easily killed by NKs than protein-pulsed cell lines (Pollara et al., 2011). We therefore examined the level of ADCC mediated by a panel of well-characterized mAbs. Our panel included 13 mAbs: 2F5 (anti-gp41); 2G12 (anti-gp120 oligomannose); b12 (anti-CD4 binding site), b12 LALA (mutated to ablate FcγR binding), b12 non-fucosylated (b12NF, mutated to enhance FcγR binding), VRC01 (anti-CD4 binding site), and VRC01 DE (mutated to enhance FcγR binding); 447-52D (anti-V3); 17B, A32, and 48D (CD4 binding induced); and 2.2C and 211C (anti-gp120 constant region). In addition, we tested a combination of mAbs, including 2F5, 2G12, b12, 447-52D, 17B, and VRC01 (Fig. 5.4.6A). MAbs were serially diluted 10-fold starting at 100 µg/ml to assess their capacity to mediate ADCC. At all dilutions, ADCC mediated by individual or pooled MAbs was low compared to that mediated by sera. Neither individual MAbs nor a combination of six MAbs mediated ADCC to the degree of Bpool serum IgG. Recent reports have cited A32 as being superior to other MAbs in directing ADCC (Ferrari et al., 2011). In this assay, the ICE mediated by A32 was relatively similar to that mediated by the other mAbs. However, it remained well below the maximum ADCC mediated by purified Bpool serum IgG (43.70%) at 100 µg/ml and was 55.5% less than the maximum ICE of purified Bpool serum at 10 µg/ml (41.8%). The level of ADCC mediated by the b12 non-fucosylated and VRC01 DE mutant, known to enhance FcγR binding, was also well below that mediated by Bpool serum IgG. Enhanced binding to FcγR was confirmed by using the b12 NF Fcγ

mutant to bind negatively isolated NKs and detecting surface IgG with a secondary antibody. In these experiments Fcγ mutants engaged a greater fraction of NKs at all dilutions than the parent mAb or Bpool IgG (Fig. 5.4.7). Thus, although Fc mutants have greater binding to NKs, these mutations did not result in the levels of ADCC observed in individual or pooled antisera.

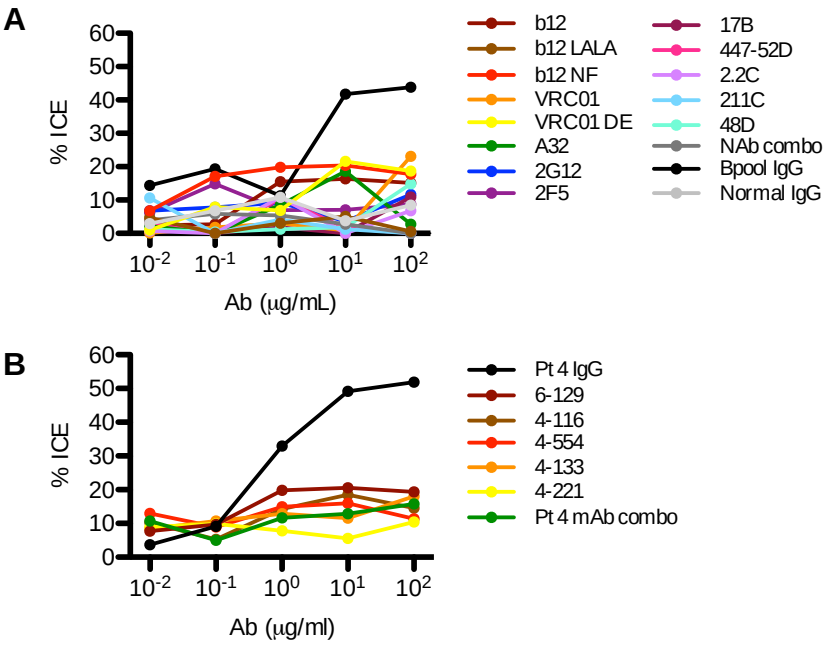


Figure 5.4.6. Serum-mediated ADCC is attributable to IgG; single anti-Env MAbs mediate only modest ADCC. (A) Standard neutralising Abs were titrated in parallel with each other and Bpool serum IgG. (B) Anti-gp120 and gp41 NAbs isolated from two patients (patients 4 and 6), a combination of the four patient 4 mAbs, and purified patient 4 IgG were titrated in parallel.

To further explore the specificities and affinity range that may mediate ADCC against primary infected targets, we selected HIV-1-specific mAbs that have been cloned from two patients whose sera exhibited ADCC activity (Scheid et al., 2009). MAbs 4-554 and 4-116 both recognized gp120, but 4-116 binding has been shown to be sensitive to deglycosylation. NAbs 4-133 and 4-221 both bind gp120 core [gp120 without V1/2/3 loops, (Kwong et al., 1998)], but

4-221 has 50-fold higher affinity (dissociation constants [K_ds], 2×10^{-8}M and 2×10^{-10}M, respectively). MAb 6-129 is an anti-gp41 non-neutralising Ab from patient 6. Purified patient 4 IgG and a combination of the four patient 4 MAbs were also tested. Consistent with the results from Fig. 5.3.6A, individual mAbs did not mediate high levels of ADCC. The killing mediated by purified IgG from patient 4 far exceeded the killing mediated by individual mAbs or patient 4 Abs combined (Fig. 5.4.6B). Thus, we did not observe a pattern of greater ADCC mediated by individual specificities or Abs of greater affinity. Taken together, these results suggested that individual MAbs or limited combinations of 6 mAbs were unable to achieve the level of ADCC observed using serum-derived IgG and this activity was not related to the level of FcR binding.

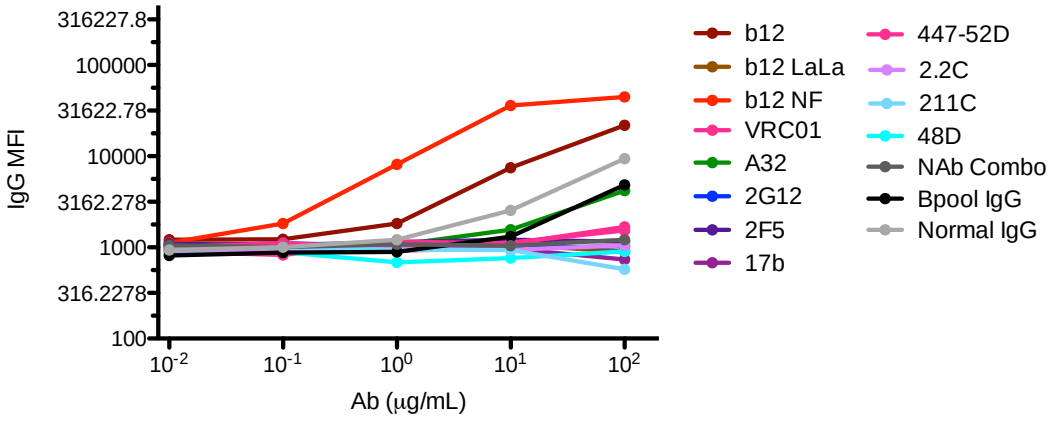


Figure 5.4.7. MFI of anti-IgG bound to MAb-coated NKs. Standard NAbs were titrated and combined with NKs to measure FcR binding. The level of IgG binding was determined by MFI. Each point represents bound IgG at a given concentration.

5.4.5 ADCC Correlates with IgG Binding

One possible explanation for the high levels of ADCC mediated by polyspecific sera is binding of larger amounts of total IgG to the surface of

HIV-1-infected cells. Large numbers of specificities may allow binding of multiple Abs per Env spike or binding of Env conformational variants. To explore the contribution of IgG binding to ADCC activity, we titrated patient sera or mAbs on infected $CD4^+$ T cells and stained for bound total IgG, IgG1, or IgG3 (Fig. 5.4.8A and B). IgG3 staining was very low at all dilutions and was excluded from further analyses (data not shown). To confirm that IgG binding was Env specific, a 1:100 dilution of patient serum was added to uninfected cells, resulting in an MFI of 9.79, whereas infected cells had an MFI of 2,846. We observed a positive correlation between bound IgG and ICE at a 1:2,500 serum dilution ($r = 0.73$, $P = 0.05$; Fig. 5.4.9A). There was also a significant correlation between bound IgG1 and ICE at a 1:100 serum dilution ($r = 0.96$, $P < 0.001$) and a 1:2,500 dilution ($r = 0.81$, $P = 0.01$) (Fig. 5.4.9B). In a parallel experiment, the amount of bound IgG was quantified using calibrated beads. We observed that at a serum dilution of 1:100, the median binding was a minimum of 1,123 molecules/cell. In a similar experiment where mAbs were titrated on infected cells and stained for IgG and IgG1, the mAbs bound less to infected cells (Fig. 5.4.8C and D). No correlation between mAb binding and ICE was found ($P > 0.5$); however, both values were extremely low in comparison to those for antisera. Taken together, reaction of serum with infected cells results in greater IgG binding than reaction of combinations of mAbs, possibly explaining the greater ADCC mediated by serum. Further, within patient sera, ADCC activity was tightly correlated with total IgG or IgG1 binding to infected cells.

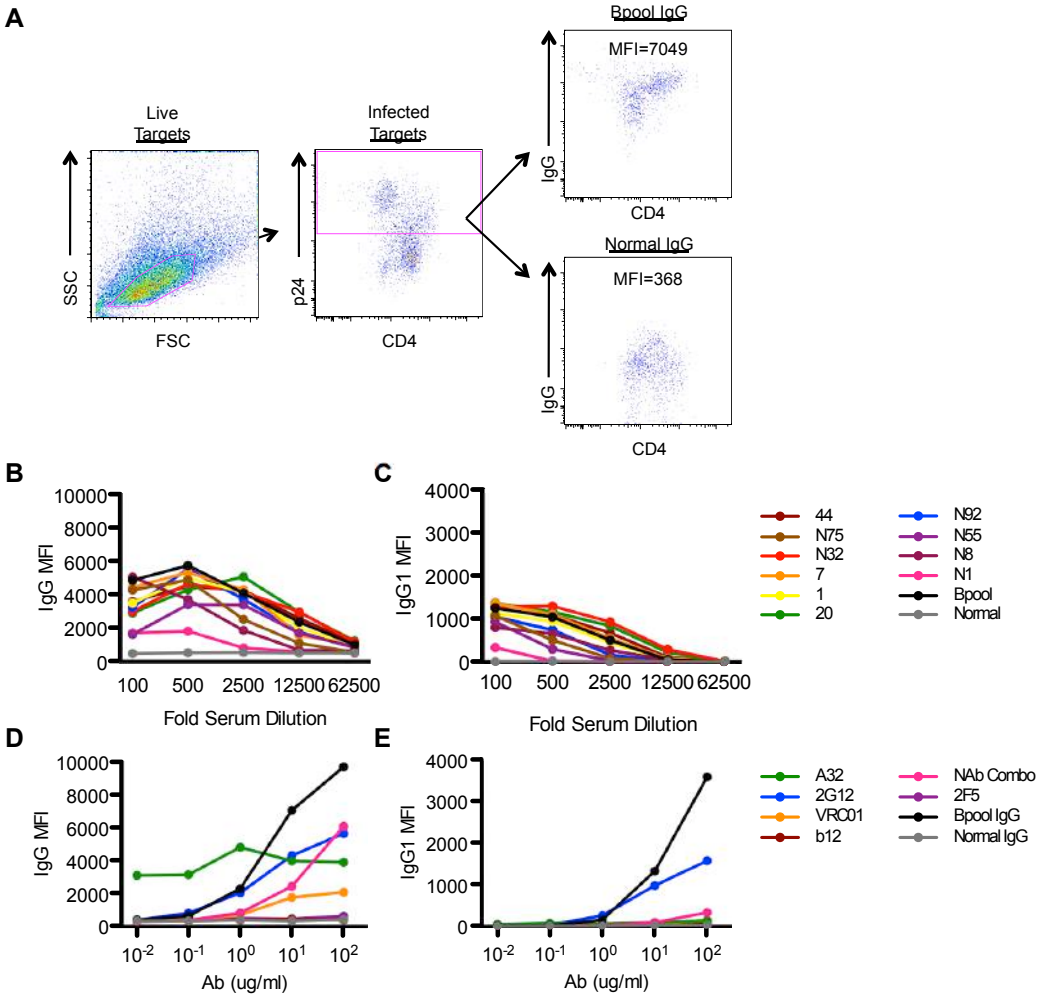


Figure 5.4.8. ADCC correlates with surface-bound IgG. (A) Gating strategy to determine surface IgG binding. MFI of anti-IgG (B) and anti-IgG1 (C) bound to serum-coated infected cells; MFI of anti-IgG (D) and anti-IgG1 (E) bound to mAbs whose titers on infected cells were determined (D). Each point represents CD4-bound IgG at a given dilution of serum or Ab.

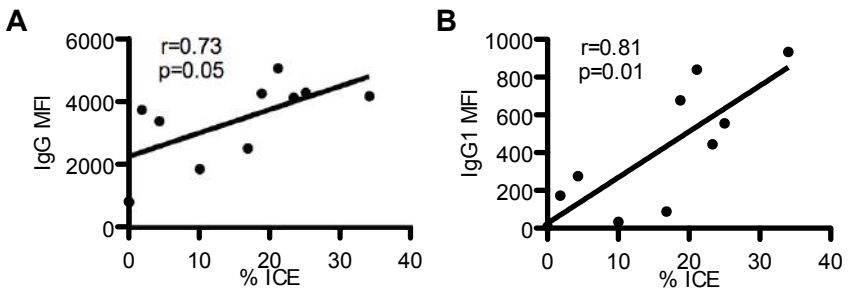


Figure 5.4.9. Correlation of ADCC with binding of serum IgG (A) or IgG1 (B) to infected targets. Cells were incubated with patient sera diluted 1:2,500. Each point represents a different subject.

5.5 Discussion

These results provide some insights regarding the qualities of sera that mediate effective ADCC against primary HIV-1-infected $CD4^+$ T cells. High levels of ADCC activity were not associated with plasma viral load or $CD4^+$ T cell count. In contrast to some prior reports (Ahmad et al., 2001; Baum et al., 1996; Johansson et al., 2011; Lambotte et al., 2009), I was unable to detect greater ADCC activity in sera from LTNPs/ECs under these experimental conditions. Further, I found no association with neutralisation of the virus used in the ADCC assay or with breadth of neutralisation. Using single mAbs I was also unable to detect a clear association with avidity or specificity. MAbs mediated very modest or low ADCC activity compared to sera from HIV-1-infected patients. The low ADCC activity of some sera and of most mAbs was associated with lower binding of HIV-1-specific Ab to the surface of infected cells. Thus, under our experimental conditions, total binding of Env-specific IgG, especially IgG1, tightly correlated with ADCC activity. These results suggest that the total amount of IgG bound to an HIV-1-infected cell is an important determinant of the level of ADCC activity.

The finding that ADCC activity is simply associated with total IgG binding may be surprising at first inspection. However, these results are potentially consistent with those of a recent a study by Sun et al. that showed a correlation between NK cell-mediated ADCC and titers of SIV_{mac251} gp140 binding antibody in the first 14 weeks of rhesus macaque SIV infection (Sun et al., 2011). Although it is unclear exactly how many Ab molecules must

bind to an infected cell to trigger degranulation of an NK, most estimates predicted that this number is relatively small. If this were the case, one might expect that binding by only a few IgG molecules of a mAb or from patient sera would mediate high levels of ADCC. If surface Env trimers were uniform, one might also expect that high levels of ADCC would be achieved by combining mAbs with different specificities. However, this was not observed even in combinations of 6 broadly neutralising MAbs with epitope specificities that are widely spaced on the Env trimer. Given the size of IgG molecules relative to the Env trimer, it seems unlikely that additional specificities would result in greater IgG binding. Although modification of the Fc to enhance FcγRIII binding increased ADCC activity, it did not attain the level of complex sera. This result suggests that an important limiting factor is not FcR binding but, rather, is at the level of antigen-specific binding on the target cell. I consistently observed the highest ADCC activity using sera from infected patients, and this was associated with greater HIV-1-specific IgG1 binding than mAb binding. Consistent with this, among patient sera, greater ADCC activity was closely associated with higher IgG1 binding to HIV-infected cells. One likely explanation for the higher ADCC activity with sera than mAbs is that Env is not homogeneous and sera containing multiple specificities are better able to recognize various surface conformations, including nonfunctional spikes. These Env forms would include Env with different levels of glycosylation, triggered Env spikes in which the conformation is altered after receptor binding, shed gp120 bound to CD4 on target cell surfaces, and residual gp41 stumps remaining after shedding of gp120. Env expression on the surface of infected cells is also suppressed to avoid

immune recognition and enhance infection by retrograde transport and regulated secretion at the virological synapse (Blot et al., 2003; Jolly et al., 2011).

One might expect that if Env-specific binding is required for ADCC, neutralisation might correlate with ADCC, as neutralisation is primarily determined by occupancy of the functional Env trimer by antibody (Parren et al., 1998; Sattentau and Moore, 1995; Yang et al., 2006). However, I did not observe a correlation between ADCC and neutralisation. A similar observation was made by (Guan et al., 2012). Guan et al. compared neutralisation and ADCC by mAbs isolated from patients against Env epitopes that become available upon CD4 binding (CD4i) and did not observe any correlation between ADCC and neutralisation. They detected potent ADCC responses against eptitopes mapping to the gp120 face occluded by gp41 in the trimer and a region near the co-receptor binding site involving the V1/V2 loops. One possible explanation is that major variations in ADCC are associated with various levels of binding of non-neutralising Abs (Florese et al., 2009).

Another possible reason for the inability to detect a correlation between ADCC and neutralisation may reflect the fact that some ADCC, measured *in vitro,* is potentially mediated by Abs recognizing non-Env epitopes (Rook et al., 1987), although my results using an *env*-deletion mutant show that ADCC responses are predominantly directed against Env. Wren et al. noted that long-term slow progressors did not exhibit a greater response to ADCC, but rather, had a broader range of ADCC responses (Wren et al., 2013).

Furthermore, only slow progressors had ADCC epitopes that mapped to Vpu peptides. While targets in this assay were whole blood that was pulsed with peptide antigens as opposed to infected cells, this could indicate that ADCC-mediating Abs recognize MHC class 1 restricted antigen.

In contrast to some prior work, I also did not observe an association between viral load and the ADCC activity of sera (Ahmad et al., 2001; Baum et al., 1996; Johansson et al., 2011; Lambotte et al., 2009). One prior study observed considerably greater ADCC activity of sera from LTNPs/ECs than progressors (Lambotte et al., 2009). That prior study used very different experimental conditions, including protein-coated cell line targets and a chromium release cell-killing assay. Likewise, in other studies, no correlation between ADCC and disease progression was observed (Dalgleish et al., 1990; Koup et al., 1989). At present, the precise reason for the disparate results remains unclear.

The results of the present study also have some implications for ADCC mediated in Ab infusion studies. Ab infusion studies examining the relative contributions of ADCC, ADCVI, or phagocytosis in protection of rhesus macaques from SIV infection have used mAbs alone or in combination (Hessell et al., 2009; Hezareh et al., 2001). However, under the presented *in vitro* conditions, the level of ADCC mediated by mAbs against primary HIV-1-infected cells was only a fraction of that mediated by most sera from chronically infected patients. Although this result is in need of confirmation *in vivo,* it suggests that compared to passive transfer of IgG from complex sera, use of mAbs may underestimate the contribution of ADCC to sterilizing immunity.

A further understanding of the parameters that dictate ADCC-mediated killing of infected cells may have implications for efforts to stimulate ADCC activity in vaccinees. Specifically, it will be important to further define the relative importance of individual specificities versus the ability to recognize structural variants on the cell surface. Isolation of additional Abs capable of recognizing a broad array of HIV-1 Envs may uncover specificities that are particularly good targets for ADCC. Alternatively, the high levels of ADCC observed using sera may not be mediated by Ab specific for conserved regions and may be mediated only by the ability to recognize variable structures. If this is the case, then immunization with multiple Env variants may be required to achieve these levels. It is encouraging that some sera from RV144 mediated ADCC in the range of those from chronically infected patients in assays using HIV-1-infected cell lines or primary $CD4^+$ T cells (A. Smalls-Mantey and G. Ferrari, unpublished observations). A further understanding of the basis of ADCC-mediated protection through Ab infusion studies in either macaques or vaccinated humans should yield important information for improving ADCC activity and possibly enhancing vaccine potency.

Chapter 6

Discussion

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This study investigated the production of PMNs from hESCs, the elimination of HIV-1-infected cells by innate immune effector cells, the modulation of this cytotoxicity by Ab, and characterized how Abs facilitate a potent ADCC response. Furthermore, I sought to define how Abs raised in the natural course of HIV-1 infection were able to mediate ADCC and whether ADCC capacity correlated with other markers of infection.

6.1 Measuring Cellular Cytotoxicity

In order to measure cytotoxicity a novel assay that specifically measures the reduction of live infected primary cells during the time of the assay was developed. It has significant advantages over many assays in the field that use cell lines, target cells pulsed with antigen, hazardous radioactive material, and markers of effector activation in that autologous, purified cell populations are used. As a corollary, fewer cells are required, and significant, discriminating levels of cell death are measured within an hour. One challenge in comparing effector cytotoxicity and ADCC among various research groups is the use of different serum dilutions and E:T ratios. Despite the other aforementioned factors these at least could be standardized. The study of neutralization in this field is much more robust given the nearly universal use of a TZM-bl reporter luciferase assay (Montefiori, 2009). In a blinded multi-lab comparison of nine ADCC assays the one reported here was among the best in precisely detecting differences in ADCC by patient sera from the RV144 vaccine trials (data not shown).

With the goal of reducing variability in studying PMN anti-viral mechanisms I derived PMNs from hESCs. I established a method by which to do so but due to low yields I was unable to perform any subsequent experiments to probe function. Another attempt to enhance yield and establish PMN chemotaxis and isolation protocols may generate cells that prove useful in the study of other diseases and cellular biology.

6.2 Effector Killing Mechanisms

I found that NKs and MCs alone mediated comparably significant cytotoxicity against HIV-1-infected cells which was significantly enhanced by HIV-1-specific Abs. PMNs alone also killed HIV-1-infected cells and killing was also significantly enhanced by HIV-1 specific Abs but to a lesser degree than NKs and MCs (Smalls-Mantey et. al., 2013, *in press*). Our data aligns with that in the field where most studies of ADCC using PBMCs, NKs, or MCs observe ADCC in the range of 30-60% or greater, (Ferrari et al., 2011; Horner et al., 2007) but considerably less when PMNs are used, sometimes a maximum of 10% even when high E:T ratios are used (Jenkins et al., 1993).

One of the outstanding questions with regards to cellular cytotoxicity is the killing mechanism mediated by MCs and PMNs. It is well established that NKs and $CD8^+$ T cells use perforin and granzyme B which activate target cell caspases but what MCs and PMNs use is completely unknown. Not only would this knowledge advance the field of HIV-1 immunobiology but it may have an even more substantial impact in the field of cancer research where mAbs are becoming a main line of treatment. While mAbs act in many different ways such as by blocking receptors for cellular growth factors,

blocking chemotactic growth signals, and conjugation to radioactive particles, many act by recruiting effector cells for targeted lysis of the cancer cell (MayoClinic, 2011; Melero et al., 2007). A recent expansion of mAb therapy is the completion of a phase III trial where Alemtuzumab, an anti-CD52 mAb indicated for treating B-cell chronic lymphocytic leukaemia, was used for treating relapsing remitting multiple sclerosis (MSSociety, 2013; Waldmann and Hale, 2005). Its therapeutic effect is believed to be via ADCC.

With regard to understanding what drives effector cell killing in conjunction with Abs there are two primary parameters to consider:

1) Molecular pathways of the effector cell which trigger target cell lysis and the downstream lysis pathway within the target cells
2) Ig and FcR types involved.

(1) The traditional method of identifying secretory granules or toxic radicals using inhibitors has only ruled out certain molecules as having a limiting role in PMN and MC cytotoxic function. Advances in immunostaining and cytometry have provided assays that allow for a broader spectrum analysis of cellular pathways. An interesting experiment might be to mix target and effectors cells for a period of 20 minutes and then sort them into individual populations again for analysis. Twenty minutes is sufficient for cell contact and ADCC but does not result in lysis of a majority of the cells (as I determined by microscopy). The addition of DNase and EDTA would ensure separation of conjugates enhancing the purity of cell populations. Then the lysates of the target and effector cells could be analyzed for relative levels of apoptosis-related proteins in a manner similar to Western blotting using for example the Proteome Profiler™ Human Apoptosis Array Kit (R&D Systems).

An alternative might be a multiplex immunoassay using Luminex magnetic beads. This initial screen would potentially indicate cytokines and proteins upregulated in cellular cytotoxicity that could be further investigated by other means. DNA microarray or RNA sequencing could also be done to look at genes upregulated and downregulated in effectors and targets.

(2) IgG, IgA, and IgE have all been implicated in mediating ADCC in various disease systems. Kramski et al. observed that NKs, MCs and PMNs bound bovine IgG with different capacity and separately identified certain FcR receptors as being important for MC ADCC (Kramski et al., 2012). However it would be interesting to compare binding of human HIV-1 antiserum (e.g. Bpool) to FcαRs and FcγRs to the levels of serum-mediated ADCC. Because cross-linking of these FcRs has different downstream signaling effects it would be another indicator of how these effector cells kill. In addition to FcR binding, to know the relative proportion of Ig isotypes in high binding/high ADCC sera would also provide clues as to which Ig isotypes should be elicited or even avoided in a vaccine response. In the RV144 HIV-1 vaccine efficacy trial higher levels of monomeric serum IgA have been associated with increased risk and IgA diminished IgG-mediated target cell killing functions, presumably by epitope competition (Tomaras et al., 2013). This immediately seems contradictory to what one would expect given the large amount of IgA in mucosal tissues and its ability to initiate cytotoxic functions in PMNs, and NKs. Perhaps IgA's role in phagocytosis and endocytosis in macrophages may be implicated (Otten and van Egmond, 2004). HIV-1 virions or even infected T cells opsonised by IgA can be phagocytised by

macrophages and then establish productive infection (Tan and Sattentau, 2013)(Sattentau lab unpublished data).

6.3 Imaging the ADCC Synapse

I was able to image the HIV ADCC synapse using LSCM having generated a high titre stock of $HIV_{NL4\text{-}3\text{-}eGFP}$ that infected primary cells; and I optimized staining, timing, and E:T ratios at which to examine effector-target conjugates. Further studies using LSCM, ImageStream™ and electron microscopy would provide more insight into structural features of the synapse such as cytoskeletal arrangement and the possible involvement of intracellular compartments.

6.4 ADCC and Clinical Status

While some studies cite ADCC as being higher in LTNPs, I did not find this to be so. I did not detect any correlation with patient status (LTNP vs. progressor), $CD4^+$ T cell count, viral load or neutralizing Abs. This remains a matter of discrepancy in the literature that is likely to remain until assays to measure ADCC are more uniform and larger patient populations are used.

6.5 HIV-1 Epitope Recognition

As certain viral epitopes prove more susceptible to neutralization, one would expect these to be similar to those that mediate ADCC. Knowing such epitopes would be essential in designing viral vaccine antigens and for mAb therapy. I found that the level of Ab binding to infected cells correlated with ADCC but using a panel of 14 NAbs I was not able to identify specific

epitopes that may mediate greater ADCC (Smalls-Mantey et al., 2012). Since this initial study more potent bNAb have been identified such as PG9, PGT121 and 10E8, which may be more functional in my assay (Corti and Lanzavecchia, 2013; Kwong and Mascola, 2012). Another method to identify important ADCC-mediating Env specificities would be to absorb specific gp120 and gp41 epitopes from high-binging/high-ADCC sera and then measure the effect this has on ADCC activity.

6.6 Conclusion

I have explored the elimination of HIV-1-infected cells by NKs, MCs, and PMNs in conjunction with Abs. These cells have complex stimulatory and inhibitory roles not only with respect to pathogens but also to each other, therefore the interplay between these cells must be taken into careful consideration when considering how to evoke an anti-viral response. In this regard, future studies need to consider analyses of innate immune cell recruitment and function against HIV-1-infected cells *in vivo*, by the use of intravital microscopy in the macaque or humanized mouse model (Murooka et al., 2012; Stolp et al., 2012). ADCC holds promise because anti-HIV-1 Abs and associated effector cells would selectively target infected cells, which by comparison is more specific than cancer mAb treatment that carries the risk of autoimmunity and systemic inflammation. Many more human vaccine and therapy trials are necessary to understand the nature of the effector cellular responses against HIV-1 infection, but my work here will provide some foundations for further work in this area.

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