Dissociated expression of granzyme B and IFN-γ by T lymphocytes in HIV-1 infected individuals and its implications for Tc1 effector diversity

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Globally, 42 million adults and children were living with HIV/AIDS at the end of 2002. Of infected adults, 48 percent were women. In 2002, the global adult HIV prevalence rate was 1.2 percent. During that year, 5 million people were newly infected with HIV and 3 million adults and children died due to HIV/AIDS (UNAIDS 2002).

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1. Introduction

1.1 Immunity

The natural environment of human beings contains a diverse variety of infectious microorganisms like viruses, bacteria, fungi, protozoa and multicellular parasites. With its ample amounts of nutrients and optimal temperature the human body represents an ideal habitat for most of these microorganisms. If these microbes enter and multiply uncontrolled within the body they can cause disease and will eventually kill their host.

Thanks to a biological phenomenon called immunity, most infections in normal individuals are short lived and cause little permanent damage. Immunity is the ability of the body to make the distinction between genetic "self" and "non-self" under foreign surroundings. Immunity may be innate (non-specific or non-adaptive) or acquired (specific or adaptive). Any immune response involves, firstly, recognition of a pathogen or other foreign material (“non-self”), and secondly, mounting a reaction in order to neutralize, eliminate, or metabolize it. Adaptive immune responses are highly specific for a particular pathogen and improve with each successive encounter with the same pathogen. The paralysis of one or more components of the immune system may lead to immunodeficiencies and loss of host defense against infections and tumors. The deregulation of the immune system may result in autoimmune diseases, allergies and cancer.

The vital role of the immune system was emphasized about twenty years ago with the emergence of AIDS (acquired immune deficiency syndrome). In the long-term,

AIDS, leaves the human body defenseless against microbes and certain cancers and leads to the death of the infected individual.

1.1.1 Components of the innate immune system

Innate immunity represents the earliest development of protection against disease causing organisms and foreign bodies and was present throughout most of the metazoa. Non-specific immune response can be mediated either by humoral factors, mainly proteins present in body fluids, or by phagocytic cells such as polymorphonuclear leukocytes. This type of immunity plays a vital part in the early containment of disease and major defects in non-specific immunity usually result in fatal, overwhelming disease. The main characteristic of non-specific immunity is that it requires no prior exposure to the organism to be effective. Physical and biochemical barriers like keratinized skin with its acid pH and a high salt concentration are the first hurdles potential pathogens have to overcome in order to gain entry into the human body. The serum also contains a variety of antimicrobial components. Cilia and mucus provide a physical barrier on the more susceptible mucus membranes thus preventing pathogens from attaching to cell surfaces. Body secretions like spermine in semen, acid in gastric secretions and lysozyme in tears are active antimicrobials. But once a pathogen has managed to colonize part of the body, humoral and cellular effector mechanisms are engaged (reviewed in ref. 75).

Non-specific humoral components include complement, a very important cascade of enzymes that induce a variety of immunological functions including inflammation, opsonisation and pathogen lysis. Acute-phase proteins are produced by liver cells in response to cytokines and contain C-reactive protein (CRP), fibrinogen and manna-

binding lectin (MBL) which aid in the opsonisation of bacteria and activate complement. There are several hormones generated by components of the immune system, these hormones are called lymphokines. Certain hormones in the body like steroids and corticosteroids (components of adrenaline) suppress the immune system. Tymosin is a hormone that encourages lymphocyte production. Interleukins are another group of hormone generated by white blood cells which play a role in innate as well as specific immunity. For example, Interleukin-1 (IL-1) is produced by macrophages (see below) after they engulfed a foreign cell. When it reaches the hypothalamus it produces fever and fatigue. Tumor Necrosis Factor (TNF), also produced by macrophages, is able to kill tumor cells and also promotes the creation of new blood vessels. Interferons (IFNs) interfere with viruses through induction of proteins that help prevent viral replication in the cell. IFNs are secreted by most cells if they are infected by certain viruses. This leads to resistance to infection of cells, in the vicinity of the infected cells, and hereby aid containment of these viruses. The two types of IFN exhibit distinct immunological properties (130). Type I IFNs include a number of IFN-α subtypes and a single species of IFN-β and can be produced by all cells under appropriate conditions. IFN-γ is a type II IFN and serves not only to induce antiviral function, but also to activate macrophages. Unlike the type I IFNs, IFN-γ is produced by a limited number of cell types. For example activated natural killer (NK) cells, activated T helper (Th) cells, and, in the presence of IL-12 and IL-18, activated dendritic cells (DCs) and macrophages (reviewed in ref. 75).

The non-specific cellular component contains two out of the three classes of Leukocytes: Granulocytes, 50% to 60% of all leukocytes and monocytes, about 7% of all leukocytes. Granulocytes (neutrophils, eosinophils and basophils) are polymorphonuclear cells produced in the bone marrow from a myeloid stem cell.

They get their name because they contain granules and these granules contain different chemicals depending on the type of cell.

Adult humans produce about a million neutrophils a second, many of them circulate in the blood, but most can be found attached to the cells lining the capillaries. Neutrophils circulate in the blood for three to four days and then migrate out into the tissues where they die after about a day. These cells are highly phagocytic and can kill pathogens by engulfing it, releasing enzymes, hydrogen peroxide and other chemicals from its granules. Eosinophils account for less than 1% of the cells in the peripheral blood, unless the individual is atopic or has a high parasitic worm burden. Although phagocytic these cells main effector function is exocytosis, which enables them to damage large organisms such as schistosomulae by releasing toxic components onto a pathogens surface. Basophils have functional similar capabilities as eosinophils and represent mediator cells like most cells because they contain histamine. Most cells whose precursors are not well defined play a part in protecting mucosal surfaces. They are better known for the release of substances that effect vascular permeability and hence play a major role in allergic responses (reviewed in ref. 75).

Monocytes circulate in the blood and upon migration into tissues evolve into macrophages which represent the second important phagocyte of the innate immune system. Most boundary tissues have its specialized macrophages, for example alveolar macrophages in the lungs or macrophages called Langerhans cells in the skin (reviewed in ref. 75).

NK cells are a population of bone marrow derived lymphocytes that function in innate immunity by direct lytic mechanisms and by secreting IFN-γ. They play a major role in innate immunity to viruses and intracellular pathogens. NK cells are an example

for the close interaction between innate and acquired immunity through their role antibody-depended cell-mediated cytotoxicity (ADCC), where the coating of pathogens by specific antibodies enables its recognition and subsequent elimination through NK cells (reviewed in ref. 75).

The innate immune system recognizes invading pathogens by germ-line-encoded pattern recognition receptors (PRR). The PRR are expressed on macrophages, dendritic cells (DC) and B lymphocytes (see below). Type I transmembrane proteins, Toll-like receptors (TLRs) are the key receptors responsible for recognizing specific conserved components of microbes (reviewed in ref. 13, 14). This includes lipopolysaccharides (LPS) from Gram-negative bacteria, CpG DNA and flagellin, thus permitting a relatively small number of receptors to recognize a large number of different microbes. Binding of pathogen-associated molecular patterns (PAMP) to TLR induces the production of reactive oxygen and nitrogen intermediates (ROI and RNI), pro-inflammatory cytokines, and up-regulates expression of co-stimulatory molecules; subsequently initiating the adaptive immunity. Ten different TLRs are identified today, with each TLR being involved in recognizing a wide variety of microorganism-derived molecular structures. TLR1 and TLR2 recognize various ligands expressed by Gram-positive bacteria (like mycobacterial lipoprotein and triacylated lipopeptides) (166, 168). TLR4 is the main receptor for LPS from Gram-negative bacteria (138, 139), while TLR5 recognizes flagellin (62). TLR6 recognizes mycoplasmal macrophage-activating lipopeptide-2 kD (MALP-2) in cooperation with TLR2 (167). TLR3 is specific for double-stranded (dsRNA) (4), a viral product, whereas TLR9 recognizes unmethylated CpG motifs (68) frequently found in the genome of bacteria and viruses, but not vertebrates. Plasmacytoid dendritic cells express TLR7 (67) as well as TLR9, they respond to TLR7 and TLR9 ligands by

producing high amounts of IFN-α. Therefore TLR3, TLR7 and TLR9 are thought to play an important role in detecting and combating viral infections (reviewed in ref. 3). The physiological role of TLR 7, TLR 8 and TLR 10 remain not well defined (14).

1.1.2 Components of the specific immune system

The principal characteristic of this type of immunity is that it is not only specific to particular components of pathogens or antigen (it recognizes pathogens by rearranged high affinity receptors), but also displays an enhanced response the second and subsequent times it comes into contact with the same organism. This memory capability makes the specific immune system so effective in dealing with many infectious agents. As in the non-specific branch of the immune response, specific immunity is mediated by both humoral and cellular responses (reviewed in ref. 75).

Lymphocytes represent 30% to 40% of all leukocytes and come in two classes: B cells, derived from the *bursa fabricii* (only in birds) or its equivalent in mammals (the fetal liver, the bone marrow and the mucosal associated lymphoid tissue) and T cells (those that mature in the thymus). All lymphocytes start in the bone marrow, but those destined to become B cells develop in the marrow before entering the bloodstream, while T cells start in the marrow but migrate through the bloodstream to the thymus and mature there. Both T cells and B cells are often found in the bloodstream but tend to concentrate in lymph tissue such as the lymph nodes, the thymus and the spleen (reviewed in ref. 75).

B cells and T cells have different functions. As with T cells millions of B cells are produced each day with a different antigen binding specificity. If the B cell comes into contact with the specific type of antigen to which its membrane bound immunoglobulin binds, it clonally expands and matures into short-lived plasma cells. These cells subsequently produce pathogen specific antibodies, which are released in high amounts into the circulation at the lymph nodes. Some of the activated B cells do not develop into plasma cells. Instead they become memory cells which continue to produce small amounts of the antibody long after the infection has been cleared. This antibody circulates as part of the gamma globulin fraction of the blood plasma. Should the same antigen enter the body again this circulating antibody acts quickly to destroy it. At the same time memory cells quickly divide to produce new clones of the appropriate type of plasma cell. Antibodies are Y shaped molecules which are responsible for the humoral immunity of the specific immune system. They eliminate infectious agents via a variety of methods such as agglutination, precipitation, neutralization, and in conjunction with complement, lysis. Certain types of immunoglobulin are specialized for particular functions such as lysis or protection at mucosal surfaces. Antibodies also collaborate in destroying disease causing organisms or toxins with lymphocytes and macrophages which are cells responsible for the cellular arm of the specific and non-specific immune response. These types of cells are able to destroy pathogens by a variety of cellular mechanisms including cytotoxicity, cytostasis, and by lymphocyte activation of macrophages which engulf and eliminate ingested organisms (reviewed in ref. 75).

T-lymphocytes play a central role in coordinating the entire specific and non-specific immune response. The defined T cell lineage marker is the T cell receptor complex

(TCR), which is a heterodimer of the disulphide linked α and β chain. The amino acid variability of the TCR resides in the N-terminal domain of the α and β subunits, which are homologous with the variable domains of the immunoglobulins. The TCR is associated with a series of polypeptides, collectively called CD3. T cells originally derive from stem cells of the bone marrow. Around the time of birth, their precursors leave the bone marrow to pass through the thymus gland. T cell diversity, the genetic information necessary to react with a multitude of possible antigens, is gained by a complex 5-stage genetic rearrangement that occurs at random in the developing T cells in the thymus. These new T cells are tested for their ability to recognize and bind to antigens in the thymus by positive and negative selection. It is postulated that positive selection eliminates T cells that do not bind tightly or strongly enough to antigen type molecules produced in the thymus, while negative selection eliminates T cells that bind too tightly or strong to self type molecules found in the thymus. This produces mature T cells that are effective against antigens but also self tolerant. Approximately 95% of all developing T cells die during this process in the thymus and as a result each lymphocyte is thus able to react with one antigen. Antigens of the body's own cells are excluded so that the population of matured T cells is potentially capable of distinguishing between “self” and “non-self” and only to react against the 'non-self' molecules. Autoimmune diseases are examples in which this recognition is defective and cells of the body may be recognized erroneously as “non-self” and subsequently destroyed. T cells each specific for one antigen circulate permanently between the blood and lymphatic systems. Several types of T cell are known which have a variety of functions (reviewed in ref. 75).

T helper cells, also know as Th cells, T4 cells or $CD4^+$ cells interact with B cells and help them to divide, differentiate and produce antibodies, or interact with

mononuclear phagocytes to help them to destroy intracellular pathogens. These are the type of cell attacked by the human immunodeficiency virus (HIV). They are activated by IL-1, produced by macrophages. $CD4^+$ cells take up residence in secondary lymphoid tissues e.g. lymph glands, Peyer's patches, mucosa-associated lymphoid tissues (MALT), gut-associated lymphoid tissues (GALT), spleen or bone marrow and produce Interleukin-2 (IL-2), interferon and other cytokines after they have been activated. These activate B cells which subsequently produce antibodies. There is a high level of complexity and interaction between neutrophils, macrophages, T cells and B cells (reviewed in ref. 75).

Cytotoxic T lymphocytes (CTL), also known as killer T cells, TC cells, T8 cells or $CD8^+$ cells, are responsible for the destruction of host cells which are infected by viruses or other intracellular pathogens, and they are able to destroy malignant host cells (reviewed in ref. 75).

Both CD4 and CD8 are glycoproteins that bind to non-polymorphic regions of the MHC molecule (see below). They perform a combination of adhesive and signaling functions, which greatly enhance the sensitivity of T cells to antigen. Since they are associated with the TCR, they are often called co-receptors. About 65% of peripheral T cells express CD4, and 35% express CD8 (reviewed in ref. 75).

1.1.3 The major histocompatibility complex

T cells recognize antigens on the surface of host cells only in association with molecules encoded within the Major Histocompatibility Complexes (MHC), also known as the Human Leukocyte Antigen (HLA). In humans MHC molecules are encoded by several genes all clustered in the same region on chromosome 6.

Because each gene has high number of alleles (alternate forms of a gene) in a population, it is very rare for two individuals to have the same set of MHC molecules, which are collectively called a tissue type. There are two major types of MHC protein molecules, MHC class I and MHC class II. MHC class I is present on most body cells, it binds peptides derived from self peptides or foreign antigens that are present in the cytoplasm of the presenting cell. Peptides of 8 or 9 amino acid length fit into the binding cleft of MHC class I. MHC class II is predominantly present on phagocytic cells and other cells of the immune system. It binds longer peptides, about 12 to 20 amino acid length, that are derived from foreign antigens that have been endocytosed and processed in the phagosomes of antigen presenting cells (APC). The binding pockets within the binding cleft of the MHC molecules are able to accommodate different peptides depending on the host's haplotype. MHC molecules are highly polymorphic and one cell expresses several different MHC molecules, thus cells can present many different antigenic peptides to T cells. MHC molecules are important components of the immune response. They allow cells that have been invaded by an infectious organism to be detected by T cells (reviewed in ref. 75).

1.1.4 Cytotoxic T lymphocyte diversity

Effector $CD8^+$ cells have been initially defined by their capability to lyse virus-infected target cells while they simultaneously produce high amounts of IFN-γ (88). Like $CD4^+$ cells (39, 82, 84, 147), $CD8^+$ cells were subsequently found in subpopulations that express type 1 and type 2 cytokine profiles (25, 35, 83, 85, 115, 120, 149, 151), one that fits the above definition and kills (cytotoxic T-cells of type 1, Tc1), and

another subset that kills, but does express interleukin 4 (IL-4) instead of IFN-γ, Tc2 cells. More recently, additional diversity was found within type 1 $CD8^+$ cells in as much that only effector $CD8^+$ cells were found to produce IFN-γ and to kill, while resting memory cells were found to produce interleukin 2 (IL-2) (60). Even within Tc1 effector cells evidence is emerging that there are discrete subpopulations. Thus, Mossman *et al*. showed that (dependent on different cytokine environments) naive murine TCR-transgenic $CD8^+$ cells can differentiate *in vitro* into effector cells that either produce IFN-γ and do not kill, or into $CD8^+$ cells that kill but do not produce IFN- γ or IL-4 (162). In mice, these different type 1 $CD8^+$ effector classes can also be induced *in vivo* via adjuvant-guided differentiation. Injection of several MHC class I-restricted peptides in incomplete Freund's adjuvant (IFA) induces $CD8^+$ cells that kill, but do not produce IFN-γ or IL-4, while the injection of the same peptides in complete Freund's adjuvant (CFA) induces IFN-γ producing $CD8^+$ cells that mediate delayed type hypersensitivity (DTH) but do not kill (P.V. Lehmann, personal communication).

$CD8^+$ cells can kill target cells via FAS-FAS ligand (FAS-L) interactions or by utilizing the perforin and granzyme B (GzB) pathway as shown schematically in Figure 1 pathway A and B, respectively. Perforin and GzB molecules are constitutively expressed and stored in intracytoplasmatic granules of $CD8^+$ effector cells, but not in naïve $CD8^+$ cells and or in resting $CD8^+$ memory cells (101, 172, 174). When activated through the TCR during antigen recognition, $CD8^+$ effector cells release the content of these granules towards the target cell. Perforin inserts itself into the target cell membrane generating pores through which GzB can penetrate into the target cell to activate the apoptotic caspase-dependent pathways (Figure 1 pathway B). However, recent evidence suggests that GzB can also exert cytolytic activity in the

absence of perforin-generated channels (11, 22, 101, 159, 174). This novel pathway involves high affinity binding of GzB to the mannose 6 phosphate/insulin-like growth factor II receptor on the target cell surface. Utilizing this receptor, GzB can penetrate into the target cell (Figure 1 pathway C and D) (116). While it has been established that the internalization of the receptor-bound GzB involves endocytotic vesicles, it is not clear how GzB translocates from these vesicles into the cytosol. There is evidence that this translocation also occurs in the absence of perforin via vesicle disruption and that non-channel forming perforin can facilitate this process (101, 172, 174).

Because of the central role of $CD8^+$ cell-mediated killing in host defense, there is considerable interest in the different cytolytic mechanisms that $CD8^+$cells employ to control infections in general and in infection with the HIV in particular. In HIV infection, antigen-specific $CD8^+$ were shown to lyse their target cells utilizing only the granule exocytosis pathway, but FAS-L was found not to be expressed on antigen specific $CD8^+$ clones, T-cells lines or circulating HIV-specific $CD8^+$ T-cells (158), suggesting the FAS-L pathway is not utilized to full potential in HIV.

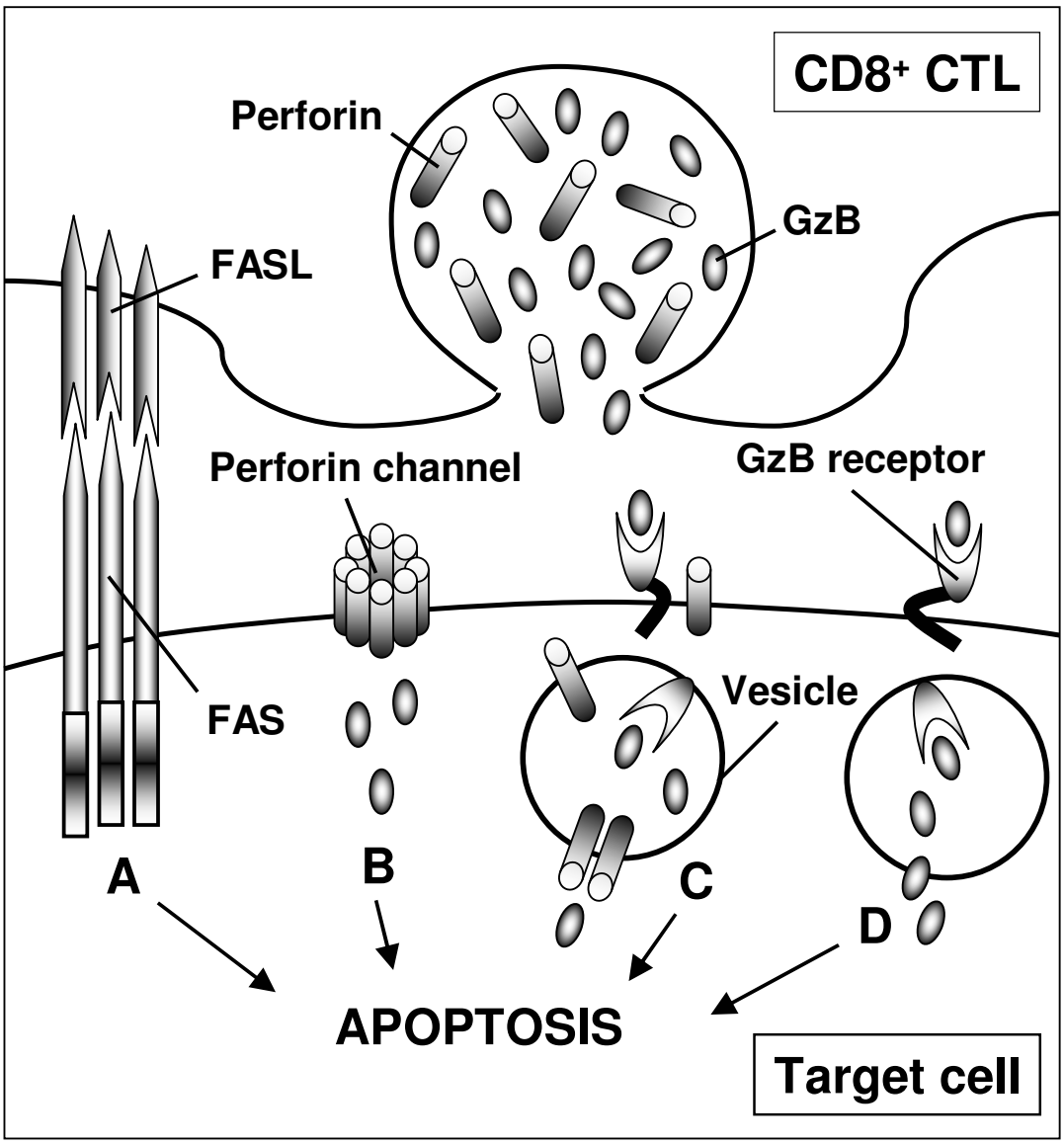

Figure 1. The various pathways by which CD8+ cells can induce apoptosis in target cells. FAS-FAS-L interaction leads to apoptosis via caspase-activation in the target cell (**A**). Perforin released from the cytolytic granules of the CD8+ cell polymerizes within the cell-membrane to form a perforin channel, through which GzB can penetrate the target cell to activate caspases (**B**). GzB enters the cells independent of perforin via the mannose-6-phosphate receptor and subsequent formation of endocytotic-vesicles. The release of the vesicle content into the cytoplasma seems to be facilitated by non channel forming perforin (**C**) or by vesicle disruption independent of perforin (**D**).

1.2 The human immunodeficiency virus

HIV, a retrovirus of the lentivirus family, is the causative agent for AIDS (51, 111). The primary targets of HIV are the cells of the immune system itself, which is gradually destroyed in the course of the natural infection. HIV infection may appear latent for years, but viral replication actively continues following initial infection and the rate of $CD4^+$ lymphocyte destruction is progressive (125, 133). During this time sufficient capacity of immune system remains intact to provide necessary immune surveillance and prevent most infections. Eventually, when a significant number of $CD4^+$ lymphocytes have been killed and production of new $CD4^+$ cells is insufficient to compensate for their ongoing dysfunction and destruction, the overall failure of the immune system leads to the appearance of clinical AIDS (70, 178).

1.2.1 Structure and genetic variability of HIV

The typical structure of the mature HIV virion consists of a bar shaped electron dense core which contains the viral genome encased in an outer lipid envelope with 72 surface projections containing an antigen, gp120, that aids in the binding of the virus to the target cells with CD4 receptors. Electron microscopy revealed that the plasma membrane of an infected $CD4^+$ lymphocyte exhibits budding virus particles approximately 80 to 100 nm in diameter. The genome consist of two short strands of ribonucleic acid (RNA) about 9200 nucleotide bases long, which is closely associated with the enzymes reverse transcriptase, protease, ribonuclease and integrase. Three structural and six regulatory genes, encoding at least 15 viral proteins orchestrate the complex mechanisms of HIV replication (57). The genetic

structure of HIV, similar to other retroviruses, contains three major genes, *gag*, *pol* and *env* (50, 173). The structural components encoded by *env* are the envelope glycoproteins, including the outer envelope glycoprotein gp120 and transmembrane glycoprotein gp41 derived from a glycoprotein precursor gp160 (50, 173). The *gag* gene encodes the capsid or core proteins p55, p40, p24, the matrix protein p17 and the nucleocapsid protein p7 (50, 173). The major proteins encoded by *pol* are the reverse transcriptase enzyme (RT) p66 and p51, the protease (PR) p11 and the integrase (IN) p32 (50, 173). Although most of the major viral proteins, including the core antigen p24 and the envelope antigen gp41, are highly immunogenic, the antibody responses can vary significantly depending on viral load and the immune competence of the host. The immunogenicity of these various viral components provides the basis for detection of antibody for most HIV testing (153).

Even within an individual HIV mutates rapidly under the selective pressure of CTLs due to the inaccuracy of reverse transcription, which introduces a single mutation approximately once per 2000 incorporated nucleotides (18, 141, 146). HIV's rapid replication rate and diversification in the infected individual enables it to adapt readily to changes in its environment (129). This leads to emergence of different HIV subtypes which are likely to account for the appearance of antiretroviral drug resistance as well as the variability in pathologic lesions as different cell types are targeted or different cytopathic effects are elicited during the course of infection (178). In perspective of different HIV variants between different individuals and geographic regions, the most common type of HIV is known as HIV-1. This is the virus responsible for the worldwide AIDS epidemic and has a number of subtypes, A through H and O, which have differing geographic distributions but all produce AIDS in a similar fashion. A second retrovirus strain HIV-2 with only 40-60% homology to

HIV-1 was discovered 1986 in West Africa (31, 59). It is less common as well as less virulent, but eventually can produce similar clinical findings as HIV-1 (180).

1.2.2 HIV infectivity and replication

HIV primarily targets cells that express CD4 cell-surface receptor molecules, with $CD4^+$ lymphocytes and macrophages playing the crucial role (37, 87). Cells of the mononuclear phagocyte system, blood monocytes and tissue macrophages, T lymphocytes, B lymphocytes, NK cells, DCs like Langerhans cells of epithelia and follicular dendritic cells in lymph nodes, hematopoietic stem cells, endothelial cells, microglial cells in brain and gastrointestinal epithelial cells have been found to be susceptible to HIV infection to various degrees. If the CD4 receptor is facilitated to enter the cell, chemokine receptors like CXCR4 and CCR5 are needed as co-receptors for HIV infection (5, 30, 40, 41, 48). Their presence on cells aids binding of the HIV envelope glycoprotein gp120. The initial binding of HIV to the CD4 receptor is mediated by conformational changes in the gp120 subunit, but such conformational changes are insufficient for completing the fusion process. The chemokine receptors lead to an additional conformational change in the gp41 subunit which enables the complete fusion of HIV. The differences in chemokine co-receptors, that are present on various cell types, are partly responsible for the selective infection of specific cells by different strains of HIV. Strains of HIV called T-tropic (X4) strains selectively interact with the CXCR4 chemokine co-receptor to infect lymphocytes, while M-tropic (R5) strains of HIV interact with the CCR5 chemokine co-receptor to infect macrophages. Rare cases of resistance to HIV infection were explained by the presence of a CCR5 mutation (38, 104, 152).

After entering the body, the viral particle encounters a cell with the appropriate CD4 receptor molecule to which it attaches and enters by fusion or by endocytosis. Subsequently the viral particle uncoats from the envelope and releases its RNA into the cell plasma (57). RT that is bound to the HIV RNA translates the RNA into proviral DNA (57). The proviral DNA is then inserted into host cell genomic DNA by IN (57). Once the HIV proviral DNA established itself within the host cell's genome, it cannot be eliminated or destroyed except by destroying the cell itself. The HIV provirus can now be replicated by the infected cell (57). The host cell releases virions by surface budding, or infected cells undergo lysis with release of new HIV virions that subsequently infect additional cells (57). Following the initial entry of HIV and establishment of infection, replication often first occurs within inflammatory cells at the site of infection or within peripheral blood mononuclear cells, but the site of replication shifts subsequently to primary and secondary lymphoid tissues of the body, including in lymph nodes, spleen, MALT, GALT and bone marrow (125). Macrophages and Langerhans cells in epithelia such as in the genital tract play a role as reservoirs and vectors for the spread of HIV in the body (21, 46, 73, 126, 140, 165). Within lymph nodes, HIV virions are trapped by follicular dendritic cells (FDCs), where they act as reservoir to infect $CD4^+$ lymphocytes that interact with the FDCs (160, 164). Primary HIV infection is followed by a viremia during which virus is easily detected in peripheral blood in mononuclear cells and plasma (133). Antibodies formed during primary infection are mostly not protective and viremia can persist despite the presence of even high antibody titers (112, 113, 137). The period of clinical latency is characterized by little detectable virus in peripheral blood, but viral active replication in lymphoid tissues (125, 133).

1.3 The role of $CD8^+$ cells in HIV

HIV triggers a vigorous $CD8^+$ cell response. Early after infection, up to several percent of the entire $CD8^+$ cell population can stain positive with a single HIV peptide-tetramer specificity (8). This initial $CD8^+$ cell response seems to play a critical role in controlling the virus (24, 92, 119, 150, 177, 182, 183). Thus, the clonal mass of HIV-specific $CD8^+$ cells correlates inversely with the viral load and disease progression (45, 121, 145). In non-human primate models, the depletion of $CD8^+$ cells leads to a dramatic rise in viral titers, while the emergence of $CD8^+$ cells coincides with the control of the virus (76, 93, 155, 184). While initially the immune response is successful in controlling HIV, it fails eventually. Various mechanisms have been attributed to this failure, but this issue remains highly controversial (102). Among the most important factors seem to be the lack of adequate $CD4^+$ cell help, the skewed maturation of HIV specific $CD8^+$ effector cells (23, 27) and the high mutation rate of the virus leading to the escape from $CD8^+$ cell recognition (6, 132, 141). Infected cells down-regulate HLA class I molecules (86, 156), and additional defects in antigen presentation by macrophages as well as dendritic cells have been observed (32, 42, 123). Inefficient lymphocyte trafficking (28, 93), and the depletion of T-cells by HIV infected macrophages and T lymphocytes via FASL-induced apoptosis has been described (9, 108, 124).

In chronic HIV infection, the $CD8^+$ cell response seems to exhaust possibly because of the continuous stimulation by the persisting virus. Only a fraction of the tetramer positive $CD8^+$ cells co-express IFN-γ (54). This common finding has been attributed to replicative senesce or anergy resulting from the extensive antigen-driven

proliferation of the specific T-cells (44, 100, 136, 157). An intriguing additional explanation for this finding might be that the IFN-γ negative, tetramer positive $CD8^+$ cells are not functionally silent, "exhausted" memory cells, but they represent the aforementioned non-classical Tc1 cells that can readily emerge *in vivo* through instructed differentiation in a Toll-like receptor neutral environment. Such $CD8^+$ cells that kill but do not produce cytokine might be a novel effector cell class which contributes to the control of infection.

1.4 Detection of cytolytic T lymphocytes at high resolution

The characterization of antigen specific immune responses at high resolution is one of the keys in understanding the mechanisms involved in immune protection. Until recently it has been challenging to gain high resolution data on antigen-specific T-cells as it is required for the understanding of the mechanisms involved in immune protection. Classic chromium release assays detect $CD8^+$ effector cells only if these occur in high frequencies (144), resting $CD8^+$ memory cells go undetected. Therefore, chromium release assays typically rely on the detection of lytic cell activity after *in vitro* expansion of $CD8^+$ cells (20). Freshly isolated peripheral blood mononuclear cells (PBMC) in general have to be cultured with the antigen of interest for 4 to 9 days, before the specific $CD8^+$ cells become detectable (20). Even after such pre-activation, frequently HIV-specific $CD8^+$ cells can not be detected by chromium release assays in infected individuals while they are clearly measurable with GzB enzyme-linked immunosorbent spot (ELISPOT) assays albeit in low frequencies (144). The *in vitro* culture leads to clonal expansion which not only obscures the frequency of the antigen-specific effector $CD8^+$ cells relative to *in vivo*,

but within a 6 days culture priming of naïve $CD8^+$ can occur which leads to the loss of the sought after information, whether the $CD8^+$ cell was indeed primed *in vivo* (143). To obtain frequency information with the chromium release approach, additionally limiting dilution assays (LDAs) need to be performed, however, LDAs frequently underestimate the actual numbers of the antigen specific $CD8^+$ cells (94, 169).

Technological advances in the measurement of antigen-specific T-cells like limiting dilution analysis/assays (LDA) in combination with fluorescence activated cell sorter FACS) analysis and intracellular cytokine staining (ICS) replaced conventional chromium release assays for detection of T lymphocytes (77, 91, 134, 142). They led to the development of new generation assays that operate at single cell resolution and hence are suitable to provide direct frequency information.

First tetrameric constructs of peptide-loaded major histocompatibility complex (MHC) antigen molecules (tetramers) (8, 122) in combination with intracytoplasmatic cytokine staining techniques (65) and the development of IFN-γ ELISPOT assays (49, 154). These techniques have been successfully used to study HIV infection (7, 36, 54, 55, 80, 81, 97, 103, 117).

Working with tetramers requires the precise knowledge of the MHC restriction element, which necessitates MHC typing of all test subjects before suitable reagents can be selected. The detection limit of any flow cytometry-based measurement, and therefore also of tetramer and intracytoplasmatic staining, is 1:1000 to 1:50,000 (43, 107). HIV-specific $CD8^+$ cells frequently occur in a frequency range lower than that, and therefore cytokine ELISPOT assays have gained an increasing role in studying $CD8^+$ cell-mediated immunity in infection and after vaccination (36, 94, 110).

Optimized ELISPOT assays detect the cytokine signature of individual T-cells even if these are as rare as one in several million of the sample cells, that is, the sensitivity of ELISPOT assays is orders of magnitudes higher than that of flow cytometry-based measurements (66, 69). This added sensitivity is frequently critical for the unambiguous detection of low frequency antigen-specific T-cells in freshly isolated peripheral blood mononuclear cells (PBMC). The ELISPOT assays' 24 hours duration avoids pitfalls of *in vitro* T-cell proliferation and differentiation (63, 69, 94, 97, 110, 154) because differentiation requires structural changes in the chromatin that are associated with proliferation (1) and the assay is terminated before the T-cells can divide and change their differentiation state relative to *in vivo*. For these reasons, ELISPOT assays have emerged as a primary tool to measure the cytokine effector lineage of antigen-specific T-cells *in vivo*. In view of the effector cell diversity within $CD8^+$ cells, it is generally assumed but not experimentally proven that IFN-γ-producing $CD8^+$ cells represent classic Tc1 cells that are also capable of killing, as opposed to the aforementioned IFN-γ positive $CD8^+$ cells that do not kill. This distinction should be of essence, however, because such different $CD8^+$ effector cell subpopulations may exert fundamentally distinct roles in the control of infections such as HIV.

1.5 Aim of research

Killing and cytokine production are distinct effector functions of $CD8^+$ cells. The release of IFN-γ and of other type 1 cytokines at the site of antigen recognition permits T-cells to recruit and locally activate macrophages and other cells of the

innate immune system. These T-cell-derived cytokines orchestrate a delayed type hypersensitivity (DTH) reaction, in which the cells of the innate immune system are instructed to become the actual effector cells (89, 105). The ability of T-cells to engage DTH is frequently essential to host defense, in particular against intracellular bacteria, but is it not known if it is also critical for the control of HIV infection. While IFN-γ measurements provide a surrogate for the T-cell's ability to induce DTH (114, 115), and while IFN-γ ELISPOT assays have become the gold standard for $CD8^+$ cell monitoring in HIV research (36), these assays might measure a function of $CD8^+$ cells that is either not even relevant for controlling HIV infection or might even have an adverse affect on virus spreading. By activating macrophages, IFN-γ has been shown to initiate transcription of latent virus in these cells that represent one of the prime targets of the infection (16, 61, 74). Not only might IFN-γ assays detect possibly irrelevant or harmful $CD8^+$ cells, they will not detect the aforementioned non-classical Tc1 cells, and Tc2 cells that might critically contribute to host defense by virtue of killing infected cells. Therefore we deemed it necessary to extend the scope of $CD8^+$ cell monitoring in HIV infection beyond IFN-γ measurements.

Our laboratory has introduced the currently used protocols for high resolution ELISPOT analysis (49) and recently we have extended this technique to measure GzB for monitoring cytolytic T-cell activity (144). GzB ELISPOT assays directly visualize the exocytotic cytolytic reaction of individual $CD8^+$ cells permitting high resolution studies in the low frequency range. The overall goal was to assess effector diversity of $CD8^+$ cells in HIV infection. In particular, we aimed at clarifying to what extent cytokine production of $CD8^+$ cells is associated with the GzB

production. Is there evidence for a $CD8^+$ effector cell class that can kill via the GzB pathway without producing type 1 or type 2 cytokine?

2. Material and methods

2.1 Definitions and Abbreviations

Ab: Antibody

Ag: Antigen

AEC: 3-Amino-9-Ethyl Carbazole

AO: Acridine Orange

APC: Antigen Presenting Cell

BSA: Bovine Serum Albumin

Ca^{2+}: Calcium kation

DMF: N, N-Dimethyl-formamide

DMSO: Dimethyl Sulfoxide

EB: Ethidium Bromide

FACS: Fluorescence Acitivated Cell Sorter

FBS: Fetal Bovine Serum

Fc: Fragment constant

F(ab): Fragment antibody binding

g: 9,81 m/sec^2

GzB: Granzyme B

H_2O: Water

H_2O_2: Hydrogen Peroxide, 30%

HAART: Highly Active Anti Retroviral Therapy

HRP: Horse Radish Peroxidase

Hu: Human

HuAB: Human AB Serum

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

M: Molar

Mg^{2+}: Magnesium kation

mM: Millimolar

PBMC: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffer Saline

PHA: Phytohemagglutinin

PPD: Purified Protein derivative of *Mycobacterium tuberculosis*

rpm: Revolution Per Minute

RPMI: RPMI 1640 Culture Medium

SD: Standard Deviation

TT: Tetanus Toxoid

2.2 Isolation and cryopreservation of PBMC

2.2.1 Equipment and Reagents

Equipment:	Vendor:	Catalog number:
Conical Tubes, 50ml, sterile	Fisher	14-432-22
Serological Pipette, 2ml	Fisher	13-678-11C
Serological Pipette, 5ml	Fisher	13-678-11D
Serological Pipette, 10ml	Fisher	13-678-11E
Pipette Aids, Drummond	Fisher	13-681-19
Centrifuge, Allegra 6R	Beckman Rotor Gh3.8A	ALR6
Variety MicroPipette (20µl)	Eppendorf	21-371-6
Pipette Tips (200µl)	VWR	53508-783
Gloves	Fisher	19-048-575A
Hemacytometer	Reichert Bright-Line	02-671-5
Hematology Mixer	Barnstead/Thermolyne	2-814-2
Vials, cryogenic, 1.8ml, sterile	Nalgene	12-565171N
Nalgene Cryo Freezing Container, filled with 2-Propanol	Nalgene	15350-50
-80 ºC Freezer	Forma scientific Inc.	55703-430
Liquid nitrogen storage tank	Thermo Forma	8030
Biological Safety Cabinet	Forma Scientific	Model 1286

Reagents	Vendor:	Catalog number:
Whole blood	Subject Source	
Ficoll, Isoprep	Robbins Scientific	1070-01-0
PBS sterile, Ca^{2+}, Mg^{2+} free	Cellgro	45000-436

Glutamine	Gibco	25030-081
AB serum	Gemini Bioproducts	100-112
RPMI 1640	Bio-Whittaker	12-167Q

2.2.2 Human PBMC donors

Blood samples were obtained from normal volunteers and from HIV patients from the Special Immunity Unit at the University Hospitals in Cleveland Ohio. All patients were under HAART therapy at the time of testing. The $CD4^+$ counts ranged from 400 to 1000 cells/ml. All studies were performed under the approval of the Institutional Review Board for Human Investigation at the University Hospitals of Cleveland.

2.2.3 Isolation of PBMC by Ficoll density gradient centrifugation

Whole blood was obtained and collected into green vacutainer tubes (sodium heparin) and processed within 24 hours of collection (best results with prompt processing). Samples were stored at room temperature, in the dark and on a rocker until processed (19). The blood (30ml) was pooled from vacutainers in 50ml conical tubes and 20ml Ca^{2+}-Free sterile PBS at room temperature was added. Subsequently the samples were mixed gently by inverting the tubes 3 times. Two sterile 50ml conical tubes were taken and 15ml of Ficoll (room temperature) added into each tube. It was avoided to mix and the Ficoll was gently overlaid with 25ml of the diluted blood using a sterile serological Pipette. It was ensured to minimize the mixing of the two phases. Subsequently the samples were centrifuged at 591g (1,800 rpm) for 30 minutes at room temperature with brake off to ensure that acceleration and deceleration did not disrupt the density gradient. Freezing Medium

A and Freezing Medium B were thawed and warmed up to room temperature. As soon as the centrifuge stopped, the mononuclear cells from the plasma/Ficoll interface were collected by a serological Pipette and the cells placed into a sterile 50ml conical tube. While collecting the cells, it was ensured to aspirate residual plasma at least one 1ml, but as little Ficoll as possible. Interface cells from maximum two 50ml tubes were combined into one wash tube. Lower cell numbers did pellet if the proportion of Ficoll was too great (> 5ml). The tube was filled with sterile Ca-free PBS at room temperature such that the final volume in the tube was 50ml. For counting of PBMC the Hemacytometer counting protocol was used (see below). With brake on, the cells were centrifuged at 330 g (1,200 rpm) for 10 minutes at room temperature (24°C). Based on the cell count the appropriate number of cryotubes (1.8m) per patient was labeled. Each cryotube did contain approximately $10x10^6$cells/ml. Labels had to indicate patient ID and date of processing. As soon as centrifugation was completed, the supernatant was decanted and discarded. The cell pellets were resuspended by tapping the tube until no clumps were visible. It was avoided to Pipette or vortex, as this would damage cells. Pelleted cells would have started dying if not resuspended promptly. If any additional washes were done, the cells were resuspended in room temperature cell-medium that contains protein (e.g. 5%HuAB).

2.2.4 Cryopreservation of PBMC

During the cooling process for cryopreservation, ice forms first external to the cell between -5°C and -15°C, leaving the cells super cooled but unfrozen. At this point an osmotic imbalance occurs across the cell membrane, leading to water flux out of the cell. To preserve the cell, it must dehydrate slowly, at a rate critical to cell survival. Fast cooling produces intracellular ice formation, which ruptures the cell wall. Cooling the cell too slowly leads to solute imbalances that are injurious to the cells. In addition, the change in water's state from the liquid to the crystalline form results in a release of energy, known as the latent heat of fusion, which can be detrimental to the cells. Because of the above phenomena, the rate of cooling should be linear over time. Cell permeability, DMSO toxicity, and cooling rate must be considered for each cell type when freezing. This protocol was specifically designed in our laboratory for freezing PBMC for subsequent testing of T-cells in cytokine ELISPOT assays. To maintain the membrane lipid fluidity of the cells it was not worked with chilled reagents and all reagents were kept at room temperature.

An appropriate amount of Freezing Medium A (Appendix A) at room temperature was added to adjust the cell concentration to about 16 to 20 x 10^6/ml. The cells were mixed gently by tapping the tube without using a Pipette. Slowly, drop by drop an equal volume of Freezing Medium B (20%DMSO) was added to Freezing Media A containing the PBMC. This was done in a time period of 2 minutes per tube. Once mixing was complete, the PBMC suspension was aliquoted into the pre-labeled cryovials, 1ml into 1.8 ml vial (not more than 10 million cells per vial). It was ensured to Pipette gently to minimize sheer forces.

For freezing the cryovials one of the following processes was used. The cryovials were placed into a Nalgene cryofreezing container filled with 2-propanol at room temperature. The freezing container was subsequently put into -80°C freezer for a minimum of 12 hours. After that the cryovials were transferred into liquid or vapor nitrogen for indefinite storage. During the freezing time it was avoided to open the freezer in order to avoid shaking or raising the freezer's temperature. If Nalgene cryofreezing containers were not available, the following "low technology" method worked well. The cryovials were placed in a Styrofoam tube container (like the racks in which the 15ml conical tubes come in) to reduce direct contact with cold surfaces and to slow the rate of freezing. Subsequently a second Styrofoam container of the same type was placed over the first one and the two containers were taped together. Afterwards they were placed in a plastic bag leaving some air in the bag before it was shut tied. The bagged container was placed with the cells in a -80°C freezer for at least 12 hours. During the freezing time it was avoided to open the freezer in order to prevent shaking or raising the freezer's temperature. Subsequently the cryovials were transferred into liquid nitrogen for indefinite storage.

2.2.5 Reagent preparation (Appendix A):

Freezing Medium A: HuAB serum (GeminiBiotech), tested for assay compatibility, heat inactivated and filtered. It can be thawed and refrozen up to 3 times or can be thawed and kept at 4℃ for up to one month.

Freezing Medium B: 20% DMSO (Sigma D2650) in heat inactivated HuAB serum, filtered. It can only be thawed one time and must be used the same day.

DSMO Hybri-Max tm Sigma D2650, graded for sensitive cells.

It is not primarily the toxicity of DMSO itself, but the osmotic shock caused by DMSO rapidly that harms the cells most. Also, the viability and functionality of the cells is much better if DMSO is added at room temperature.

Media
for human PMBC

- 940ml of 1640 RPMI (w/o glutamine).
- Add 50ml heat inactivated HuAB (5%).
- Add 10ml glutamine (equivalent to 1%) for a final 2mM solution.
- Filter through 0.2μm filter and store in 4℃ for up to a month wrapped in aluminum foil to protect it from light.
- Resupplement glutamine before usage if it had been stored for more than a week.

2.3 Cell counting

2.3.1 Equipment and reagents

Equipment:	Vendor:	Catalog number:
Olympus Reflected Fluorescence System	Olympus	Model BX41TF
Kimwipes EX-L	Kimberly-Clark	34155
MicroPipette 20µl	Eppendorf	21-371-6
Pipette Tips 1-200µl	VWR	53508-783
Gloves	Fisher	19-048-575A
Hemacytometer	Reichert Bright-Line	02-671-5
Hematology Mixer	Barnstead/Thermolyne	12-814-2
Parafilm	American National Can	13-374-10
Hand Tally Counter	Fisher	07-905-6
Lab Counter –Two Units	Fisher	02-670-12

Reagents:	Vendor:	Catalog number:
Phamco. Ethyl Alcohol	Polysciences Inc	16020
Acridine Orange	Molecular Probes	A3568
Ethidium Bromide	Fisher Biotech	BP1302-10
Ethanol 70%	Laboratory	
Bleach 20%	Clorox	
PBS sterile, Ca^{2+}, Mg^{2+} free	Cellgro	45000-436

2.3.2 Preparing and counting with the hemacytometer

The hemacytometer was cleaned with H_2O and 70% ethanol then dried off with a ply of Kim wipes and placed on a microscope stage and subsequently focused using white light at 10X. In order to count the cells the grid had to be visible and the UV light had to be turned on, which was best accomplished by having the white light just barely turned up, what enables to see the grid in relationship to the stained cells, which the UV light caused to light up. The stain was prepared ahead of time (preparing working stain see Appendix B). In order to stain cells 20µl of cell suspension were combined with 20µl of working stain (1:1). This was done on a piece of parafilm, were the cells were mixed well with a Pipette. The goal was to achieve an accurate cell distribution with cell clumping kept to a bare minimum. The hemacytometer was placed on the counter and the counting chamber covered with a hemacytometer glass cover slip. Using a MicroPipette 9 µl of the stained cells carefully was introduced into the notch of the hemacytometer. The goal was to cover the slide completely and to limit air bubbles to zero, avoiding flooding of the hemacytometer.

AO/EB staining was used. ORANGE cells were DYING and GREEN cells were ALIVE. Living and dead cells were counted within two opposing squares, while only two sides of each square were included. The numbers were added up and if total cell count did not reach a hundred cells then the two leftover squares needed to be counted as well for accuracy.

2.3.3 Formula used to determine cell counts

- Viable cells/ml = (total number of viable cells divided by the squares counted) x 10^4 x dilution factor

- Total viable cells = viable cells/ml x volume of original cell suspension

- The percentage of viable cells can also be calculated using the following formula:

% viability = (number of total viable cells counted / total number of cells (live and dead) counted) x100%

2.3.4 Solution preparation (Appendix B)

Working stain:

Note: AB and AO are biological hazards obey proper safety protection.

To 10ml sterile 1x PBS:

- Add 2µl EB 1% Solution.

- Add 2µl AO (10mg/ml).

Aliquot, wrap in aluminum foil to protect from light. Store at 4ºC for up to 2 months.

2.4 Antibody titration, antigen testing and ELISPOT

2.4.1 General principle

Until recently, it has been a considerable challenge to determine the frequencies and the effector classes of antigen-specific T-cells *in vivo*. The lack of direct T-cell measurements affected progress in all major fields of immune-mediated diseases and their therapies, including allergies, autoimmunity, cancer, transplantation and infectious disease/vaccines in clinical trials and animal models. ELISPOT assays (Figure 1) have been developed to provide the frequency and cytokine signature of antigen-specific T-cells, directly *ex vivo*, reflecting immunity *in vivo* (66, 69, 79, 170). The cytokine production of as few as one in a million antigen-specific T-cells can be readily detected by this technology that combines improved ELISPOT assay protocols with advanced image analysis tools. While operating at the highest possible resolution (single cell resolution assays), this technology is also best suited for high throughput analysis like scanning peptide libraries (64, 128, 171, 185). In comparison to other methods it requires minimal amounts of cell material and can utilize frozen samples (163), which make it particularly suited for clinical trials.

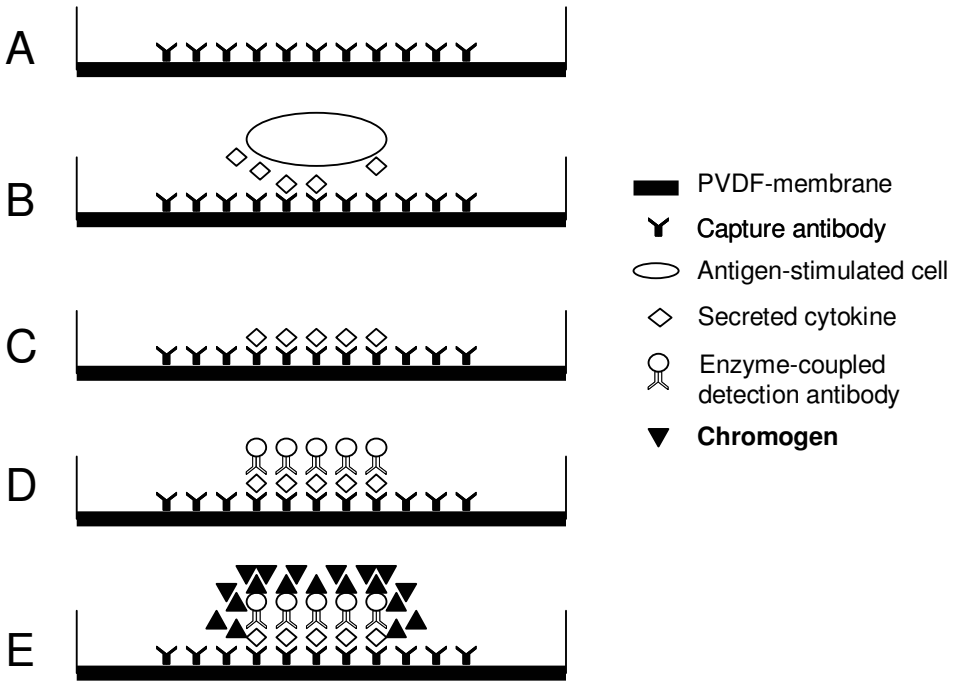

Figure 2. The principle of the enzyme-linked immunosorbent spot assay (**ELISPOT**) for cytokine detection is shown. An ELISPOT plate with a PVDF-membrane is coated with a monoclonal cytokine specific antibody (**A**). Freshly isolated or cultivated cells are loaded together with the antigens of interest and incubated to allow for activation and cytokine secretion. Antigen specific memory cells will secrete the cytokine (**B**). The cells are washed away and only to the antibody bound cytokine molecules remain in the well (**C**). A second enzyme-coupled monoclonal antibody specific for a different epitope of the to be detected cytokine is added (**D**). The addition of a chromogen leads to an enzyme catalyzed spot development (**E**).

2.4.2 Equipment and reagents

Equipment:	Vendor:	Catalog number:
MicroPipette (10µl)	Eppendorf	21-371-5
MicroPipette (20µl)	Eppendorf	21-371-5
MicroPipette (100µ)	Eppendorf	21-371-5
MicroPipette (200µl)	Eppendorf	21-371-5
MicroPipette (1000µl)	Gilson	K18347K
Pipette Tips1-200µL, large orifice	VWR	53503-616
Pipette Tips1-200µL small orifice	VWR	53509-009
Pipette Tips 101-1000µL, blue	Fisher	21-197-8A
Pipette Tips 5-300µL, sterile	Lab.Prod.Sale	L110803
Repeater Syringe Dispenser	Oxford	S/N 0Z00J
Repetitive Syringe (1.5ml)	VWR	53498-974
Repetitive Syringe (3ml)	VWR	53519-458
Repetitive Syringe (6ml)	VWR	53498-976
Gloves	Fisher	19-048-575A
ImmunoSpot M200 plates	Cellular Technology Ltd	M200/50
ImmunoSpot P50 plates	Cellular Technology Ltd	P50/50
Biological Safety Cabinet	Forma Scientific	Model 1286
Refrigerator	Danby	Model DCR122W
Incubator	Forma Scientific	Model 320
Conical Tubes , 15ml	Fisher	14-959-70C
Conical tubes, 50ml	Fisher	14-432-22

Various racks		
Water bath 6.7x14.6 liter	Lab-Line	22-2997-36
-80 ºC Freezer	Forma scientific Inc.	55703-430
Ice buckets		
Dry-ice container, Cooler		
Convective warm-air flow	Remington	
Centrifuge, Allegra 6R	Beckman Rotor Gh3.8A	ALR6
Solution Basin Sterile	Fisher	NC9490002
Plate washer	BioTek Instruments Inc.	Model Elx405R
0.2 um filter unit 1000ml	Nalgene	09-740-3A
0.2um filter unit 250ml	Nalgene	09-740-2A
Pipette Aids, Drummond	Fisher	13-681-19
Serological Pipette, 1ml	Fisher	13-678-11B
Serological Pipette, 2ml	Fisher	13-678-11C
Serological Pipette, 5ml	Fisher	13-678-11D
Serological Pipette, 10ml	Fisher	13-678-11E
Serological Pipette, 25ml	Fisher	13-678-11F
8 channel (50-300µl)	Labsystem	13-688-502
12 channel (50-300µl)	Labsystem	13-688-505
ImmunoSpot Software	Cellular Technology Ltd	
ImmunoSpot Analyzer	Cellular Technology Ltd	Series 1, 2001120

Reagents:	**Vendor:**	**Catalog number:**
Liquid nitrogen		

Acetic acid glacial	Fisher	A38S-500
BSA, fraction 5, RIA grade	Sigma-Aldrich	A-7888
DMF	Sigma-Aldrich	D8654
Sodium Acetate	Fisher	S210500
Glutamine	Gibco	25030-081
AB serum	Gemini Bioproducts	100-112
RPMI 1640	Bio-Whittaker	12-167Q
1x PBS	Cellgro	VWR 45000-432
10x PBS 500ml	Cellgro	VWR 45000-428
Antibodies see Appendix D		

2.4.3 Day 0

Preparation of reagents and all solutions refer Appendix C. After the design of the plate layout the plates were coated with anti-cytokine antibodies diluted in sterile PBS under a biological safety cabinet, with 100µl/well for M200 plates and 50µl/well for P50 plates with concentrations according to the titration specified before. The titration concentration was done around the values of the previous testing, since those values proved to be the best previously (see example of cross titration in Figure 3). The plates were tapped gently so that the solution spreads uniformly all over the well and then the plates were incubated at 4ºC overnight in a humid chamber (this is the minimum amount of time required, plates can be keep for up to a week at 4ºC).

2.4.4 Day 1

The plates were washed 3 times with PBS (Solution 1), 200µl/well and the PBS removed by flicking the plate. Afterwards the plates were blocked for at least 1 hour at room temperature with 200µ/well of the blocking solution (Solution 3) and subsequently washed 3 times with PBS (Solution 1), 200µl/well. The cells were incubated at 37ºC in a humidified incubator, 24 hours for IFN-γ, granzyme B and 48 hours for IL-4 and IL-5.

In case of using frozen cells the media for the cells was warmed up in an incubator with loosened caps or warmed to room temperature by using the water bath with bottles tightly closed, before use. Antigens were diluted in the biological safety cabinet with cold media in labeled tubes. Antigens were plated according to the in each case applicable titration layout (e. g. Figure 3). To plate the antigens filled

reservoirs with multi-channel Pipettes or filled from dilution tube with a repeat Pipette were used. The antigen or mitogen solution for desired T-cell activation was plated in 100µl/well for M200 plates and 25µl/well for P50 plates. Leftover antigens were stored at 4°C in the refrigerator until the end of the testing and were discarded the following day. Once all antigens were plated, the plates were placed at 4°C in a refrigerator within a humid chamber. About 15 minutes before cells were ready to be plated, Ag coated plates were moved in the 37°C incubator to warm up. Fresh PBMC were added cells in 100 µl/well specified medium (for whole blood processing see procedure above). If frozen samples were handled "to be tested cell samples" were moved from liquid nitrogen to -80℃ freezer being transported in dry ice-filled containers. Right before thawing the samples were placed on dry ice. The samples were thawed by convective warm airflow or by hand if desired. After thawing, each vial was poured into the pre-labeled sample tube. Each cryovial was rinsed with 1ml of washing medium and the rinse was added to the sample tube. The sample tube was then brought up to a volume equal to 4 times the original frozen volume of the batch. These steps were designed to dilute the frozen cells over several minutes so as to minimize osmotic shock. Centrifugation followed at 1200 rpm for 10 minutes, brake on, at 24°C. As soon as the centrifuge stopped the supernatant was decanted and the cells were resuspended by tapping the tube. Then the appropriate amount of media for counting/washing was added. This amount depended on the expected cell count. After total cells counts had been determined (see procedure above), the cells were centrifuged again at 1200rpm, 24ºC with brake on. As soon as the centrifuge stopped, they were decanted and resuspended in the amount of media that depended on the cell count and the cell concentration needed for plating. Based on the plate layout the cells were pipetted with a multichannel pipette from a

reservoir with large-orifice tips or with a repeater Pipette with a large orifice tip into the well. 100µl/well were plated in M200 plates and 25µl/well in P50 plates. The plate was then placed in the CO_2 incubator where it was left undisturbed for 24 or 48 hrs, depending on the cytokine that was tested.

2.4.5 Day 2

Depending on the cytokine or cytotoxin tested incubation time varied from 4h to 48h before the 2nd Ab could be added. For granzyme B the minimum incubation time was 4 to 24 hours, for human IFN-γ it was 20 to 24 hours and for human IL-4 and IL-5 incubation between 44 and 48 hours were required. With a BioTek Plate Washer, the plates were washed 3 times with 200µl/well PBS (Solution 1) and 3 times with PBS-Tween (Solution 2). In PBS-BSA-Tween (Solution 4) the appropriate dilution of the 2nd biotin labeled Ab was prepared based on previous lot's titration. After flicking the plates empty, 100µl/well was added to M200 plates and 50µl/well to P50 plates and subsequently incubated at 4°C overnight in a humid chamber.

2.4.6 Day 3 or 4

Depending on the cytokine or cytotoxin investigated after 4 to 48 hours, plates were washed 4 times with PBS-Tween with a BioTek plate washer (200µl/well). Then 100µl/well of diluted tertiary reagent (3rd Ab) in PBS-BSA (1%) to M200 plate wells were added and 50µl/well to P50 plates. Streptavidin-HRP (DAKO) at 1/2000 was used and an incubation at room temperature for 2 hours followed. For development plates were washed 3 times with PBS-Tween with a BioTek plate washer (200µl/well for M200, or 150µl/well for P50). After that an additional wash of 3 times with PBS in

a BioTek plate washer (200µl/well for M200, or 150µl/well for P50 followed. Fresh developing solution was prepared (see Appendix C) and 200µl/well (M200 plate) or 150µ/lwell (P50 plate) of freshly made solution was added to the plates. Afterwards the plates were incubated at room temperature for 3 to 45 minutes depending on cytokine or cytotoxin, until obvious spot development was seen. The reaction was then stopped by flushing plate 3 times with tap water and the plates flicked dry. Subsequently the plates were dried while being from light protected for 24 hours in an upright position, to avoid staining artifacts.

2.4.7 Day 5 up to 3 months

The wells of every plate were scanned, counted and digitally archived using an ImmunoSpot image analyzer. If results of the cross titration were unsatisfactory additional titration steps were necessary. A decision was made on what the optimal antibody or antigen concentration for the given lot was and depending on the results anti-cytokine antibody or antigen aliquots were made and stored appropriately.

2.4.8 Reagent preparation (Appendix C):

Sterile PBS (Solution 1)

- Fill a beaker with 900ml distilled H_2O.
- Add 100ml 10x PBS.
- Filter through (0.2µm) and store in 1l bottle at room temperature for up to 1 year.

Non-Sterile PBS

- Fill a beaker with 900ml distilled H_2O.
- Add 100ml 10x PBS.
- Store in 1l bottle at 4ºC for up to 1 month.

PBS-Tween (0.05%) (Solution 2)

- Fill a beaker with 895ml distilled H_2O.
- Add 100ml 10x PBS to beaker.
- Add 5ml 10%Tween 20 to the solution.
- Mix solution together.
- Pour in 1l bottle and store at 4ºC for up to 1 month.

PBS-BSA (1%) (Solution 3)

- 1l of PBS
- 10g of BSA
- Pour PBS into beaker and add BSA on top.
- Do not mix. Let solution sit for approximately 30 minutes/until completely dissolved.
- Once completely dissolved, filter (0.2um) reagent.
- Store at 4℃ for up to 6 months.

PBS-Tween (0.05%)-BSA (1%) (Solution 4)

- Fill beaker with 895ml of distilled H_2O.
- Add 100ml of 10X PBS.
- Measure out 10g of BSA and add on top of mixture.
- Let solution sit for BSA to completely dissolve.
- Add 5ml of 10%Tween 20.
- Filter (0.2µm), pour in 1l bottle and store in 4℃ up to 6 months.

AEC Buffer (0.1M Acetate)

- Place 900ml of distilled H_2O in a beaker.
- Stir in 1.702ml of glacial acetic acid (17.4N).
- Add 5.775g of Na acetate (FW 82.03).
- Mix well.
- pH to 5.0
- Bring the solution up to a total of 1l.
- Store in 4℃ up to 6 months.

AEC Solution
Note: Chemical hazard!

- Prepare with a glass tube, in a fume hood while wearing proper glove and lab coat.
- 100mg AEC (3-Amino-9-Ethyl Carbazole)
- 10ml DMF (N, N, Dimethylformamide).
- Store at room temperature, away from light up to a year.

Developing Solution

- Prepare within 10min of use.
- Measure out 24ml AEC buffer and place in 50ml tube.
- Measure out 800µl AEC solution, in glass Pipette, and add to 24ml of AEC buffer; mix.
- Filter solution through a 0.45µm filter
- Add 12µl H_2O_2. (30%).
- This amount is enough for one plate.
- Use immediately.

Media
for human PMBC

- 940ml of 1640 RPMI (w/o glutamine).
- Add 50ml heat inactivated HuAB (5%).
- Add 10ml glutamine (equivalent to 1%) for a final 2mM solution.
- Filter through 0.2µm filter and store in 4℃ for up to a month wrapped in aluminum foil to protect it from light.
- Resupplement glutamine before usage if it had been stored for more than a week.

10% Tween 20

- Measure out 55g of Tween 20 to beaker.
- Use a total of 450ml of distilled H_2O to dissolve and rinse out the beaker. Pour the solution from the beaker into a bottle.
- Store the total solution of 500ml at 4℃.

Note: Tween 20 has a specific gravity of 1.1 Divide 55g by 1.1 = 50ml.

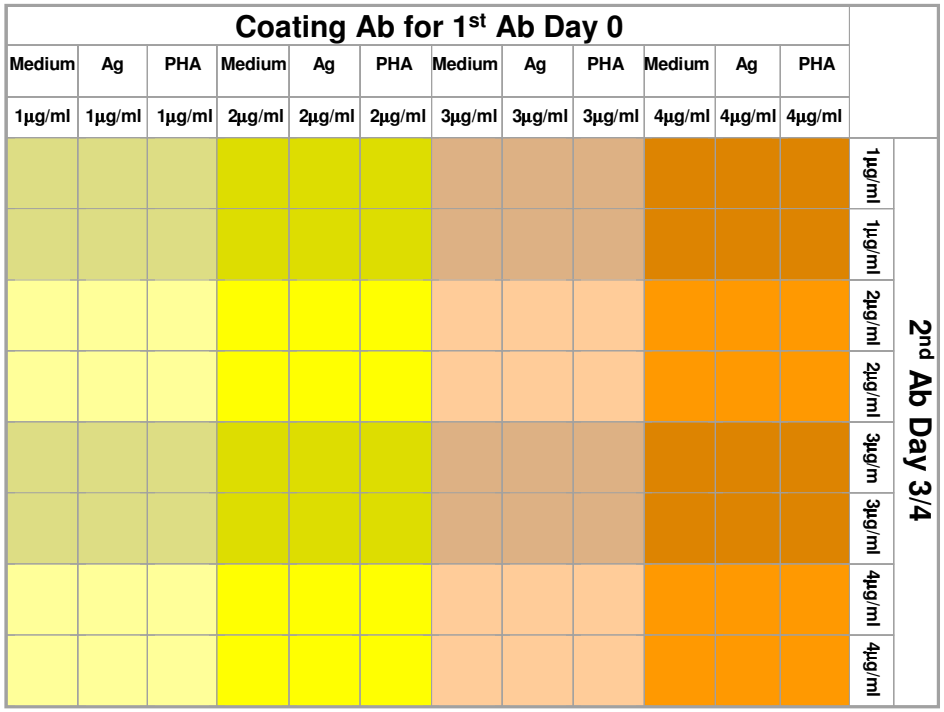

Figure 3. Example of cross-titration. It is critical that every antibody pair used in the assay is carefully titrated. This applies to every new batch of antibodies used. The first (coating on day zero) and second antibody (on day 3 or 4) are titrated against each other in different concentrations. To obtain proper information about spot density and spot morphology a medium control, an antigen (like tetanus toxoid (TT) or purified protein derivative of *mycobacterium tuberculosis* (PPD) and a mitogen (like PHA) should be used for stimulation.

2.4.9 Antibodies for human ELISPOT (Appendix D):

Coating Ab:

Hu-GzB	Hoelzel Diagnostika (GB-11)	4µg/ml
Hu-IFN-γ	Endogen (M-700A, 2G1)	4µg/ml
Hu-IL-4	PharMingen (#18651, 8D4-8)	5µg/ml
Hu-IL-5	PharMingen (#18051A, TRFK5)	5µg/ml

Secondary Ab:

Hu-GzB-biotin	Hoelzel Diagnostika (GB-10)	3µg/ml
Hu-IFN-γ-biotin	Endogen (M-701, B133.5)	2µg/ml
Hu-IL-4-biotin	PharMingen (#18502D, MP4-25D2)	2µg/ml
Hu-IL-5-biotin	PharMingen (#18522D, JES1-5A10)	2µg/ml

Tertiary Reagent:

Streptavidin-HRP	Dako	1:2000

Note: All concentrations given above are estimates. Due to batch-to-batch variations, each new batch is tested to establish the ideal concentration of a new reagent batch.

2.5 Biotinylation of antibodies

In order to biotinylate a minimum of 1 mg of antibody the antibody solution, if bought, had first to be dialyzed. Before use the dialyzing membrane was washed 30 minutes with deionised water. Afterwards a clip was put at the bottom and the antibody was filled in with a Pasteur Pipette. While leaving a little bit of air above the antibody the second clip was put on. Subsequently this antibody solution was dialyzed against buffer (Appendix E) overnight in cold room 4°C while being stirred. The following day 150µg biotin per mg antibody, i.e. 15µl biotin solution per mg antibody, was added and shaken on a rocker wrapped in aluminium foil for four hours at room temperature. The reaction was stopped by adding 1M ammonium chloride, i.e. 20µl per 250µg added biotin. The biotinylated antibody was dialyzed in the cold room against a total of 10l PBS, five times exchanging the solution approximately every 6 hours.

2.5.1 Equipment and reagents

Equipment:	Vendor:	Catalog number:
Dialyze membrane: Spectra/Por, molecularporus membrane tubing, (MWCO 12.000 - 14.000, flat width 10 +/- 1mm, diameter 6,4mm, vol/length 0,32ml/cm, length 15m)	Spectrum medical industries	132-676
Plastic clips		
MicroPipette (20µl)	Eppendorf	21-371-5
MicroPipette (200µl)	Eppendorf	21-371-5
MicroPipette (1000µl)	Gilson	K18347K

Pipette Tips1-200µl small orifice	VWR	53509-009
Pipette Tips 101-1000µl, blue	Fisher	21-197-8A
Stirrer	Thermolyne	Nuova 2
Pasteur Pipette		
Serological Pipette, 2ml	Fisher	13-678-11C
Serological Pipette, 5ml	Fisher	13-678-11D
Serological Pipette, 10ml	Fisher	13-678-11E
Pipette Aids,	Drummond	Fisher#13-681-19
Biological Safety Cabinet	4-000-100 Forma Scientific	Model 1286

Reagents:	**Vendor:**	**Catalog number:**
N-Hydroxysuccinimido-Biotin (stored at –20°C)	Sigma	H-1759
PBS sterile, Ca^{2+}, Mg^{2+} free,	Cellgro	45000-436
Ammonium chloride (NH_4Cl)	Fisher	A661-500
Sodium bicarbonate	Fisher	BP357-1
Sodium hydroxide	Fisher	BP359-212
Boric Acid: (H_3BO_3)	Sigma	B-6768
Borax ($Na_2B_40_7$ x 10 H_2O)	Sigma	B-9876.

2.5.2 Solution preparation (Appendix E)

Buffer: 1l 250ml Boric Acid 0,2M (12,4g/l)

150ml Borax 0,05M (19,03g/l)

Diluted with Aqua dest., pH adjusted to 8,8.

Biotin solution: Warm the biotin vial up in your hands before opening to avoid inwards air suction. The prepared biotin solution is unstable use immediately. 1mg biotin in 100µl DMSO (glass vial), let stand for 10 minutes before use.

2.6 Cell separation

In order to separate sub-populations of human $CD8^+$, $CD4^+$ or $CD3^+$ cells for studies, two different methods were employed the purification via high affinity selection in enrichment columns (15) form R&D after prior isolation of PBMC and the enrichment out of the whole blood via the tetrameric antibody complex method (TAC) from RosetteSep™ (96). In both cases the purification was achieved by negative selection in order to prevent any potential involuntary activation or signaling of cells through cross linking of cell surface molecules.

2.6.1 Equipment and reagents

Equipment:	Vendor:	Catalog number:
Conical Tubes, 50ml, sterile	Fisher	14-432-22
Serological Pipette, 2ml	Fisher	13-678-11C
Serological Pipette, 5ml	Fisher	13-678-11D
Serological Pipette, 10ml	Fisher	13-678-11E
Pipette Aids, Drummond	Fisher	13-681-19
Centrifuge, Allegra 6R	Beckman Rotor Gh3.8A	ALR6
MicroPipette (200µl)	Eppendorf	21-371-5
Pipette Tips 1-200µL	VWR	53508-783
Biological Safety Cabinet	Forma Scientific	Model 1286

Reagents::	Vendor:	Catalog number:
Whole blood	Subject source	
Frozen blood sample	Laboratory source	
Ficoll, Isoprep	Robbins Scientific	1070-01-0

PBS sterile, Ca^{2+}, Mg^{2+} free,	Cellgro	45000-436
Human T-cell $CD8^+$ subset column kit	R&D Systems	HCD8C-1000
Human T-cell $CD4^+$ subset column kit	R&D Systems	HCD4C-1000
Depletion cocktail for $CD3^+$ T-cells RosetteSep™	Stemcell Technologies	15661
Depletion cocktail for $CD4^+$ T-cells RosetteSep™	Stemcell Technologies	15662
Depletion cocktail for $CD8^+$ T-cells RosetteSep™	Stemcell Technologies	15663

2.6.2 Column separations

Leukocytes were isolated by standard density gradient separation (see above). Subsequently 2 x 10^6 cells in 1ml of sterile 1 x column buffer were mixed with 1ml of the respective monoclonal antibody cocktail, which was gently mixed and incubated at room temperature for 15 minutes. The cells were washed twice in 10ml 1x column buffer with centrifugation at 300 x g for 10 minutes and decanting of the supernatant in between these steps. The final cell pellet was resuspended in 2ml of the 1 x column buffer. Columns were placed in a column rack and the top cap removed first to avoid drawing of air into the bottom of the column. Afterwards the bottom cap was removed and the column fluid was allowed to drain into a waste receptacle. A 70% ethanol solution was used to rinse the outside tip of the column to ensure sterile cell processing. Subsequently the column was washed with 10ml of 1 x column buffer and the eluat drained again into a waste receptacle. Sterile 15ml conical centrifuge tubes were used to replace the waste receptacle and the prepared antibody treated cells were added to the column. After the solution was allowed to enter the column,

the now in the column suspend cells were incubated for 15 minutes at room temperature. 10ml of 1 x column buffer was used to elute the cells from the column after the incubation, making sure the eluat is collected until it appears clear. The within the eluat collected cells were centrifuged at 250 x g for 5 minutes and the supernatant was decanted. The cell pellet was resuspended in the appropriate buffer or culture medium and subsequently counted (see above).

The non selected T-cell subsets and B cells were bound to anti-Ig coated glass beads through F(ab)-surface immunoglobulin (Ig) interactions, while monocytes bind to Ig coated glass beads via the Fc receptor. The column eluat contained highly enriched T-cell subsets with only minimal amounts of non selected T-cell subsets, B cells or monocytes (see control by FACS).

2.6.3 Spin on cell separation

In order to isolate APC or as alternative method to obtain purified T-cell subsets from full blood samples by negative selection respective cell depletion cocktail (Stemcell Technologies) was used. 50µl of RosetteSep cocktail was added per ml of the whole blood sample and mixed well. Unwanted cells were cross linked to red blood cells (rosette) via the tetrameric antibody complex (TAC) reagent. After 20 minute incubation at room temperature the sample was diluted with an equal volume of PBS + 2% FBS and again mixed gently. The diluted sample was then layered on top of Ficoll or the Ficoll carefully layered underneath the diluted sample, while it was ensured to avoid mixing of the two layers. Subsequently the samples were centrifuged for 20 minutes at 1200 rpm at room temperature without brake. The enriched cells were removed from the Ficoll plasma interface and washed with PBS + 2 % FBS. This step was repeated once before the enriched cells were used.

2.7 Flow cytometric analysis of immunostained cells

2.7.1 Equipment and reagents

Equipment:	Vendor:	Catalog number:
MicroPipette (20µl)	Eppendorf	21-371-5
MicroPipette (200µl)	Eppendorf	21-371-5
MicroPipette (1000µl)	Gilson	K18347K
Pipette Tips1-200µl small orifice	VWR	53509-009
Pipette Tips 101-1000µl, blue	Fisher	21-197-8A
Refrigerator	Danby	Model DCR122W
Conical Tubes, 15ml	Falcon	14-959-70C
Conical tubes, 50ml	Falcon	Fisher 14-432-22

Reagents:	Vendor:	Catalog number:
Frozen blood sample	Laboratory source	
Anti-huCD3 FITC	Laboratory source	
Anti-huCD4$^+$ FITC	BD Biosciences	340133
Anti-huCD8$^+$ PE	BD Biosciences	340046
IgG_1FITC/IgG_1PE	BD Immunocytometry Systems	349526
PBS sterile, Ca^{2+}, Mg^{2+} free,	Cellgro	45000-436

$CD4^+$ and $CD8^+$ cells were obtained by negative selection passing PBMC though affinity columns (see above). The efficacy of enrichment was controlled by FACS analysis. APC were isolated from PBMC by negative selection using the T-cell Depletion Cocktail (see above). Flow cytometry was performed using a Becton Dickinson FACScan staining with labeled anti-$CD4^+$, anti-$CD8^+$, and anti-$CD3^+$

antibodies and proper isotype controls. The staining was performed as previously published (95, 109). At least 5,000-10,000 live cells were analyzed per sample. 100 µl of well-mixed sample was added to the bottom of a labeled tube and afterwards the appropriate primary antibody. After mixing well the samples were incubated in the dark at room temperature for 20-30 minutes. Subsequently the tubes were removed from dark chamber and each tube was mixed well. After incubation the samples were centrifuged for 3 minutes at 1,800 rpm, 4℃ with break on after adding 1ml of washing solution. The supernatant were decanted, palette mixed and 1ml washing solution was added to each tube centrifuged for 3 minutes at 1,800 rpm, 4℃ with break on. This was repeated two more times. For analysis on the same day 500µl washing solution were added to each tube, vortexed and analyzed within 8 hours. In cases were analyses had to be done the next day 2% formaldehyde buffer were added to each tube, vortexed and the sampled stored in the refrigerator at 2 to 8℃ for up to 36 hours.

2.7.2 Solution preparation (Appendix F)

Washing Solution: PBS + 0.1% sodium azide + 1% fetal bovine serum.

Formaldehyde buffer: 2% formaldehyde in PBS.

2.8 ^{3}H-Thymidine proliferation assay

2.8.1 Equipment and reagents

Equipment:	Vendor:	Catalog number:
MicroPipette (20µl)	Eppendorf	21-371-5
MicroPipette (200µl)	Eppendorf	21-371-5
Multichannel, 12 channel (50-300µl)	Labsystem	13-688-505
Pipette Tips1-200µl small orifice	VWR	53509-009
Conical tubes, 50ml	Fisher	14-432-22
96 well cell culture plate	Costar	3596
Printed Filtermat A	Wallac	1205-401
Sample Bag	Wallac	1205-411
Incubator	Forma Scientific	Model 320
Refrigerator	Danby	Model DCR122W
Cell harvester	Tomtec	
Beta Counter	Wallac	1205 Betaplate

Reagents:	Vendor:	Catalog number:
Glutamine	Gibco	25030-081
AB serum	Gemini Bioproducts	100-112
RPMI 1640	Bio-Whittaker	12-167Q
PBS sterile, Ca^{2+}, Mg^{2+} free	Cellgro	45000-432
[^{3}H]-Thymidine	ICN Biomedicals	012406005

The PBMC were stimulated under the same conditions and with the same antigens as in the ELISPOT assay in a separate 96 well flat bottom plate on the same day. Classical proliferation assay was performed as previously described (78). The cells were cultured for 5 days in an incubator at 37°C and 7.2 % CO_2. [^{3}H]-Thymidine was added to each well. 18 hours later, the plates were removed from the incubator wrapped in plastic foil and stored in the refrigerator for at leas 4 h before the cells where harvested and [^{3}H]-Thymidine incorporation was counted by liquid scintillation. The results are expressed as incorporation of radiolabel by the cells (arithmetic mean $\pm$ SD).

2.9 Peptides and antigens

2.9.1 Equipment and reagents

Equipment:	Vendor:	Catalog number:
MicroPipette (20µl)	Eppendorf	21-371-5
MicroPipette (200µl)	Eppendorf	21-371-5
Multichannel, 12 channel (50-300µl)	Labsystem	13-688-505
Pipette Tips1-200µl small orifice	VWR	53509-009
Conical tubes, 50ml	Fisher	14-432-22

Reagents:	Vendor:	Catalog number:
Glutamine	Gibco	25030-081
AB serum	Gemini Bioproducts	100-112
RPMI 1640	Bio-Whittaker	12-167Q
PBS sterile, Ca^{2+}, Mg^{2+} free	Cellgro	45000-432
PHA	Sigma	L9132
TT	Chiron Behring GmbH & Co	
PPD	Staten Serum Institute Copenhagen	

The HIV 8 to 9-mer peptides used (Interactiva, Ulm, Germany) were previously defined, class I-restricted epitopes with different HLA binding properties as described in detail in the HIV molecular Immunology Database (http://hiv-web.lanl.gov and see Table 1 and 2). These antigens were a generous gift of Dr. Bernhard Boehm. The 20-mer peptides with 10 aminoacid-overlaps spanning the entire gp120 sequence from HIV 1 substrain 89.6 were a generous gift of Dr. Samuel Landry (see Table 3

and 4 for details). Control wells contained medium with PBMC or effector cells alone. The peptide-concentration of 10µM was defined as optimal after titration in ELISPOT assays. As control antigen Tetanus Toxoid (TT), a generous gift of Chiron Behring GmbH & Co, was used at 25µg/ml or purified protein derivative of *mycobacterium tuberculosis* (PPD) was used at 5µg/ml. The mitogen control was phytohemagglutinin (PHA) at 10µg/ml final**.**

2.10 Safety measures

All work was performed under a biological safety cabinet observing biosafety regulations and sterile techniques whenever working with open containers. It was worked with sterile materials and solutions and universal precautions against blood borne pathogens were observed. Gloves and lab coats were worn at all times. Safe centrifuge techniques were practiced, opposite tubes or buckets were balanced within 0.1 g of each other. When working with liquid nitrogen cryo-gloves, a lab coat and a face shield were worn for protection.

3. Results

3.1 HLA-Class I restricted 8-10-mer HIV-peptides induce production of GzB and IFN-γ in PBMC of HIV infected donors

In the first set of experiments, PBMC of four healthy HIV negative donors and of nine HIV infected individuals were tested repeatedly. The latter were HIV antibody positive for more then two years, were clinically stable and had $CD4^+$ counts between 400 and 1070 cells/ml. All HIV infected individuals were on highly active antiretroviral therapy (HAART) at the time of testing. PBMC were tested directly ex vivo by challenging them for 24 hours with a library of HIV peptides that entailed 21 previously defined $CD8^+$ determinants (80, 81) of HIV proteins gp120, nef, p17, pol, gp41 and p24 (the peptides are specified in Table 1 and 2). For each tested peptide and patient, the secretion of GzB and of IFN-γ was measured in parallel. As shown in Table 1, seven of these nine HIV patients responded to at least one of the HIV peptides with GzB producing cells at a frequency larger than 5:100,000 PBMC. Three patients responded to 4, 8 and 10 peptides, respectively. By adding up the frequencies of cells that responded to individual peptides (each reflecting the clonal size of cells responding to the individual peptide), the cumulative clonal mass of HIV reactive GzB producing cells for each patient was calculated (Table 1). This cumulative clonal mass of GzB producing cells was 46, 6, 22, 146, 12, 14 and 224 for the seven of the nine HIV patients that tested positive for at least one peptide (using the > 5:100,000 cut off criterion for a positive result). In contrast, none of the four healthy controls responded to any of the peptides with > 5:100,000 GzB

producing cells (Table 1). Similar results were obtained when HIV-peptide-induced IFN-γ-production was measured in HIV infected subjects and in healthy control donors (Table 2). While therefore the numbers of positive $CD8^+$ peptides permitted to distinguish between infected and non-infected individuals, an even better distinction could be made calculating the total clonal mass of peptide-reactive GzB and IFN-γ-producing cells.

Table 1: Previously defined 8-10-mer CD8$^+$-epitopes-induce granzyme B production in PMBC of HIV patients but not in healthy controls

Peptides	HLA restriction	Sequence	HIV patients									Controls			
			A	B	C	D	E	F	G	H	I	J	K	L	M
gp120															
53-62	A24	LFCASDAKAY	-*	nd†	nd	-	**15**	-	-	**16**	-	-	-	-	-
312-320	B2705	GRAFVTIGK	-	nd	nd	-	-	-	-	**18**	-	-	-	-	-
nef															
72-82	B35, B51	PQVPLRPMTY	**12**	-	-	-	**7**	-	-	**15**	-	-	-	-	-
73-82	A11, A301	QVPLRPMTYK	-	-	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
86-94	A11, A301	DLSHFLKEK	-	-	-	**22**	**19**	-	-	-	-	-	-	-	-
136-145	A201	PLTFGWCYKL	-	nd	nd	-	**30**	-	**14**	**18**	-	-	-	-	-
180-189	A201	VLEWRFDSRL	-	nd	nd	-	**16**	**8**	-	**17**	-	-	-	-	-
186-194	A24	DSRLAFHHM	**17**	nd	nd	-	**7**	-	-	-	-	-	-	-	-
190-198	A2, B52	AFHHVAREL	**6**	nd	nd	-	**11**	-	-	**8**	-	-	-	-	-
p17															
18-26	A301	KIRLRPGGK	**11**	nd	nd	-	**41**	-	-	-	-	-	-	-	-
pol															
171-180	B8	GPKVKQWPL	-	nd	nd	-	-	-	-	**41**	-	-	-	-	-
325-333	A11, A301, A33	AIFQSSMTK	-	nd	nd	-	-	-	-	**26**	-	-	-	-	-
342-350	B35	HPDIVIYQY	-	-	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
346-354	A201	VIYQYMDDL	-	nd	nd	-	-	**6**	-	-	-	-	-	-	-
587-596	B35, B51	EPIVGAETFY	-	nd	nd	-	-	-	-	-	-	-	-	-	-
gp41															
591-598	A24	YLKDQQLL	-	nd	nd	-	-	-	-	**38**	-	-	-	-	-
751-759	B2705	GRRGWEALK	-	nd	nd	-	-	-	-	**27**	-	-	-	-	-
p24															
148-156	B7	SPRTLNNAWV	-	nd	nd	-	-	-	-	-	-	-	-	-	-
202-211	A25	ETINEEAAEW	-	-	**6**	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
259-267	B8	GEIYKRWII	-	-	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
260-268	B35	PPIPVGDIY	-	-	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Clonal mass		**46**	**0**	**6**	**22**	**146**	**12**	**14**	**224**	**0**	**0**	**0**	**0**	**0**
	Mean of induced spots		**52**									**0**			

* ≤5 SFC/ 10^5 cells per well, † not done

Table 2: Previously defined 8-10-mer $CD8^+$-epitopes-induce IFN-γ production in PMBC of HIV patients but not in healthy controls

Peptides	HLA restriction	Sequence	HIV patients									Controls			
			A	B	C	D	E	F	G	H	I	J	K	L	M
gp120															
53-62	A24	LFCASDAKAY	-*	nd†	nd	**6**	-	-	-	-	-	-	-	-	-
312-320	B2705	GRAFVTIGK	-	nd	nd	-	-	-	-	**8**	-	-	-	-	-
nef															
72-83	B35, B51	PQVPLRPMTY	**6**	-	-	**6**	**41**	-	-	**48**	-	-	-	-	-
73-82	A11, A301	QVPLRPMTYK	**47**	**12**	**8**	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
86-94	A11, A301	DLSHFLKEK	**27**	-	**18**	-	**8**	-	-	-	-	-	-	-	-
136-145	A201	PLTFGWCYKL	**7**	nd	nd	**26**	**21**	-	-	-	-	-	-	-	-
180-189	A201	VLEWRFDSRL	-	nd	nd	-	**7**	-	-	-	-	-	-	-	-
186-194	A24	DSRLAFHHM	-	nd	nd	-	-	-	-	-	-	-	-	-	-
190-198	A2, B52	AFHHVAREL	**50**	nd	nd	-	-	-	-	-	-	-	-	-	-
p17															
18-26	A301	KIRLRPGGK	-	nd	nd	-	-	-	-	-	-	-	-	-	-
pol															
171-180	B8	GPKVKQWPL	**7**	nd	nd	-	-	**24**	-	-	-	-	-	-	-
325-333	A11, A301, A33	AIFQSSMTK	-	nd	nd	-	-	-	-	-	-	-	-	-	-
342-350	B35	HPDIVIYQY	-	**8**	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
346-354	A201	VIYQYMDDL	-	nd	nd	-	-	-	-	-	-	-	-	-	-
587-596	B35, B51	EPIVGAETFY	-	nd	nd	-	**21**	**6**	-	-	-	-	-	-	-
gp41															
591-598	A24	YLKDQQLL	**33**	nd	nd	-	-	-	-	-	-	-	-	-	-
751-759	B2705	GRRGWEALK	-	nd	nd	-	-	-	**6**	-	-	-	-	-	-
p24															
148-156	B7	SPRTLNNAWV	-	nd	nd	-	-	-	-	-	-	-	-	-	-
202-211	A25	ETINEEAAEW	**25**	**8**	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
259-267	B8	GEIYKRWII	**13**	-	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
260-268	B35	PPIPVGDIY	-	**52**	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Clonal mass		**215**	**88**	**26**	**45**	**91**	**30**	**6**	**56**	**0**	**0**	**0**	**0**	**0**
	Mean of induced spots		**62**									**0**			

* ≤5 SFC/ 10^5 cells per well, † not done

3.2 Systematic 20-mer gp120 peptide library induces GzB and IFN-γ secretion in PMBC of HIV patients but not in healthy controls

In a second set of experiments seven HIV infected and four control individuals were tested for responses to an overlapping series of gp120 peptides (47 peptides of 20 amino acid length, progressing through the sequence in increments of 10 amino acids). The data for each individual peptide are shown in four representative patients in Figures 4-7, the data for all donors are summarized in Table 3 for GzB and in Table 4 for IFN-γ. Like the 4 patients shown in Figures 4-7, all seven HIV infected individuals responded with GzB producing cells > 5:100,000. The HIV patients displayed responses to at least 3 different peptides and up to 16 different peptides (Table 3 and Figure 4 A). This cumulative clonal mass of GzB producing cells was 335, 1273, 1899, 337, 181, 113 and 66 (Table 3 and Figure 8). Out of the four healthy individuals two did not display any reactivity to the peptides tested, while two did display minor reactivity (Table 3 and Figure 8). These two control subjects (K & M) were health care workers who might have been environmentally sensitized to HIV (12, 33, 135, 189). They responded to few peptides (4 and 2, respectively) with low frequency GzB producing cells (< 46/100,000). The HIV infected individual's cumulative response (with the exception of patient I) where 2.5 to 41 times the mass of the highest responding control (control K) as shown in Figure 8. Overall, therefore, clear-cut GzB reactivity to HIV peptides were seen only in HIV infected individuals**.**

IFN-γ measurements done in parallel gave similar results (Figures 4-7, and Table 4). The HIV peptides did not elicit IFN-γ responses in non-infected controls including the health care workers who responded weakly with GzB release (Table 4 and Figure 8).

The frequencies of the IFN-γ producing cells elicited by individual peptides were in the < 62/100,000 range, comparable to the frequencies of peptide-induced GzB producing cells.

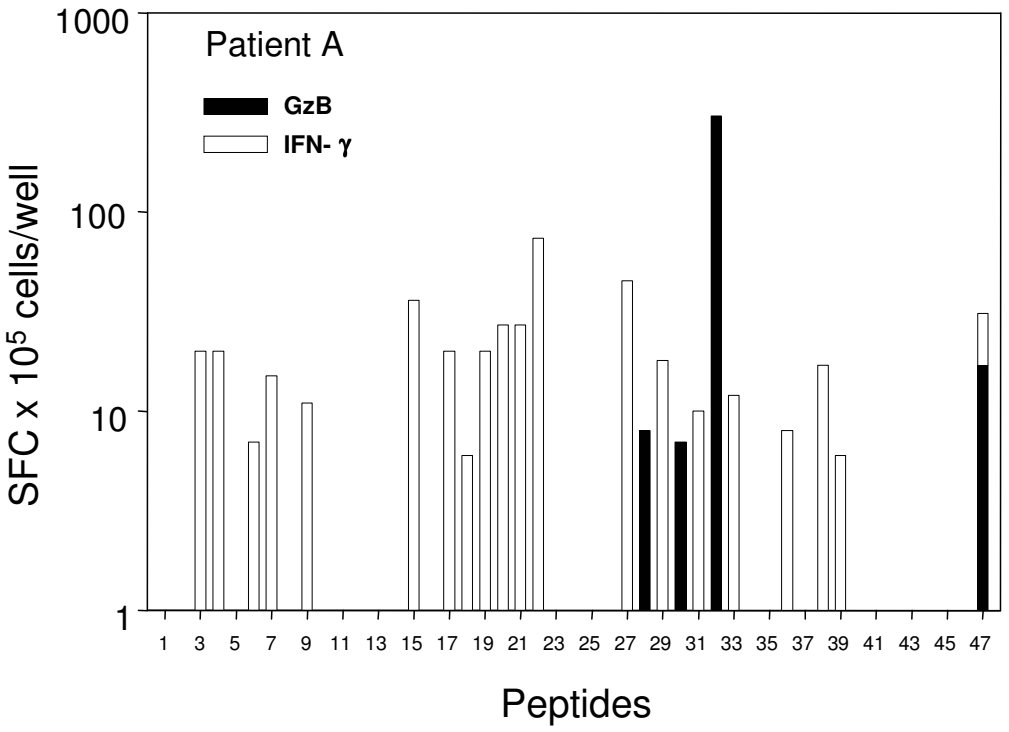

Figure 4. The GzB and IFN-γ responses of Patient "A" towards the 47 overlapping 20-mer peptides of the entire HIV gp120 protein. The data for GzB and IFN-γ are represented in closed and open bars, respectively. The results are expressed as the number of spot forming cells (SFC) induced by each peptide in 10^5 cells/well with the medium background subtracted.

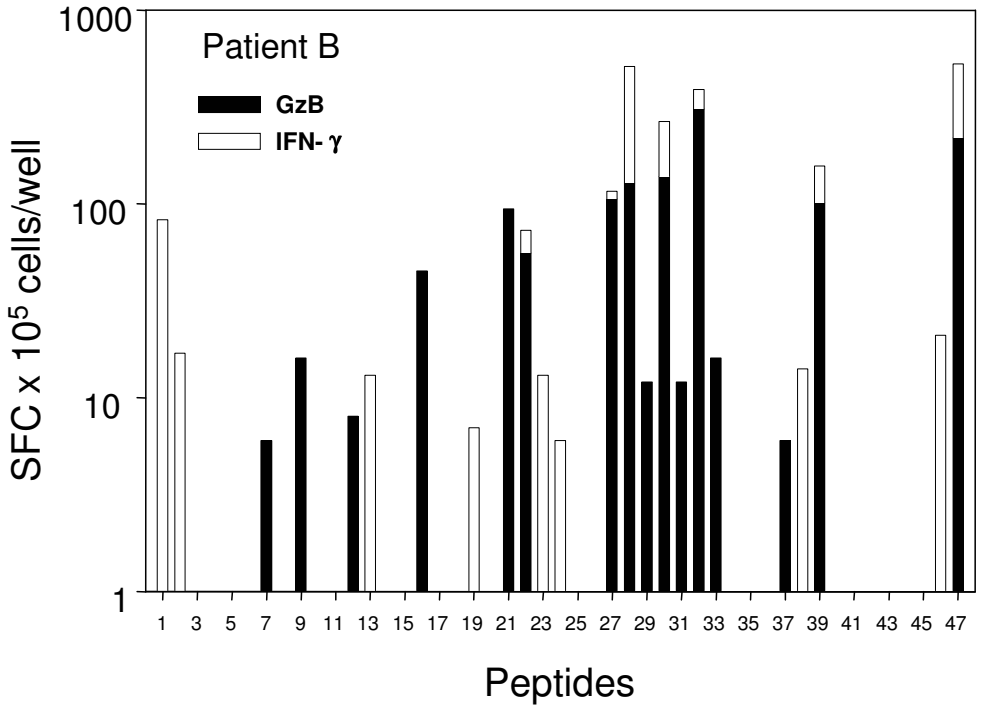

Figure 5. The GzB and IFN-γ responses of Patient "B" towards the 47 overlapping 20-mer peptides of the entire HIV gp120 protein. The data for GzB and IFN-γ are represented in closed and open bars, respectively. The results are expressed as the number of spot forming cells (SFC) induced by each peptide in 10^5 cells/well with the medium background subtracted.

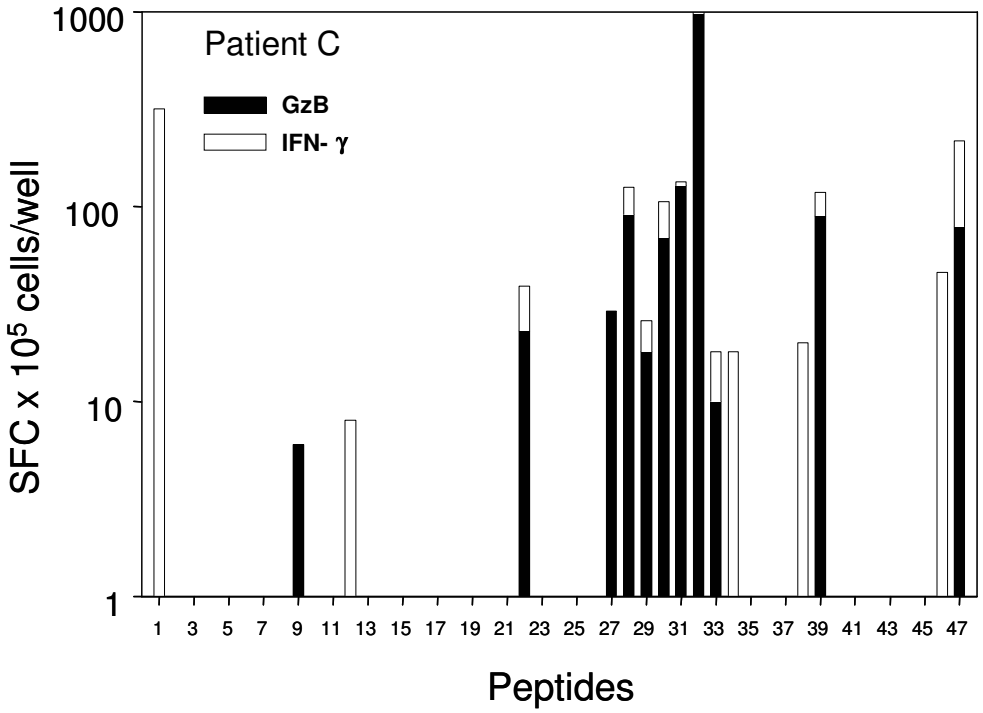

Figure 6. The GzB and IFN-γ responses of Patient "C" towards the 47 overlapping 20-mer peptides of the entire HIV gp120 protein. The data for GzB and IFN-γ are represented in closed and open bars, respectively. The results are expressed as the number of spot forming cells (SFC) induced by each peptide in 10^5 cells/well with the medium background subtracted.

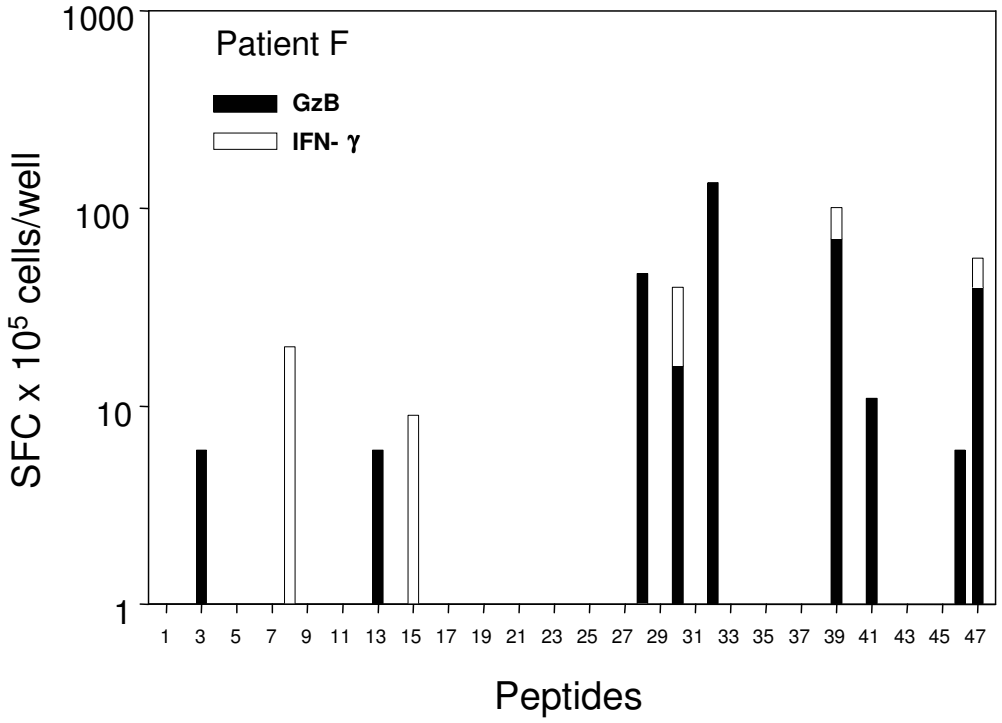

Figure 7. The GzB and IFN-γ responses of Patient "F" towards the 47 overlapping 20-mer peptides of the entire HIV gp120 protein. The data for GzB and IFN-γ are represented in closed and open bars, respectively. The results are expressed as the number of spot forming cells (SFC) induced by each peptide in 10^5 cells/well with the medium background subtracted.

Table 3: Granzyme B induced in PBMC by gp120 peptide series

20mers	Peptides	HIV patients							Controls			
		A	B	C	F	G	H	I	J	K	L	M
(030-049)	01	-*	-	-	-	-	-	-	-	-	-	-
(040-059)	02	-	-	-	-	-	-	-	-	-	-	-
(050-069)	03	-	-	-	6	12	-	-	-	-	-	-
(060-079)	04	-	-	-	-	-	-	-	-	-	-	-
(070-089)	05	-	-	-	-	-	-	-	-	-	-	-
(080-099)	06	-	-	-	-	-	-	-	-	-	-	-
(090-109)	07	-	6	-	-	-	-	-	-	-	-	-
(100-119)	08	-	-	-	-	-	-	-	-	-	-	-
(110-129)	09	-	16	6	-	7	-	-	-	-	-	-
(120-139)	10	-	-	-	-	-	-	-	-	-	-	-
(130-149)	11	-	-	-	-	-	-	-	-	19	-	-
(140-159)	12	-	8	-	-	-	-	-	-	-	-	-
(150-169)	13	-	-	-	6	-	-	-	-	-	-	-
(160-179)	14	-	-	-	-	-	-	-	-	-	-	-
(170-189)	15	-	-	-	-	-	-	-	-	-	-	-
(180-199)	16	-	45	-	-	-	-	-	-	-	-	-
(190-209)	17	-	-	-	-	-	-	-	-	9	-	-
(200-219)	18	-	-	-	-	-	-	-	-	-	-	-
(210-229)	19	-	-	-	-	6	-	-	-	-	-	-
(220-239)	20	-	-	-	-	8	-	-	-	-	-	-
(230-249)	21	-	94	-	-	7	-	-	-	-	-	-
(240-259)	22	-	56	23	-	6	-	-	-	-	-	13
(250-269)	23	-	-	-	-	6	-	15	-	6	-	-
(260-279)	24	-	-	-	-	-	-	-	-	-	-	-
(270-289)	25	-	-	-	-	7	-	-	-	-	-	-
(280-299)	26	-	-	-	-	15	-	6	-	-	-	-
(290-309)	27	-	106	29	-	-	-	-	-	-	-	-
(300-319)	28	8	128	91	47	6	-	12	-	-	-	-
(310-329)	29	-	12	18	-	8	-	-	-	-	-	-
(320-339)	30	7	138	69	16	-	13	-	-	-	-	11
(330-349)	31	-	12	128	-	-	-	-	-	-	-	-
(340-359)	32	303	309	1357	135	48	18	-	-	-	-	-
(350-369)	33	-	16	10	-	-	-	-	-	-	-	-
(360-379)	34	-	-	-	-	6	-	-	-	-	-	-
(370-389)	35	-	-	-	-	-	-	-	-	-	-	-
(380-399)	36	-	-	-	-	-	-	-	-	-	-	-
(390-409)	37	-	6	-	-	-	-	-	-	12	-	-
(400-419)	38	-	-	-	-	-	-	-	-	-	-	-
(410-429)	39	-	101	89	70	18	-	-	-	-	-	-
(420-439)	40	-	-	-	-	-	-	-	-	-	-	-
(430-449)	41	-	-	-	11	-	-	-	-	-	-	-
(440-459)	42	-	-	-	-	-	-	-	-	-	-	-
(450-469)	43	-	-	-	-	7	-	-	-	-	-	-
(460-479)	44	-	-	-	-	-	-	-	-	-	-	-
(470-489)	45	-	-	-	-	-	-	-	-	-	-	-
(480-499)	46	-	-	-	6	-	-	-	-	-	-	-
(490-508)	47	17	220	79	40	14	82	33	-	-	-	7
Clonal mass		335	1273	1899	337	181	113	66	0	46	0	24
Mean of induced spots					601					18		

* ≤5 SFC/ 10^5 cells

Table 4: Interferon-γ induced in PBMC by gp120 peptide series

20mers	Peptides	HIV patients							Controls			
		A	B	C	F	G	H	I	J	K	L	M
(030-049)	01	-*	83	317	-	-	-	-	-	-	-	-
(040-059)	02	-	17	-	-	-	-	-	-	-	-	-
(050-069)	03	20	-	-	-	-	-	-	-	-	-	-
(060-079)	04	20	-	-	-	-	-	-	-	-	-	-
(070-089)	05	-	-	-	-	-	-	-	-	-	-	-
(080-099)	06	7	-	-	-	-	-	-	-	-	-	-
(090-109)	07	15	-	-	-	-	-	-	-	-	-	-
(100-119)	08	-	-	-	20	-	-	-	-	-	-	-
(110-129)	09	11	-	-	-	-	-	-	-	-	-	-
(120-139)	10	-	-	-	-	-	-	-	-	-	-	-
(130-149)	11	-	-	-	-	-	-	-	-	-	-	-
(140-159)	12	-	-	8	-	-	-	-	-	-	-	-
(150-169)	13	-	13	-	-	-	-	-	-	-	-	-
(160-179)	14	-	-	-	-	-	-	-	-	-	-	-
(170-189)	15	36	-	-	9	-	-	-	-	-	-	-
(180-199)	16	-	-	-	-	-	-	-	-	-	-	-
(190-209)	17	20	-	-	-	-	-	-	-	-	-	-
(200-219)	18	6	-	-	-	-	39	-	-	-	-	-
(210-229)	19	20	7	-	-	-	-	-	-	-	-	-
(220-239)	20	27	-	-	-	-	-	-	-	-	-	-
(230-249)	21	27	-	-	-	-	-	-	-	-	-	-
(240-259)	22	74	17	16	-	-	-	-	-	-	-	-
(250-269)	23	-	13	-	-	-	-	-	-	-	-	-
(260-279)	24	-	6	-	-	-	-	-	-	-	-	-
(270-289)	25	-	-	-	-	6	-	-	-	-	-	-
(280-299)	26	-	-	-	-	-	-	-	-	-	-	-
(290-309)	27	45	10	-	-	-	-	-	-	-	-	-
(300-319)	28	-	385	34	-	15	10	-	-	-	-	-
(310-329)	29	18	-	8	-	-	-	-	-	-	-	-
(320-339)	30	-	128	37	24	-	21	7	-	-	-	-
(330-349)	31	10	-	6	-	-	-	-	-	-	-	-
(340-359)	32	-	80	107	-	22	12	-	-	-	-	-
(350-369)	33	12	-	8	-	-	-	-	-	-	-	-
(360-379)	34	-	-	18	-	-	-	-	-	-	-	-
(370-389)	35	-	-	-	-	-	-	-	-	-	-	-
(380-399)	36	8	-	-	-	-	-	-	-	-	-	-
(390-409)	37	-	-	-	-	-	-	-	-	-	-	-
(400-419)	38	17	14	20	-	-	-	-	-	-	-	-
(410-429)	39	6	56	29	31	19	8	-	-	-	-	-
(420-439)	40	-	-	-	-	-	-	-	-	-	-	-
(430-449)	41	-	-	-	-	-	-	6	-	-	-	-
(440-459)	42	-	-	-	-	-	-	-	-	-	-	-
(450-469)	43	-	-	-	-	-	-	-	-	-	-	-
(460-479)	44	-	-	-	-	-	-	-	-	-	-	-
(470-489)	45	-	-	-	-	-	-	-	-	-	-	-
(480-499)	46	-	21	46	-	-	7	-	-	-	-	-
(490-508)	47	14	307	138	16	18	36	-	-	-	-	-
Clonal mass		413	1157	792	100	74	133	13	0	0	0	0
Mean of induced spots		383							0			

* ≤5 SFC/ 10^5 cells

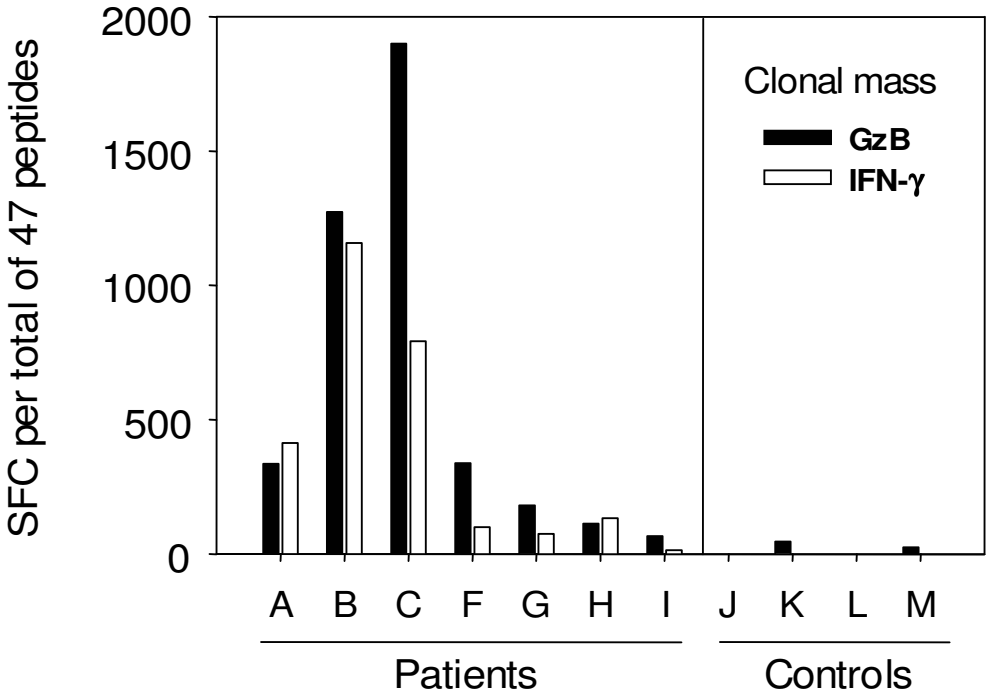

Figure 8. The clonal mass of the overall response from patients towards the 47 overlapping 20-mer peptides of the entire HIV gp120 protein allows an assessment of the total immune response mounted towards gp120. The peptide-induced GzB production (closed bars) and IFN-γ response (open bars) is shown for all seven patients in comparison to the four controls.

3.3 GzB induced by the HIV gp120 peptides is derived from $CD8^+$ cells

Peptides of 8-10 amino acid length are of ideal length for binding to HLA Class I molecules, and are too short for binding to HLA-Class II molecules (47). The here used peptides have been functionally characterized as $CD8^+$ cell determinants in the HIV Molecular Immunology Database (http://hiv-web.lanl.gov) and in different publications (80, 81). In contrast, 15-20-mer peptides are of ideal length for Class II binding and for eliciting $CD4^+$ cells (47, 53, 148). However, such longer peptides can activate $CD8^+$ cells as well, because they frequently degrade to shorter peptides during storage, handling or incubation (29, 72). In order to determine to what extent $CD8^+$ cell activity is elicited by the 20-mer peptides, T-cell subsets were purified and tested. Such separations typically lead to < 95% pure $CD8^+$ populations in HIV patients as well as healthy controls (Figure 9 and 10). Despite the often impaired $CD4^+$ numbers in HIV patients even during HAART therapy < 85% pure $CD4^+$ cell populations could usually be achieved (Figure 9), whereas in healthy controls the resulting purities were about 95% (Figure 10). In order to provide autologous antigen presenting cells (APC), $CD3^+$ cell depletions were done in parallel which resulted in cell fractions void of $CD4^+$ or $CD8^+$ cells (Figure 11). The purified $CD4^+$ and $CD8^+$ cells were tested on these T-cell depleted autologous APC for reactivity to the 20-mer peptides; in parallel, to the unpurified PBMC. A representative example of such experiment is shown in Figure 12. Peptides 8, 30, and 47 (37 being a negative control) that induced GzB secretion in the unseperated PBMC of the HIV positive donor shown also induced GzB release in the purified $CD8^+$ cell fraction, but not in the $CD4^+$ cells (Figure 12 A) confirming that GzB producing cells reside

exclusively $CD8^+$ fraction. In contrast, 20-mer peptide-induced IFN-γ production was recovered from $CD4^+$ and $CD8^+$ cells (Figure 12 B).

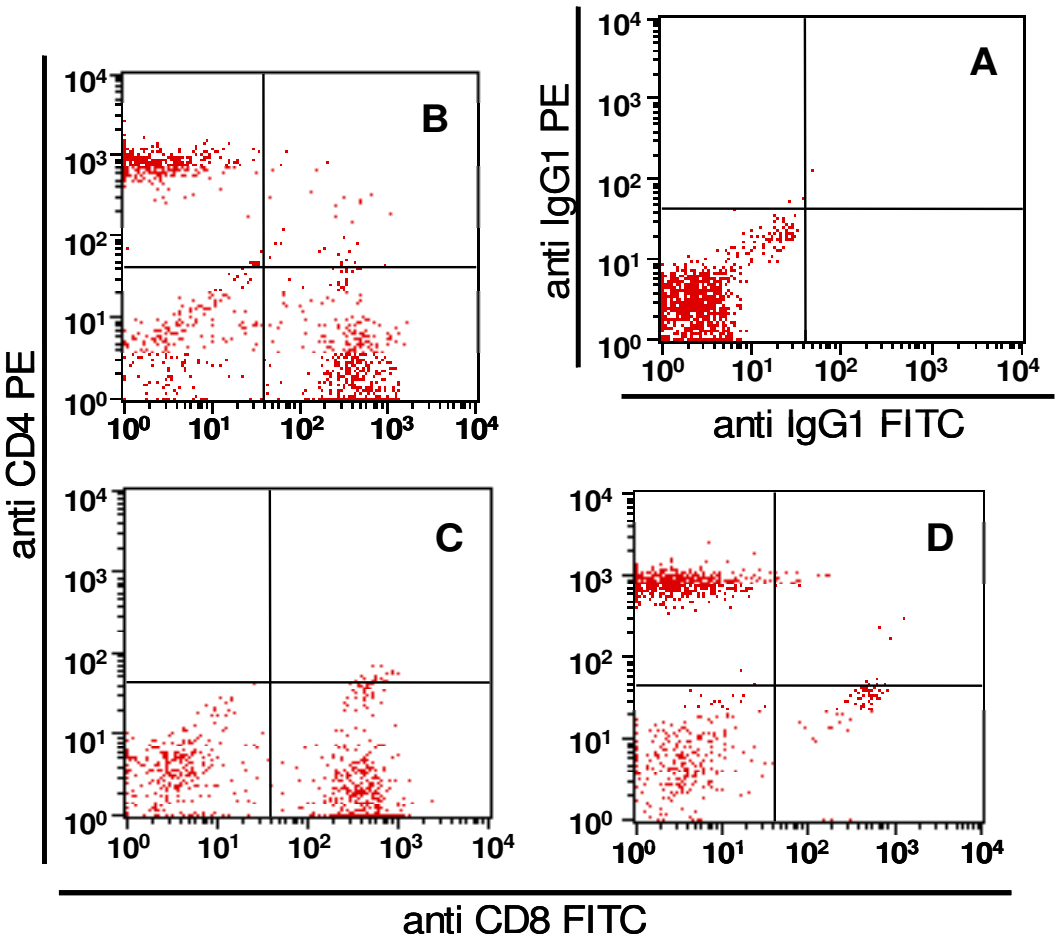

Figure 9. Representative results of the separation experiments for a HIV patient. The $CD8^+$ fraction is depicted on the X-axis and the CD4+ on the Y-axis. With panel **A** being the negative control and panel **B** the whole blood sample Panel **C** shows the result of the $CD8^+$ separation that lead to a 96% pure $CD8^+$ population while panel **D** is representing the $CD4^+$ separation that yielded a 87% pure $CD4^+$ population.

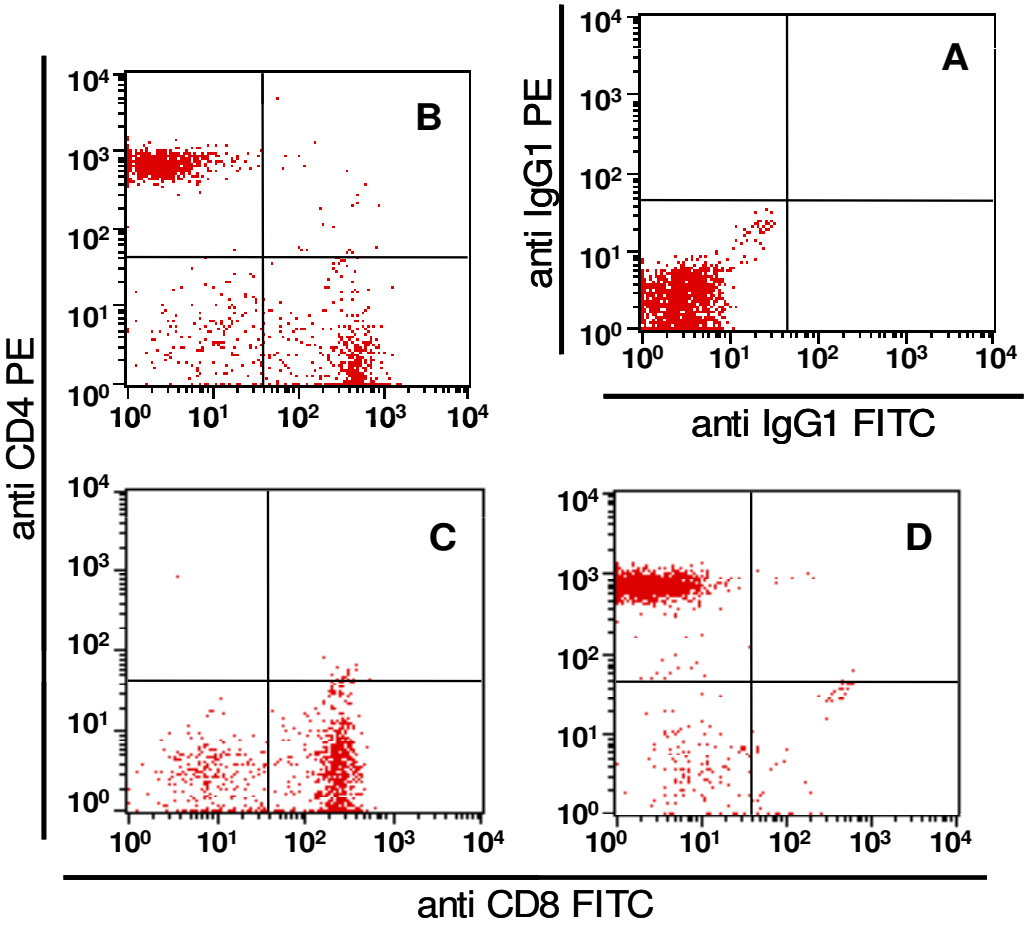

Figure 10. Representative results of the separation experiments for one of the healthy controls. The $CD8^+$ fraction is depicted on the X-axis and the $CD4^+$ on the Y-axis. With panel **A** being the isotype control and panel **B** the whole blood sample Panel **C** shows the result of the $CD8^+$ separation that lead to a 96% pure $CD8^+$ population while panel **D** is representing the $CD4^+$ separation that yielded a 94% pure $CD4^+$ population.

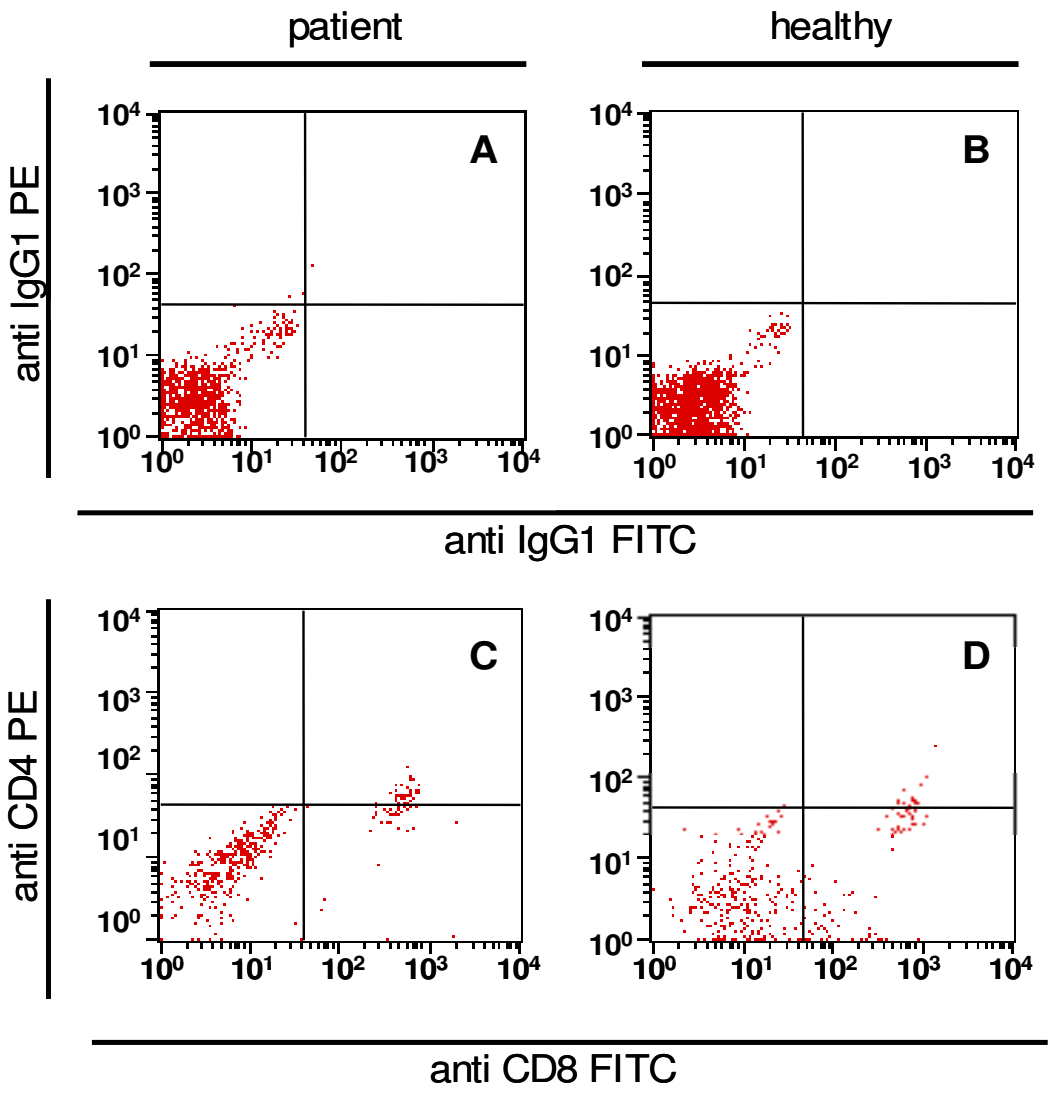

Figure 11. Representative results of the $CD3^+$ depletion experiments for a HIV patient and a healthy control. The $CD8^+$ fraction is depicted on the X-axis and the CD4+ on the Y-axis. With panel **A** being the isotype control for the patient and panel **B** the one for the control. Panel **C** shows the result of the $CD3^+$ depletion for the patient while panel **D** is representing depletion for the control, that both yielded a clean $CD8^-/CD4^-$-APC population.

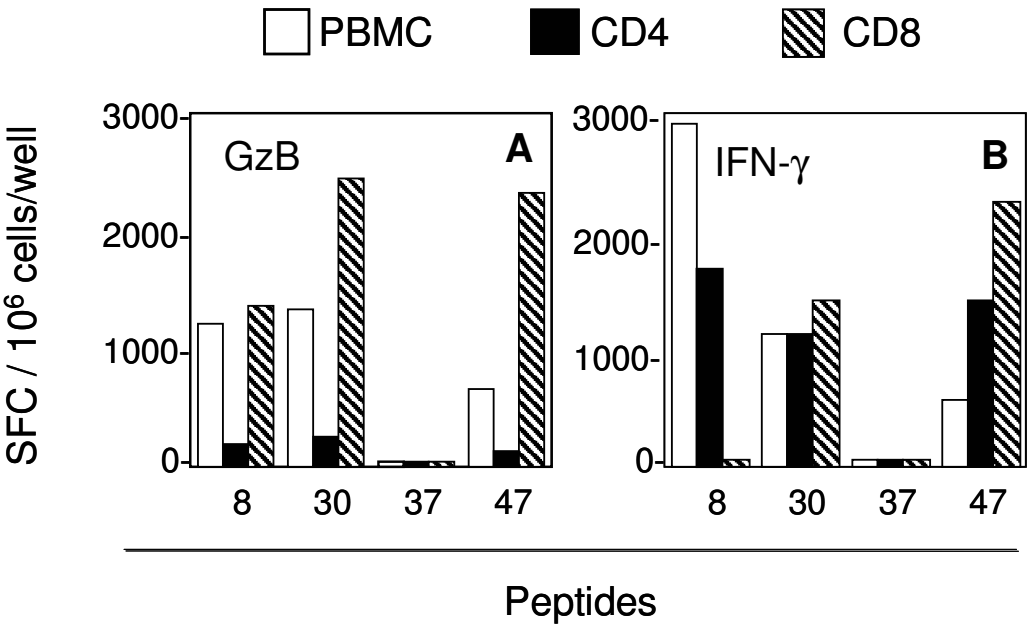

Figure 12. GzB producing cells reside exclusively $CD8^+$ fraction (**A**), while IFN-γ producing cells exist in both, the $CD4^+$ and $CD8^+$ cell compartment (**B**). Either unseparated PBMC or enrichment column purified T cell fractions where tested. Representative peptides of the gp120 molecule from HIV-1 substrain p896 were chosen, that were positive eliciting GzB and IFN-γ. PBMC are depicted in open bars, $CD4^+$ cells in closed bars and $CD8^+$ in dashed bars.

3.4 HIV specific $CD8^+$ cells occur in different subpopulations with respect to GzB and IFN-γ secretion

Canonical Tc1 cells capable of killing via the GzB/perforin pathway should also express IFN-γ. Approximately 18% (17.8%) of the 8-9-mer peptides that induced GzB production also triggered IFN-γ (Table 5). Among the 20-mer peptides that triggered $CD8^+$ cells to produce GzB, ~27% elicited IFN-γ as well (Table 5). A representative example for peptide induced coexpression of GzB and IFN-γ is shown for unpurified PBMC in Figure 13, and for $CD8^+$ cells in Figure 14. Strikingly however, frequently complete dissociation of GzB and IFN-γ was seen, as illustrated in Panels A and B of Figures 13 and 14 for unpurified and purified $CD8^+$ cells, respectively. Overall, ~ 40% of the peptide-induced GzB-producing cells did not secrete detectable IFN-γ and about the same number of IFN-γ producing cells did not secrete GzB (Table 5). Some of the peptides elicited GzB producing cells in the frequency range higher 1000/100,000 while triggering about 100/100,000 IFN-γ producing cells (e.g. Figures 4 -7, peptide 32). Because these measurements were done at single cell resolution (66), the polarization could reach values as high as ten fold.

Because the IFN-γ negative $CD8^+$ cells that are capable of GzB secretion could represent Tc2 cells (that secrete type 2 cytokines), we tested whether they would produce IL-4 or IL-5. Single cell resolution ELISPOT assays were performed to measure production of the latter cytokines. Unlike in mitogen (PHA)-stimulated control cultures, no IL-4 or IL-5 was triggered by peptides that elicited GzB

production (Figure 15 vs. Figure 12). The peptides that elicited GzB or IFN-γ production did not induce detectable proliferative responses (Figure 16). The different GzB/IFN-γ phenotypes of the peptide-reactive cells were found to be rather stable when retesting the patients 5 weeks later (Figure 17).

Table 5: Summary of the three different types of responses

	Number of positive peptides					
	HLA Class I restricted 9-mers			gp120 20-mers		
Patient	**IFN-γ**	**GzB**	**IFN-γ+GzB**	**IFN-γ**	**GzB**	**IFN-γ+GzB**
A	06	02	02	19	03	01
B	04	00	00	08	09	07
C	02	01	00	04	01	09
D	03	01	00	nd*	nd	nd
E	01	04	04	nd	nd	nd
F	02	01	00	02	06	03
G	01	01	00	00	10	05
H	00	08	02	05	01	02
I	00	00	00	02	04	00
∑	**19**	**18**	**08**	**40**	**34**	**27**
%	**42.2**	**40.0**	**17.8**	**40.2**	**33.3**	**26.5**
Control	**IFN-γ**	**GzB**	**IFN-γ+GzB**	**IFN-γ**	**GzB**	**IFN-γ+GzB**
J	00	00	00	00	00	00
K	00	00	00	00	04	00
L	00	00	00	00	00	00
M	00	00	00	00	02	00
∑	**00**	**00**	**00**	**00**	**06**	**00**

* not done

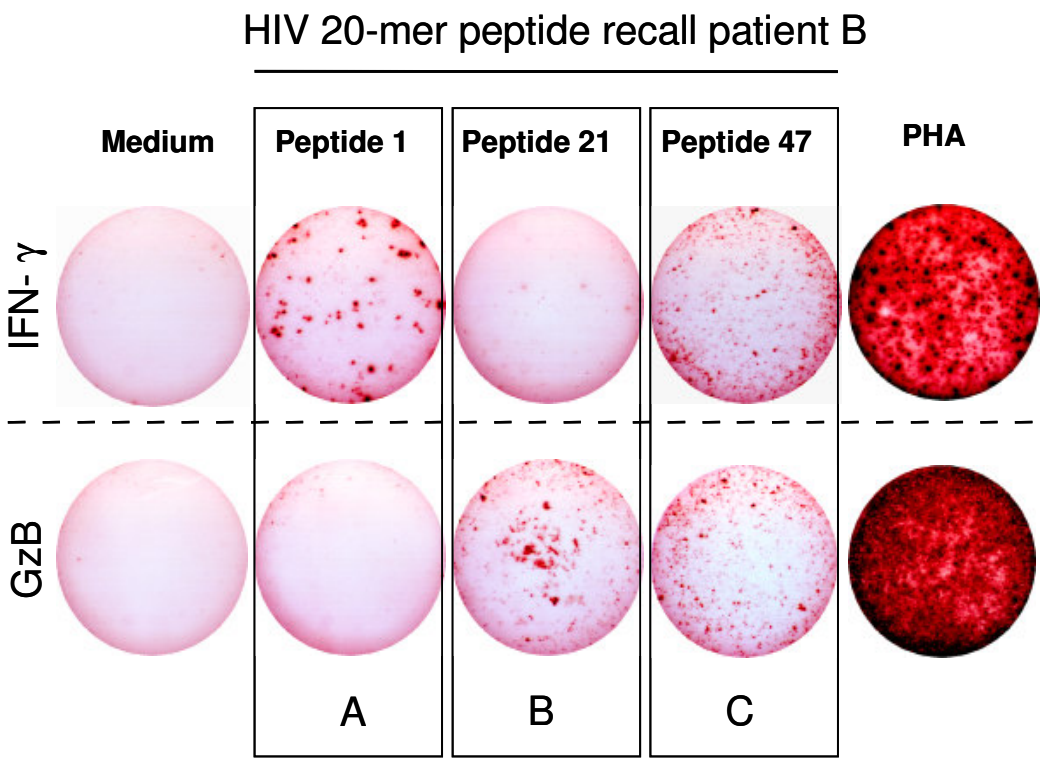

Figure 13. Within an individual patient, IFN-γ and GzB production in response to specific 20-mer peptides can occur in an associated or dissociated fashion. Peptides trigger either an IFN-γ response alone (**A**), only a GzB response (**B**) or can induce IFN-γ as well as GzB production (**C**). Depicted is the representative response of patient B to peptides 1, 21 and 47 from HIV-1 substrain p896. The negative control (medium) and mitogen control (PHA) are shown as reference.

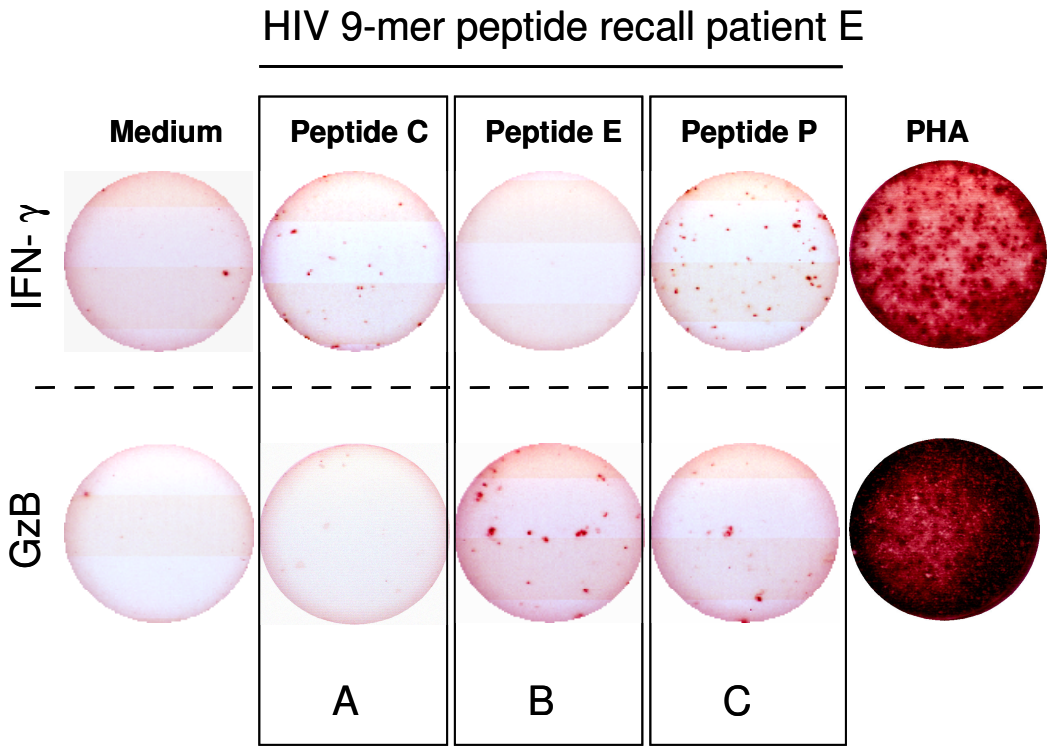

Figure 14. IFN-γ and GzB production of purified $CD8^+$ cells in response to single 9-mer peptides can occur in an associated or dissociated fashion within an individual patient. Peptides induce either only an IFN-γ response (**A**), a GzB response alone (**B**) or can trigger IFN-γ as well as GzB production at the same time (**C**). Depicted is the representative response of patient E to peptides C, E and P from HIV-1. The negative control (medium) as well as mitogen control (PHA) are shown as reference.

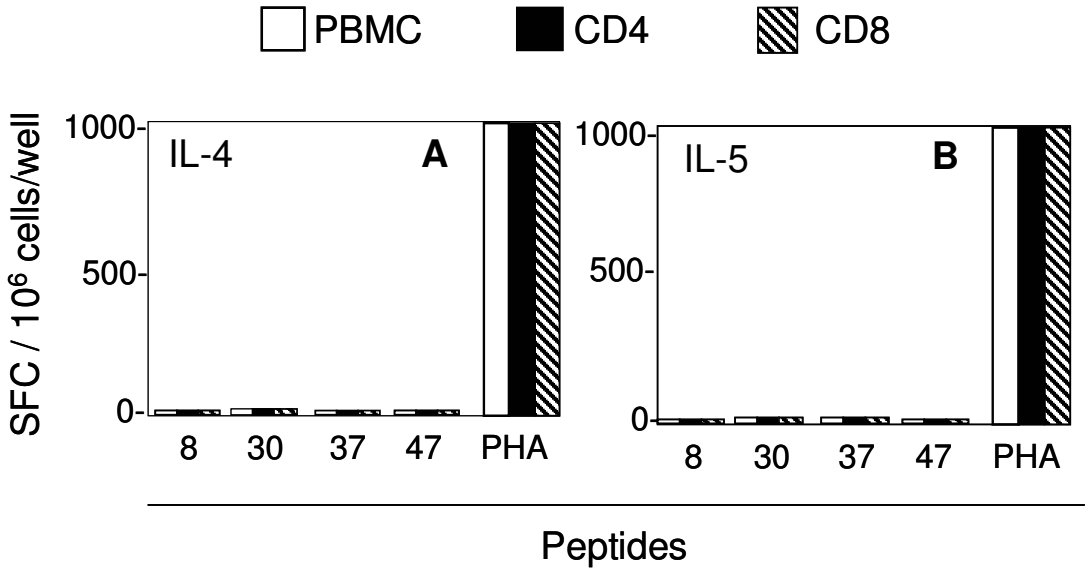

Figure 15. GzB and IFN-γ producing cells do not secrete IL-4 (**A**) or IL-5 (**B**). Either unseparated PBMC or column purified T cell fractions were tested. Representative peptides of the gp120 molecule from HIV-1 substrain p896 were chosen, that were double positive in eliciting a GzB and IFN-γ. PBMC are depicted in open bars, $CD4^+$ cells in closed bars and $CD8^+$ in dashed bars. The mitogen control (PHA) is shown as reference.

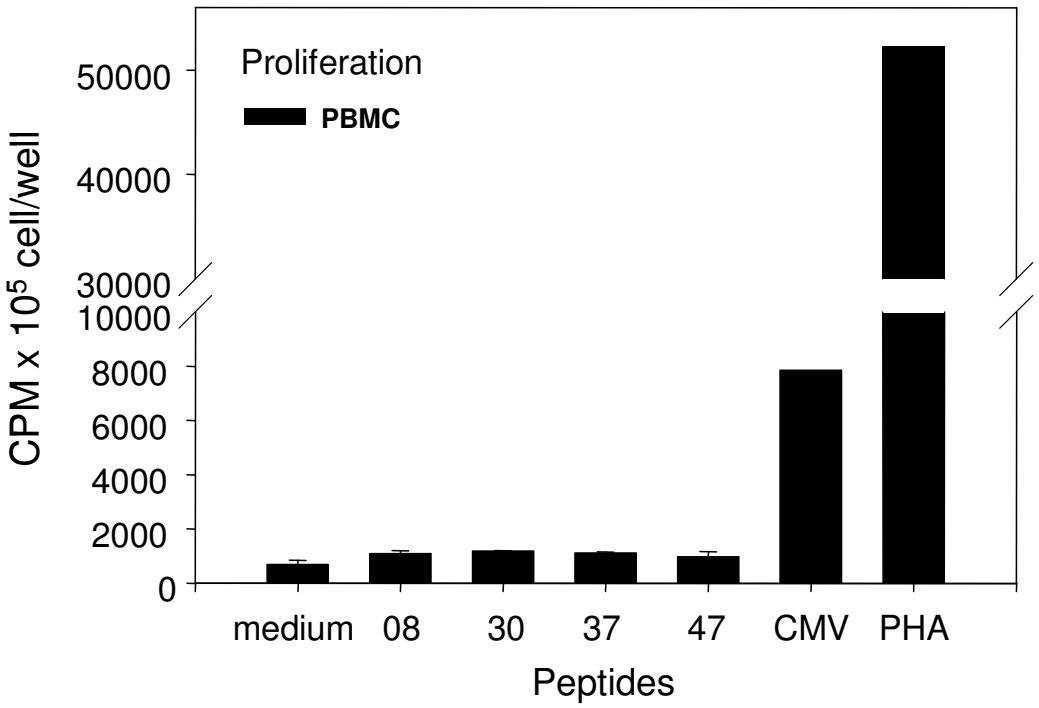

Figure 16. PBMC (closed bars) showed no proliferative response towards peptides that triggered GzB or IFN-γ production, while they were clearly able to respond to cytomegalovirus antigen (CMV) and mitogen (PHA). Peptides were derived from the gp120 molecule from HIV-1 substrain p896. Counts are shown in counts per minute (CPM) per 10^5 cells per well.

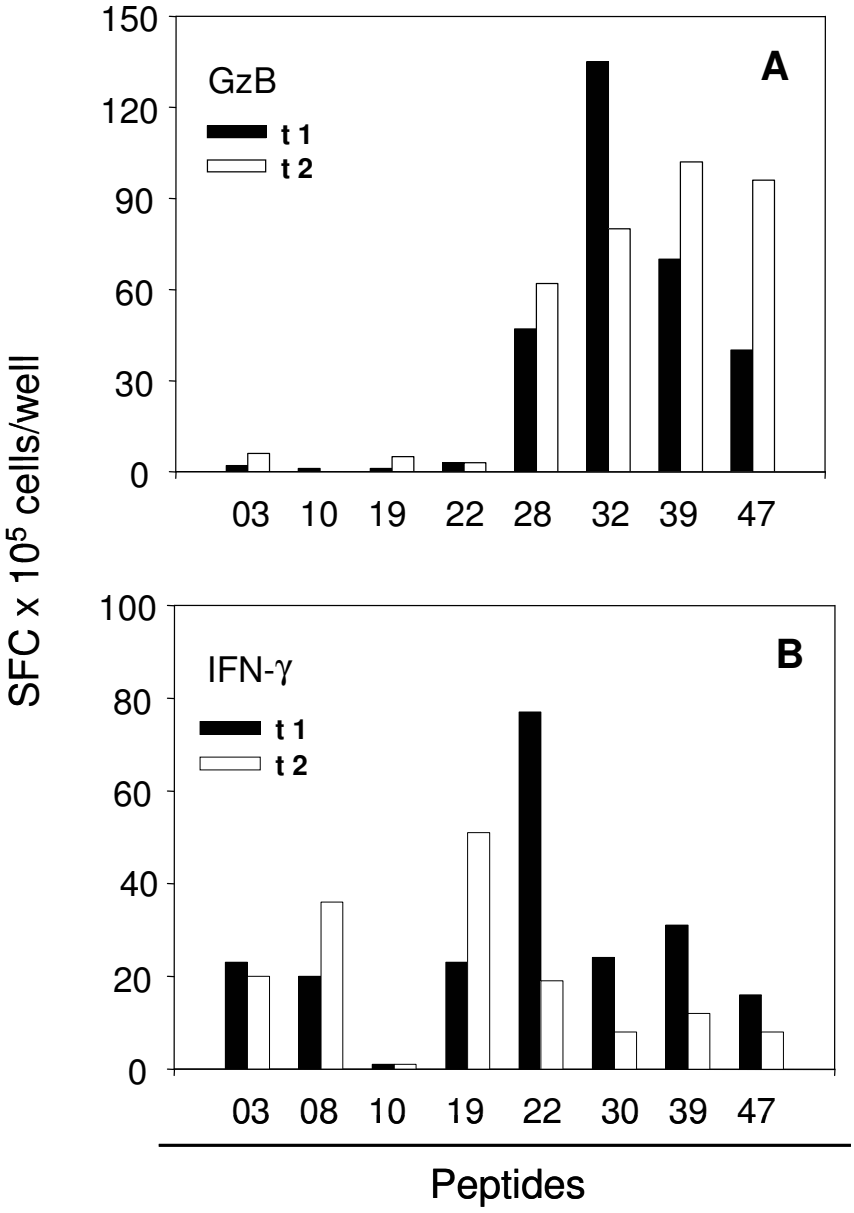

Figure 17. The production of GzB and IFN-γ in response to single 20-mer peptides from HIV-1 are reproducible features of the T-cell response. The peptide-induced GzB production (**A**) and IFN-γ response (**B**) is shown for time point 1 (closed bars) and the five weeks later time point 2 (open bars).

4 Discussion

Our data support the emerging evidence that a greater effector T-cell diversity exists than predicted by the type1/type 2 dichotomy (54, 60, 103). Frequently only one cytokine per T-cell is expressed, and even the expression of the produced cytokine underlies allelic exclusion (26, 71, 79, 79). The data presented here suggest that in the HIV response, IFN-γ production by $CD8^+$ cells is linked with the ability to produce GzB for no more then ~20% of the peptides recognized. Therefore, only ~20% of the $CD8^+$ cells in HIV correspond to the classic Tc1 double positive phenotype (60).

The experimental setting does not permit to conclude whether simultaneous GzB and IFN-γ production to these ~20% of the peptides represent individual $CD8^+$ cells coexpressing these molecules at the single cell level or reflects a population phenomenon with one subpopulation of $CD8^+$ cells producing GzB, and the other one producing GzB. Two color Elispot assays will permit to make this distinction. There is abundant evidence that $CD8^+$ cells coexpress these two molecules when studied by flow cytometry (174). It could be envisioned that GzB or IFN-γ single positive $CD8^+$ cells arise through instructed differentiation from double positive precursors (like for CD4 cells, Th1 and Th2 cells arise from Th0 precursor cells that coexpress Th1 and Th2 cytokines). While therefore our data does not clarify the very nature of the (apparently) double positive $CD8^+$ cells, they clearly show that in chronic HIV infection double positive cells seem to be more an exception than the rule. More importantly, our data clearly shows the existence of novel, single positive $CD8^+$ cell types.

We have identified two additional subpopulations that each occur at a higher frequency, constituting 30-40% of the HIV specific $CD8^+$ cell pool. One of these subpopulations secretes IFN-γ only. We propose to term this subpopulation Tc1b, as opposed to the classic Tc1 cell (Tc1a) (Table 6). The primary function of Tc1b cells might be to exert indirect effector functions by secreting IFN-γ and hereby attracting and activating macrophages to engage in local DTH reactions. Tc1b cells therefore are likely to operate similar to Th1 $CD4^+$ cells in being pro-inflammatory.

In contrast, the $CD8^+$ cell subpopulation that secretes GzB but no IFN-γ (Tc1c) might kill without inducing inflammation. Evidence for Tc1c mediated effector functions may have been observed in melanoma trials. Autoimmune, $CD8^+$-cell mediated destruction of normal melanocytes is a frequent side effect of this therapy, resulting in vitiligo-like depigmentation (99, 127). However, no sign of inflammation is seen in these lesions (106) and strikingly GzB and perforin was detected in T-cells in the vicinity of disappearing melanocytes (175).

Table 6: Effector cell diversity within Tc1 cells

	Secretory activity		Effector function	
$CD8^+$	**IFN-γ**	**GzB**	**Killing**	**Inflammation**
Tc1a	+	+	+	+
Tc1b	+	−	−	+
Tc1c	−	+	+	−

The $GzB^-/IFN\text{-}\gamma^+$ (Tc1b) and $GzB^+/IFN\text{-}\gamma^-$ (Tc1c) $CD8^+$ effector cell populations might represent separate effector cell lineages arising from instructed differentiation.

These two phenotypes can be selectively engaged *in vitro* when naïve TCR-tg $CD8^+$ cell are cultured in different cytokine microenvironments (162), and also by immunizing mice with MHC class I restricted peptides in different adjuvants (P.V. Lehmann, personal communication). It is unclear why within the $CD8^+$ cell response of individual HIV patients the $GzB^+/IFN\text{-}\gamma^-$ and the $GzB^-/IFN\text{-}\gamma^+$ phenotype is segregated to different peptides. One possibility is that the $CD8^+$ cells secreting $GzB^+/IFN\text{-}\gamma^-$ or $GzB^-/IFN\text{-}\gamma^+$ are primed in different microenvironments, for example in different tissues, or at different time points in the course of the disease, at stages when the net cytokine environment shifts. An additional explanation for the distinct phenotypes induced in response to challenge with different peptides, could be variation in signal strength during the TCR receptor engagement together with differential costimulatory receptor involvement (2, 118, 131).

It needs to be elucidated whether Tc1b or Tc1c cells are better suited to control HIV infection. While IFN-γ mediated inflammation is critical for host defense against many intracellular pathogens, including mycobacterium tuberculosis, it is unclear whether it is also beneficial for controlling HIV. Macrophages are a primary target of HIV infection (52, 74, 90, 179, 181) and it has been shown that IFN-γ reactivates the expression of HIV in persistently infected promonocytic cells (16). Furthermore, IFN-γ up regulates the expression of the HIV coreceptor CCR5 in macrophages (61, 187) and increases the susceptibility of these cells to infection with HIV-1 (186). Therefore, IFN-γ secreting Tc1a and Tc1b cells may have an adverse effect on the control of HIV replication. In contrast, $GzB^+/IFN\text{-}\gamma^-$ $CD8^+$ cells (Tc1c) could be more suitable for controlling this infection. Tc1c effector cells are likely to destroy infected

target cells including macrophages without activating viral replication in macrophages and without attracting additional cells susceptible to infection.

In addition to killing infected target cells, $CD8^+$ T lymphocytes are believed to be able to control HIV infection through the release of various HIV-suppressive factors (177). A subset of chemokines, including regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein 1α (MIP-1α), MIP-1β and other yet to be clearly defined factors were identified as major HIV inhibitory factors produced by $CD8^+$ T-cells (5, 10, 17, 34, 41). Recombinant human RANTES, MIP-1α, and MIP-1β were shown to inhibit different strains of HIV-1, HIV-2, and SIV in a dose-dependent manner (34). This finding becomes particularly intriguing in the light of the observations that MIP-1α, MIP-1β and RANTES are co-stored with GzB and perforin in the same cytolytic granula of HIV specific $CD8^+$ cytotoxic T lymphocytes (176). Therefore, the "silent killer" cells seem to be capable of lysing HIV-infected cells while at the same time inhibiting new infections of bystander cells by free virus. The association of GzB/perforin with virus inhibitory chemokines and their dissociation from the HIV replication promoting IFN-γ might make Tc1c cells ideal effector cells for the control of HIV. It will require further investigation how to selectively induce this effector cell class and to exploit it for the control of primary and chronic HIV infection.

The exact mechanism of how HIV spreads after the initial infection is still unclear. Following mucosal infection, the initial virus replication occurs locally in cells of the mucosal innate immune system, primarily macrophages, and dendritic cells (56, 73, 165, 188). It is conceivable that the subsequent spread occurs because of the

contribution of Tc1b cells whose IFN-γ secretion recruits additional target cells and promotes in them the replication of the virus. In contrast, one could envision that if Tc1c cells would be the prevalent class engaged early in the course of infection or after vaccination, they could successfully control the infection by killing the infected cells in addition to inhibiting the replication of the virus.

Unlike traditional chromium release assays, ELISPOT assays permit the testing of large peptide libraries with limited amount of PBMC (45, 58, 98, 144, 185), and importantly for clinical trials, ELISPOT assays can also be performed with freeze-thawed material (36, 161). For these reasons, the trend has developed in the field to substitute ^{51}Cr with IFN-γ ELISPOT assays (7, 36, 54, 55, 80, 81). This approach assumes that killing and IFN-γ production are linked effector functions. Our data clearly show that this simplistic approach does not do justice to the $CD8^+$ effector cell diversity, possibly detecting the irrelevant class of response while neglecting the relevant one.

Monitoring of cellular immunity to HIV frequently relies on longer peptides, such as 20-mers. It is well established that such peptides can not directly bind to HLA-class I molecules, and therefore they should not be ideal for detecting $CD8^+$ cells. Yet, the limitation of blood and of peptides available for testing has made testing of 20-mer peptide libraries main stream. As seen in Table 3, 9-mer peptides and 20-mer peptides induced the IFN-γ/GzB single positive $CD8^+$ cells in similar proportions, that is, 30-40% in each category. Therefore, the 9-mer and 20-mer peptides seem to stimulate the same subsets of $CD8^+$ cells. However, while the 20-mer peptides triggered IFN-γ production in $CD4^+$ and $CD8^+$ cells, they induced GzB only in the

$CD8^+$ cell fraction. Also the cell separation experiments that we performed confirmed that peptide-induced GzB production was confined to $CD8^+$ cells. While $CD4^+$ and $CD8^+$ cells are capable of producing IFN-γ (and were therefore detected in IFN-γ assays), only $CD8^+$ cells are capable of producing GzB. Therefore, GzB assays done with long peptides are suited for detecting Tc1c cells.

We show here that GzB^+/IFN-γ^- $CD8^+$ cells constitute a major effector cell class in HIV infection and that this cell type can be readily monitored ex vivo using GzB ELISPOT assays (144). Further studies of GzB^+/IFN-γ^- versus GzB^-/IFN-γ^+ $CD8^+$ cells in HIV and other infections should yield insights as to which of these is the class of response that affords higher protective value and thus should aid management of the infections and vaccine design.

5 Summary

A $CD8^+$ cell-mediated host defense relies on cognate killing of infected target cells and on local inflammation induced by the secretion of IFN-γ. Using assays of single cell resolution, it was studied to what extent these two effector function of $CD8^+$ cells are linked. Granzyme B (GzB) is stored in cytolytic granules of $CD8^+$ cells and its secretion is induced by antigen recognition of these cells. Following entry into the cytosol GzB induces apoptosis in the target cells. It was measured whether GzB release by individual $CD8^+$ cells is accompanied by the secretion of IFN-γ and of other cytokines. HIV peptide libraries were tested on bulk peripheral blood mononuclear cells and on purified $CD4^+$ and $CD8^+$ cells obtained from HIV infected individuals. The library included a panel of previously defined HLA class I restricted HIV peptides and an overlapping 20-mer peptide-series that covered the entire gp120 molecule. To characterize the *in vivo* differentiation state of the T-cells, freshly isolated lymphocytes were tested in assays of 24h duration. The data showed that only ~20% of the peptides triggered the release of both GzB and IFN-γ from $CD8^+$ cells. The majority of the HIV peptides induced either GzB or IFN-γ, ~40% in each category. The GzB positive, IFN-γ negative $CD8^+$ cells did not produce IL-4 or IL-5, which suggests that they do not correspond to Tc2 cells but represent a novel Tc1 subclass, which was termed Tc1c. Also the IFN-γ positive, GzB negative $CD8^+$ cell subpopulation represents a yet undefined $CD8^+$ effector cell lineage that was termed Tc1b. Tc1b and Tc1c cells are likely to make different, possibly antagonistic contributions to the control of HIV infection. Since IFN-γ activates HIV replication in latently infected macrophages, the secretion of this

cytokine by Tc1b cells in the absence of killing may have adverse effects on the host defense. In contrast, cytolysis by Tc1c cells in the absence of IFN-γ production might represent the protective class of response. Further studies in the field of Tc1 effector cell diversity should lead to valuable insights for management of infections and developing rationales for vaccine design.

6 Zusammenfassung

Im Verlauf von Infektionskrankheiten basiert die Verteidigung durch $CD8^+$-Zellen auf der direkten Tötung von infizierten Zellen über die Perforin/Granzyme-Kaskade und auf der Entzündungsbildung verursacht durch die Sekretion von Interferon-Gamma (IFN-γ). In der vorliegenden Arbeit wurde die Granzyme B (GzB) Sezernierung als direkter Nachweis für das Abtöten von Zellen und parallel die Produktion von IFN-γ durch HIV peptid-spezifische T-Zellen in chronisch HIV-infizierten Patienten gemessen. Eine Auswahl von in vorhergehenden Arbeiten definierten HLA-Klasse-I spezifischen HIV-Peptiden und eine HIV-Peptide-Bibiliotek, bestehend aus sich überlappenden 20-mer Peptiden, die das gesamte Gp120 Molekül von HIV-1 darstellten, wurden benutzt, um T-Lymphozyten aus frisch von HIV-infizierten Patienten gewonnenen mononukleären Zellen aufgereinigte $CD8^+$-Zellen zu stimulieren. ELISPOT-Assays wurden benutzt, um die Sekretion von GzB und IFN-γ mit einer Auflösung auf der Ebene der Einzelzelle zu messen. Nur ~20% der Peptide lösten die Freisetzung von sowohl GzB als auch IFN-γ von $CD8^+$ Zellen aus. Die Mehrheit der Peptide induzierte entweder GzB oder IFN-γ, je ~40% in der jeweiligen Kategorie. Granzyme B-positive Zellen produzierten in parallel gemessen kein IL-4 oder IL-5. Da sie aus diesem Grunde keine Tc2 Zellen darstellen, wurden sie als neue Untergruppe Tc1c definiert. Diese GzB^+/IFN-γ^- $CD8^+$ Zellen vermutlich eine durch Apoptose induziertes Absterben von infizierten Zellen auslösen, ohne gleichzeitig eine Entzündungsreaktion zu verursachen. Die GzB^-/IFN-γ^+ $CD8^+$-Zellen stellen ebenfalls eine neue bisher unbeschriebene Zelluntergruppe dar und wurden als Tc1b Zellen bezeichnet. Diese könnten Entzündungsreaktion

verursachen, ohne gleichzeitig direkt das Abstreben von Zellen zu induzieren und im Verlauf der HIV Infektion eine antagonistische Rolle zu den Tc1c Zellen einnehmen. Diese Tc1-$CD8^+$ Effektorzell-diversität könnte die Implementierung und Feineinstellung von fundamental verschieden Verteidigungsstrategien gegen HIV und andere Infektionskrankheiten ermöglichen. Die hier vorgelegten Studien liefern eindeutige Indizien dafür, dass es in HIV-Infizierten eine signifikante Population von GzB sezernierenden $CD8^+$-Zellen gibt, die weder IFN-γ noch IL-4 oder IL-5 produzieren und daher in der Lage sind, mit Hilfe der Perforin/Granzyme-Kaskade zu töten, ohne dabei klassische Tc1 oder Tc2-Zellen darzustellen. Die höhere Sensitivität des GzB ELISPOT-Assays Zytotoxizität im Vergleich zum Chromium-Release-Assay zu messen, bietet eine hilfreiche Methode zum besseren Verständnis $CD8^+$-Zell vermittelter Immunität. Die Ergebnisse führen zu der Schlussfolgerung, dass es im Menschen die von uns erstmals beschriebenen $GzB^+/IFN\text{-}\gamma^-$ und $GzB^-/IFN\text{-}\gamma^+$ Untergruppen von $CD8^+$-Zellen gibt. Aufgrund der verschiedenen Effektorfunktionen die diese vermutlich ausüben, erscheint es wichtig ihre jeweiligen Anteile im Kampf gegen HIV und andere Infektionskrankheiten zu ermitteln. Bei der Beurteilung von Immunantworten, ausgelöst durch experimentelle HIV-Impfstoffe, dürfte es sich als ausgesprochen wichtig erweisen, diese GzB produzierenden $CD8^+$-Zellen neben den konventionell gemessenen IFN-γ sezernierden $CD8^+$-Zellen zu berücksichtigen. Diese Vorgehensweise sollte wertvolle Einblicke gewähren, in wie weit der jeweilige Phänotyp von Effektorzellen den höheren oder effektiveren Schutz gewährt und daher wertvolle Hilfe beim Management von Infektionen und im Bereich des Impfstoffdesign liefern.

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9 Curriculum Vitae

Persönliche Daten

Name:	Thomas-Oliver Kleen
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Ausbildung

1980-1993	Besuch von Vorschule, Grundschule und Gymnasium in Bremen
1993	Abitur am Gymnasium an der Hermann-Böse Straße, Bremen
1993-1998	Studium der Biologie an der Universität Bremen
1995	Vordiplom Biologie, Gesamtnote „Sehr Gut“ verliehen von der Universität Bremen
1998	Externe Diplomarbeit am deutschen Primatenzentrum (DPZ) in Göttingen, Abteilung für Virologie und Immunologie unter der Betreuung von Prof. Dr. Gerhard Hunsmann und Dr. Wolfgang Lueke Thema: "Charakterisierung der Immunantwort gegen virus-ähnliche Partikel (VLP’s) des Hauptstrukturproteins VP1 des humanen Polyomavirus JCV in Kaninchen ";
1998	Diplom Biologie, Gesamtnote „Sehr Gut“ verliehen von der Universität Bremen

1998-1999	Praktikumsaufenthalt bei New Zealand Forest Research, Abteilung Molecular Genetics, Rotorua, Neuseeland, unterstützt durch die Mühlfenzel-Stiftung
1999-2003	Promotionsstudium and der Case Western Reserve University in Cleveland, Ohio, USA

Stipendien

1996-1998	Studienstiftung des Deutschen Volkes
1998-1999	Mühlfenzel-Stipendium

Wettbewerbe

1992	Landeswettberwerb Jugend forscht in Bremen, Preis: Römpp-Lexikon Biotechnologie

Zusätzliche Qualifikationen

1998	Bescheinigung über die Qualifikation zum Umgang und Transport von Gefahrstoffen gemäß der Gefahrstoffverordung des Landes Bremen
1999	New Zealand Lifetime Private Pilot License Aeroplane
1999	United States of America Private Pilot License Airplane Single Engine Land Instrument Airplane

Veröffentlichungen:

Paper: Kleen T.O., Assad R., Landry S., Boehm B.O. and Tary-Lehmann M. Tc1 effector diversity shows dissociated expression of IFN-γ and of granzyme B in HIV infection. *AIDS 2004, 18: 383-392*

Kadereit S., Junge G.R., Kleen T.O., Kozik M.M., Welsh B.A., Daum-Woods K., Fu P., Tary-Lehmann M. and Laughlin M.J. Deficient IFN-γ expression in umbilical cord blood (UBC) T cells can be rescued by IFN-γ-mediated increase in NFATc2 expression. *Journal of Clinical Immunology 2003, 23: 485-497*

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Poster: GOLDMANN, C., AST, O., KRÄMER, M., KLEEN, T., JENTSCH, K.D., HUNSMANN, G., WEBER, T., PETRY, H., LÜKE, W. Development of an efficient and safe gene delivery system based on virus-like particles. 1st Annual Meeting of the American Society of Gene Therapy 1998, Seattle, USA

GOLDMANN, C., AST, O., MARX, D., KRÄMER, M., KLEEN, T., JENTSCH, K.D., HUNSMANN, G., WEBER, T., MEDEN, H., PETRY, H., LÜKE, W.: Development of a novel gene therapy for cervical cancer based on virus-like particles. 1st International Conference on Human Papillomavirus Infections and Cervical Cancer 1998, Montreal, Canada

LÜKE, W., GOLDMANN, C., AST, O., MARX, D., KRÄMER, M., KLEEN, T., JENTSCH, K.D., HUNSMANN, G., PETRY, H., MEDEN, H.: Development of a new method for the gene therapy on the basis of VLPs (virus-like particles) in patients with mammary cancer. 18th Annual Meeting of the German Society for Serology 1998, Düsseldorf, Germany

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KLEEN T.O., ASSAD R., LANDRY S. AND TARY-LEHMANN M. Direct ex vivo characterization of Gp120-specific $CD4^+$ and $CD8^+$ T-cells in HIV-infected and healthy donors using ImmunoSpot assay. Clinical Immunology Society 2000, Seattle, USA

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KLEEN T.O., ASSAD R., LANDRY S. AND TARY-LEHMANN M. Direct ex vivo characterization of Gp120-specific $CD4^+$ and $CD8^+$ T-cells in HIV-infected and healthy donors using ImmunoSpot assay. 2nd International Conference on Vaccine Development and Immunotherapy in HIV 2001, San Juan, Puerto Rico

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KLEEN T.O., ASSAD R., LANDRY S. AND TARY-LEHMANN M. Dissociated expression of IFN-γ and of Granzyme-B by HIV specific $CD8^+$ cells in infected individuals. 238.8 Experimental Biology 2002 New Orleans, USA, *the FASEB J. 16: A299*

KLEEN T.O., KRUSE C., LEHMANN P.V. AND TARY-LEHMANN M. Dissociated expression of IFN-γ and of Granzyme-B by HIV specific $CD8^+$ cells in infected individuals. FOCIS 2002 San Francisco, USA

Vorträge:

KLEEN T.O., ASSAD R., LANDRY S. AND TARY-LEHMANN M. Direct ex vivo characterization of Gp120-specific $CD4^+$ and $CD8^+$ T-cells in HIV-infected and healthy donors using ImmunoSpot assay. Autumn Immunology Conference 2000, Chicago, USA. *Minisymposium speaker*

KLEEN T.O., ASSAD R., LANDRY S., AND TARY-LEHMANN M. Dissociated expression of IFN-γ and of Granzyme-B by HIV specific $CD8^+$ cells in infected individuals. 238.8 Experimental Biology 2002 New Orleans, USA, the FASEB J. 16: A299. *Minisymposium speaker*

Lehre:

Facilitator of the WHO - UNAIDS sponsored workshop on "Application of ELISPOT assay in HIV Vaccine Research" organized in collaboration with Duke University Medical Center and U.S. Military HIV Research Program 2001, Duke University Medical in Durham , NC, USA

Facilitator of the WHO - UNAIDS Training Workshop on "Application of ELISPOT assay to monitor cellular immune response in HIV Vaccine - related research in Latin America and the Caribbean" 2002, Rio De Janeiro, Brazil

Konferenzen:

Keystone Symposia, Novel Biological Approaches on New Insights into HIV Biology 2000, Keystone, USA

Clinical Immunology Society Meeting 2000, Seattle, USA

Autumn Immunology Conference 2000, Chicago, USA,

Keystone Symposia, AIDS vaccines in the New Millennium 2001, Keystone, USA

2nd International Conference on Vaccine Development and Immunotherapy in HIV 2001, San Juan, Puerto Rico

FASEB, Experimental Biology 2001, Orlando, USA

FOCIS Boston 2001, USA

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Keystone Symposia HIV-1 Protection and Control by Vaccines 2002 Keystone, USA

FASEB, Experimental Biology 2002 New Orleans, USA

FOCIS 2002 San Francisco, USA

10 Ehrenwörtliche Erklärung

Hiermit erkläre ich ehrenwörtlich, dass die vorliegende Arbeit von mir selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde. Weiterhin habe ich noch keinen Promotionsversuch unternommen oder diese Dissertation in gleicher oder ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt.

Cleveland den 10. October 2003

Thomas O. Kleen