Immunology of Viral Disease, How to Curb Persistent Infection

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1. Summaries

1.1. Preamble and extended abstract

The present thesis dissertation addresses the question of antiviral immunity from the particular standpoint of the adaptive T cell-mediated immune response. The experimental work is presented in the form of three published articles (two experimental articles and one review article, see sections 4.1, 4.2 and 4.3 on pages 73, 81 and 91, respectively), describing advances both in our understanding of viral control by CD8 T lymphocytes, and in vaccine development against the Human Immunodeficiency Virus Type 1 (HIV-1).

Because the articles focus on rather specialized areas of antiviral immunity, the article sections are preceded by a general introduction (section 3) on the immune system in general, and on four viruses that were addressed in the experimental work, namely HIV-1, Cytomegalovirus (CMV), Epstein Barr Virus (EBV) and Influenzavirus (Flu). This introduction section is aimed at providing a glimpse on viral molecular biology and immunity, to help the hypothetical non-expert reader proceeding into the experimental part. For this reason, each section is presented as individual entity and can be consulted separately.

The four viruses described are of peculiar relevance to immunity because they induce an array of opposite host responses. Flu causes a self limiting disease after which the virus is eradicated. CMV and EBV cause pauci-symptomatic or asymptomatic diseases after which the viruses establish lifelong latency in the host cells, but are kept in check by immunity. Eventually, HIV-1 establishes both latency – by inserting its genome into the host cell chromosome – and proceeds in destroying the immune system in a poorly controlled fashion. Hence, understanding the fundamental differences between these kinds of viral host interactions might help develop new strategies to curb progressive diseases caused by viruses such as HIV-1.

Publication #1: The first article (section 4.1, page 73) represents the main frame of my laboratory work. It analyses the ability of CD8 T lymphocytes recovered from viral-infected patients to secrete interferon \(\gamma\) (IFN-\(\gamma\)) alone or in conjunction with interleukin 2 (IL-2) when exposed \textit{in vitro} to their cognate viral antigens. CD8 T cells are instrumental in controlling viral infection. They can identify infected cells by detecting viral antigens presented at the surface of the infected cells, and eliminate both the cell and its infecting virus by triggering \textit{apoptosis} and/or lysis of the infected cell. Recognition of these antigens triggers the cognate
CD8 cells to produce cytokines, including IFN-γ and IL-2, which in turn attract and activate other pro-inflammatory cells. IFN-γ triggers both intrinsic antiviral activity of the infected cells and distant activation of pro-inflammatory cells, which are important for the eradication of infection. IL-2 is essential for clonal expansion of the antigen (Ag)-specific CD8 T cell. Hence the existence of Ag-specific CD8 cells secreting both IFN-γ and IL-2 should be beneficial for controlling infection.

In this first work we determined the percentage of IFN-γ/IL-2 double positive and single IFN-γ secreting CD8 T cells against antigens HIV-1, CMV, EBV and Flu in three groups of subjects: (i) HIV-1 infected patients progressing to disease (progressors), (ii) HIV-1-infected subjects not progressing to disease (long-term non progressors or LTNP), and (iii) HIV negative blood donors. The results disclosed a specific IFN-γ/IL-2 double positive CD8 response in all subjects able to control infection. In other words, IFN-γ/IL-2 double positive CD8 cells were present in virus-specific CD8 T cells against Flu, CMV and EBV as well against HIV-1 in LTNP. In contrast, progressors only had single IFN-γ secreting CD8 T cells. Hence, the ability to develop an IFN-γ/IL-2 double positive response might be critical to control infection, independently of the nature of the virus.

Additional experiments helped identify the developmental stage of the missing cells (using different markers such as CD45RA and CCR7) and showed a correlation between the absence of IL-2 secreting CD8 T cells and a failure in the proliferation capacity of virus-specific CD8 T cells. Addition of exogenous IL-2 could restore clonal expansion of HIV-1 specific CD8 T cells, at least in vitro. It could further been shown, that IL-2 secreting CD8 T cells are sufficient to support proliferation even in absence of CD4 help. However, the reason for the missing IFN-γ/IL-2 double positive CD8 T cell response in HIV-1 progressors has yet to be determined.

Publication #2: The second article (section 4.2, page 81) explores new strategies to trigger CD8 T cell immunity against specific HIV-1 proteins believed to be processed and exposed as "infection signal" at the surface of infected cells. Such signals consist of peptide fragments (8-13 amino acids) originating from viral proteins and presented to CD8 T cells in the frame of particular cell surface molecules of the major histocompatibility complex class I∗ (MHC I). To mimic "natural" viral infection, the HIV-1 polyprotein Gagpolnef was inserted and expressed in either of two attenuated viruses i.e. vaccinia virus (MVA) or poxvirus (NYVAC). Mice

* words in italic (except for names of pathogens) are explained in the glossary section 7 p. 103
were infected with these recombinant viruses and specific CD8 T cell response to Gagpolnef peptides was sought.

Mice could indeed mount a CD8 T cell response against the HIV-1 antigens, indicating that the system worked, at least in this animal model. To further test whether peptides from Gagpolnef could also be presented in the frame of the human MHC class I proteins, a second round of experiments was performed in "humanized" transgenic mice expressing human MHC molecules. The transgenic mice were also able to load Gagpolnef peptides on their human MHC molecule, and these cells could be detected and destroyed by Ag-specific CD8 T cells isolated from HIV-1-infected patients. Therefore, expressing Gagpolnef on attenuated recombinant viruses might represent a valid strategy for anti-HIV-1 immunization in human.

Publication #3: This is a review paper (section 4.3, page 91) describing the immune response to CMV and newly developed methods to detect this cellular immune response. Some of it focuses on the detection of T cells by using *in vitro* manufactured tetramers. These consist of four MHC class I molecules linked together and loaded with the appropriate antigenic peptide. The tetramer can be labeled with a fluorochrome and analyzed with a fluorescence-activated cell sorter.

Taken together, the work presented indicates that (i) an appropriate CD8 T cell response consisting of IFN-γ/IL-2 double positive effectors, can potentially control viral infection, including HIV-1 infection, (ii) such a response might be triggered by recombinant viral vaccines, and (iii) CD8 T cell response can be monitored by a variety of techniques, including recently-developed MHC class I tetramers.
1. 2. Préambule et résumé élargi

Le présent travail de thèse s'intéresse à l'immunité antivirale du point de vue particulier de la réponse adaptative des cellules T. Le travail expérimental est présenté sous la forme de trois articles publiés (2 articles expérimentaux et 1 article de revue, voir sections 4.1, 4.2 et 4.3, pages 58, 66 et 77, respectivement), décrivant des progrès dans la compréhension du contrôle de l'infection virale par les lymphocytes T CD8, ainsi que dans le développement de nouveaux vaccins contre le Virus d'Immunodéficience de Humaine de type 1 (VIH-1).

En raison du caractère spécialisé de l'immunité antivirale de type cellulaire, les articles sont précédés par une introduction générale (section 3), dont le but est de pourvoir le lecteur non avisé avec des bases nécessaire à une meilleure appréhension du travail expérimental. Cette introduction présente les grandes lignes du systèmes immunitaire, et décrit de façon générale les 4 virus utilisés dans le travail expérimental: à savoir le virus VIH-1, le Cytomégalovirus (CMV), le virus Epstein Barr (EBV) et le virus Influenza A (Flu).

Toutes les sections sont présentées de façon individuelle et peuvent être consultées séparément.

La description des 4 virus a une pertinence particulière quant à leur interaction avec le système immun. En effet, ils induisent une panoplie de réponses immunitaires s'étendant aux extrêmes de la réaction de l'hôte. Influenza A est à l'origine d'une maladie cytopathique aiguë, au décours de laquelle le virus est éradiqué par l'hôte. CMV et EBV sont classiquement à l'origine d'infections pauci-symptomatiques, voire asymptomatiques, après lesquelles les virus persistent de façon latente dans la cellule hôte. Cependant, ils restent sous le contrôle du système immunit, qui peut prévenir une éventuelle réactivation. Enfin, VIH-1 s'établit à la fois en infection latente – par l'insertion de son génome dans le chromosome des cellules hôtès – et en infection productive et cytopathique, échappant au contrôle immunitaire et détruisant ses cellules cibles. La compréhension des différences fondamentales entre ces différents types d'interactions virus-hôte devraient faciliter le développement de nouvelles stratégies antivirales.

Article 1 : Le premier article (section 4.1 Page 58) représente l'objet principal de mon travail de laboratoire. Il analyse la capacité des lymphocytes T CD8 spécifiques de différent virus à sécréter de l’interféron gamma (IFN-γ) et/ou de l’interleukine 2 (IL-2) après stimulation par leur antigène spécifique. Les cellules T CD8 jouent un rôle crucial dans le contrôle des infections virales. Elles identifient les cellules infectées en détectant des antigènes viraux
présentés à la surface de ces mêmes cellules, et éliminent à la fois les cellules infectées et les virus qu'elles contiennent en induisant l'apoptose et/ou la lyse des cellules cibles. Parallèlement, l'identification de l'antigène par la cellule T CD8 la stimule à sécréter des cytokines. L'IFN-γ en est un exemple. L'IFN-γ stimule les cellules infectées à développer une activité antivirale intrinsèque. De plus, il attire sur place d'autres cellules de l'inflammation, et active leur fonction d'éradication des pathogènes. L'IL-2 est un autre exemple. L'IL-2 est essentielle à l'expansion clonale des cellules T CD8 spécifiques à un virus donné. Elle est donc essentielle à augmenter le pool de lymphocytes antiviraux. En conséquence, la double capacité de sécréter de l’IFN-γ et de IL-2 pourrait être un avantage pour le contrôle antiviral par les cellules T CD8.

Dans ce travail nous avons comparé les proportions de lymphocytes T CD8 doubles positifs (IFN-γ/IL-2) et simples positifs (IFN-γ) chez trois groupes de sujets: (i) des patients infectés par VIH-1 qui ne contrôlent pas l’infection (progresseurs), (ii) des patients infectés par VIH-1, mais contrôlant l’infection malgré l’absence de traitement ("long term non progressors“ [LTNP]) et (iii) des donneurs de sang négatifs pour l’infection à VIH-1. Les résultats ont montré que les individus capables de contrôler une infection possédaient des cellules T CD8 doubles positifs (IFN-γ/IL-2), alors que les patients ne contrôlant pas l'infection procédaient prioritairement des CD8 simples positifs (IFN-γ). Spécifiquement, les lymphocytes T spécifiques pour Flu, CMV, EBV, et VIH-1 chez les LTNP étaient tous IFN-γ/IL-2 doubles positifs. Au contraire, les lymphocytes T CD8 spécifique à VIH-1 étaient IFN-γ simples positifs chez les progresseurs.

La capacité de développer une réponse IFN-γ/IL-2 pourraient être primordiale pour le contrôle de l'infection, indépendamment de la nature du virus. En effet, il a été montré que l'absence de sécrétion d'IL2 par les lymphocytes T CD8 corrélait avec leur incapacité de proliférer. Dans nos mains, cette prolifération a pu être restaurée in vitro par l'adjonction exogène d’IL-2. Toutefois, la faisabilité de ce type de complémentation in vivo n'est pas clair.

Des expériences additionnelles ont permis de préciser de stade de développement des lymphocytes doubles positifs et simples positifs par le biais des marqueurs CD45RA et CCR7. Il reste maintenant à comprendre pourquoi certains lymphocytes T CD8 spécifiques sont incapables à sécréter de l’IL-2.

**Article 2:** Le deuxième article explore des nouvelles stratégies pour induire une immunité T CD8 spécifique aux protéines du VIH-1, qui sont éditées et exposées à la surface des cellules
infectées. Ces signaux consistent en fragments de peptide de 8-13 acide aminés provenant de protéines virales, et exposées à la surface des cellules infectées dans le cadre des molécules spécialisées d'histocompatibilité de classe I (en anglais "major histocompatibility class I" ou MHC I). Pour mimer une infection virale, la polyprothèque Gagpolnef du VIH-1 a été insérée et exprimée dans deux vecteurs viraux atténués, soit MVA (provenant de vaccinia virus) ou NYVAC (provenant d'un poxvirus). Ensuite des souris ont été infectées avec ces virus recombinants et la réponse T CD8 aux peptides issus de Gagpolnef a été étudiée. Les souris ont été capables de développer une réponse de type CD8 T contre ces antigènes du VIH-1. Pour tester si ces antigènes pouvaient aussi être présentés par dans le cadre de molécules MHC humaines, des expériences supplémentaires ont été faites avec des souris exprimant un MHC humain. Les résultats de ces manipulations ont montré que des cellules T CD8 spécifiques aux protéines du VIH pouvaient être détectées. Ce travail ouvre de nouvelles options quant à l'utilisation des virus recombinants exprimant Gagpolnef comme stratégie vaccinale contre le virus VIH-1 chez l'homme.

**Article 3:** Ces revues décrivent la réponse immunitaire à CMV ainsi que des nouvelles méthodes pouvant servir à sa détection. Une partie du manuscrit décrit la détection de cellule T à l'aide de tétramères. Il s'agit de protéines chimériques composées de 4 quatre molécules MHC liées entre elles. Elles sont ensuite "chargées" avec le peptide antigénique approprié, et utilisée pour détecter les cellules T CD8 spécifiques à ce montage. Elles sont aussi marquées par un fluorochrome, qui permet une analyse avec un cytomètre de flux, et l'isolement ultime des CD8 d'intérêt.

En résumé, le travail présenté dans cette thèse indique que (i) une réponse T CD8 appropriée – définie par la présence des cellules effectrices doublement positives pour l’IFN-γ et l’IL-2 – semble indispensable pour le contrôle des infections virales, y compris par le VIH-1, (ii) une telle réponse peut être induite par des vaccin viral recombinant, et (iii) la réponse T CD8 peut être analysée et suivie grâce à plusieurs techniques, incluant celle des tétramères de MHC class I.
1.3. Résume pour un large public

Le système immunitaire humain est composé de différents éléments (cellules, tissus et organes) qui participent aux défenses de l’organisme contre les pathogènes (bactéries, virus). Parmi ces cellules, les lymphocytes T CD8, également appelés cellules tueuses, jouent un rôle important dans la réponse immunitaire et le contrôle des infections virales. Les cellules T CD8 reconnaissent de manière spécifique des fragments de protéines virales qui sont exposés à la surface des cellules infectées par le virus. Suite à cette reconnaissance, les cellules T CD8 sont capables de détruire et d’éliminer ces cellules infectées, ainsi que les virus qu’elles contiennent.

Dans le contexte d’une infection par le virus de l’immunodéficience humaine (VIH), le virus responsable du SIDA, il a pu être montré que la présence des cellules T CD8 est primordiale. En effet, en l’absence de ces cellules, les individus infectés par le VIH progressent plus rapidement vers le SIDA.

Au cours de la vie, l’Homme est exposé à plusieurs virus. Mais à l’opposé du VIH, certains d’entre eux ne causent pas des maladies graves : par exemple le virus de la grippe (Influenza), le cytomégalovirus ou encore le virus d’Epstein-Barr. Certains de ces virus peuvent être contrôlés et éliminés de l’organisme (p. ex. le virus de la grippe), alors que d’autres ne sont que contrôlés par notre système immunitaire et restent présents en petite quantité dans le corps sans avoir d’effet sur notre santé.

Le sujet de mon travail de thèse porte sur la compréhension du mécanisme de contrôle des infections virales par le système immunitaire : pourquoi certains virus peuvent être contrôlés ou même éliminés de l’organisme alors que d’autres, et notamment le VIH, ne le sont pas.

Ce travail a permis de démontrer que les cellules T CD8 spécifiques du VIH ne sécrètent pas les mêmes substances, nécessaires au développement d’une réponse antivirale efficace, que les cellules T CD8 spécifiques des virus contrôlés (le virus de la grippe, le cytomégalovirus et le virus d’Epstein-Barr). Parallèlement nous avons également observé que les lymphocytes T CD8 spécifiques du VIH ne possèdent pas la capacité de se diviser. Ils sont ainsi incapables d’être présents en quantité suffisante pour assurer un combat efficace contre le virus du SIDA. La (les) différence(s) entre les cellules T CD8 spécifiques aux virus contrôlés (grippe, cytomégalovirus et Epstein-Barr) et au VIH pourront peut-être nous amener à comprendre comment restaurer une immunité efficace contre ce dernier.
### 2. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cells</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BMT</td>
<td>bone marrow transplanted</td>
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<tr>
<td>C</td>
<td>constant region</td>
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<td>CA</td>
<td>capsid</td>
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<td>CC</td>
<td>cystein-cystein</td>
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<td>CCR7</td>
<td>chemokine receptor 7</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CFSE</td>
<td>5- (and 6-) carboxyfluorescein diacetate succinimidyl ester</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CTL</td>
<td>cytolytic/cytotoxic T lymphocytes</td>
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<td>cystein-X-cystein</td>
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<tr>
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<tr>
<td>DNA</td>
<td>deoxypribonucleic acid</td>
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<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>EA</td>
<td>early antigen (EBV)</td>
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<tr>
<td>EBERs</td>
<td>Epstein-Barr encoded small RNAs</td>
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<td>EBNA</td>
<td>EBV nuclear antigens</td>
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<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
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<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
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<td>FACS</td>
<td>fluorescence activated cell sorter</td>
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<td>Influenza</td>
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<td>Human immunodeficiency virus 1</td>
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<td>immunoglobuline</td>
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<td>Infectious mononucleosis</td>
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<td>IN</td>
<td>integrase</td>
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<tr>
<td>ITAMs</td>
<td>immunoreceptor tyrosine-based activation motifs</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>latent membrane protein</td>
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<td>long term non progressor</td>
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<td>LTR</td>
<td>long terminal repeat (sequence)</td>
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<td>M</td>
<td>matrix (Influenza)</td>
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<td>MA</td>
<td>matrix (HIV-1)</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MIP-1α/β</td>
<td>macrophage inflammatory protein 1α/β</td>
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<tr>
<td>m.o.i.</td>
<td>multiplicity of infection</td>
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<td>messenger RNA</td>
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<td>nucleoprotein</td>
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<td>basic polymerase 1/2</td>
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<td>virus capsid antigen (EBV)</td>
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3. Introduction

3.1. General principles of immunology

3.1.1 The principle of innate and adaptive immunity

Microbes (viruses, prokaryotes, fungi and parasites) represent the vast majority of the earth's biomass (1). Most of them are indifferent to higher eukaryotes because they never engage contact with them. In contrast, higher eukaryotes live in the middle of microbes and constantly interact with them. Therefore they have developed specialized molecules that can recognize conserved bacterial components and activate intracellular cascades that kill and digest the microbes. This is a feature of the so-called innate immunity. However, recognition of conserved microbial molecules by the host has pushed microorganisms to surround themselves with anti-phagocytic structures, including surface polysaccharids and proteins. These structures hide the microbial sensitive structures from detection by the host. To cope with this evolution, higher eukaryotes have developed an additional layer of defense, called adaptive immunity. Adaptive immunity allows detecting specific molecules of the non-self, including microbial molecules that are used to trick the first line innate immunity. Typical examples of such molecules are the pneumococcal antiphagocytic capsule, and the antiphagocytic M protein on the surface of *Streptococcus pyogenes*.

3.1.2 The origin of the cellular partners of innate and adaptive immunity

The cells of the immune system are generated in the bone marrow (BM). Most of these cells mature in the BM before they circulate in the blood and lymphatic system to guard peripheral tissues. All the cellular elements from the blood arise from the same pluripotent hematopoietic stem cell in the bone marrow (Fig. 1). Each of them carries specific surface molecules, called cluster differentiation (CD) markers, which define special functions and help classify them in different "CD" types.

*Innate immunity cells:* The myeloid progenitor is the precursor cells of the innate immune system such as granulocytes, macrophages, dendritic cells and mast cells. Macrophages, the mature form of monocytes, are distributed in all the tissues. Dendritic cells (DC) are specialized in uptaking and displaying Ag to adaptive immunity T cells. Before encountering a pathogen DC are "immature" and continuously traffic from the blood to tissues. They are both phagocytic and macropinocytic. After engulfing an intruder they mature, express cytokines and chemokines, and migrate to lymph nodes.
Adaptive immunity cells: The common lymphoid progenitor gives rise to lymphocytes, natural killer cells and some dendritic cells, although most of dendritic cells arise from the myeloid progenitors. Most lymphocytes are small, resting cells with few cytoplasmic organelles and most of their chromatin is inactive. They have no functional activity until they encounter their specific antigen in combination with co-stimulatory molecules. Lymphocytes can be divided into B and T lymphocytes. B lymphocytes mature in the bone marrow. Once they are activated, they differentiate into plasma cells and produce antibodies. In contrast, T cell precursors are produced in the bone marrow and migrate then to the thymus for their differentiation into mature T cells (see section 3.2.1, p. 25).

The lymphoid organs: Lymphocytes are generated in the bone marrow and the thymus, which are referred to as the primary lymphoid organs. They then migrate to the secondary lymphoid organs, which include the lymph nodes, spleen and lymphoid tissues associated with mucosa (e.g. gut-associated tonsil, Peyer patches and appendix), where they mature, and initiate and maintain the adaptive immunity. Lymphoid organs are composed of large numbers of lymphocytes gathered in a framework of nonlymphoid cells (e.g. macrophages, granulocytes and mature dendritic cells displaying the Ag to the lymphocytes).
Figure 1. All the cellular elements of the blood arise from a pluripotent hematopoietic stem cell. Figure from Immunobiology 6th edition chapter 1.
### 3.1.3 Innate immunity

Innate immunity is present in newborns, and does not need pre-exposure to foreign microbial materials to react. It has an intrinsic ability to recognize conserved microbial molecules, also referred to as PAMPs (pathogen-associated molecular patterns). Hence, it can directly react against a large number of viruses, bacteria, fungi and parasites (for review see (2-5)).

Innate immunity is always involved in the primary detection of invading microbes, and in the inflammatory response that aims at calling in other players of the host defense system. It can kill microbes by itself. Moreover, after having digested a pathogen, professional phagocytes (which are part of innate immunity) present small fragments of the microbe to cognate T-lymphocytes from adaptive immunity. This is achieved by a sophisticated intercellular signaling implying the surface receptors of the major MHC class II family on the side of the innate immunity partner cells, and the T-cell receptor on the side of adaptive immunity partner cell. The cognate T-lymphocytes then expand and can promote the production of either cytotoxic cells (T<sub>H1</sub> pathway) or antibody (Ab) production (T<sub>H2</sub> pathway) to get rid of the invader. This sophisticated defense network allows both responding to unknown intruders, and preventing re-infection with a previously known one (see also section 3.1.4., page 17).

The cellular and humoral components in innate immunity have been extensively reviewed (for instance, see (6)). Cellular factors primarily include macrophages and dendritic cells (Fig. 1), which are abundant in tissues, and polymorphonuclear neutrophils and monocytes (the precursors of macrophages) that are abundant in the blood. Soluble factors comprise all the cellular mediators (cytokines and chemokines) that amplify the inflammatory response to infection, the complement cascade, as well as alternative soluble components such as the C-reactive protein, mannan-binding lectin proteins, platelet-activating factor, and lipopolysaccharide-binding protein.

Thank to their array of PAMPs-recognizing receptors, the cellular components of innate immunity can recognize a vast number of microbes, including viruses, bacteria, fungi and parasite (6). One important antiviral innate protein is INF-γ, which acts mainly as a cytokine, but can also modify transcription and translation viral polynucleotides by the host cell.

### 3.1.4 Adaptive immunity

Adaptive immunity results from an adaptation to infection with a specific pathogen. Recall that the cells of innate immunity recognize only a limited number of microbial molecules, the repertoire of which is constrained by germ line-encoded receptors. To ensure coverage of the
whole palette of pathogens, including viruses, lymphocytes of adaptive immunity have evolved Ag receptors specific for infinity of molecules, excluding only those carried by the host itself. These receptors are determined by a unique genetic mechanism that operates during lymphocyte development and not described in details here (for review see (6)).

Lymphocyte maturation and survival are regulated by signals received through their very Ag receptors. A strong signal causes immature lymphocytes to die or to undergo further receptor rearrangement. The complete absence of a signal can also lead to cell death. This process is called clonal selection and is a central principle of the adaptive immune system (Fig. 2).

If the Ag is recognized as foreign to the host, then lymphocytes undergo clonal expansion. On the other hand, if the Ag is recognized as belonging to the self, the particular clone is eliminated.

**Figure 2. Clonal selection.** (A) A single progenitor cell gives rise to a large number of lymphocytes each with a different specificity. (B) Lymphocytes with specificity to ubiquitous self antigens are eliminated before the finish maturation. This ensures tolerance to self antigens. (C) The remaining mature naïve lymphocytes can recognize foreign antigens. When an antigen is recognized and bound to the T cell receptor on a naïve lymphocyte, the cell becomes activated and (D) starts to proliferate. The resulting clone of progeny has all the same receptor binding the same antigen. Activated lymphocytes differentiate into effector cells and eliminate the antigen. Figure adapted from Immunobiology 6th edition chapter 1.
3.1.5 The innate and adaptive immunity cooperation

The innate immune response is important for the initiation and subsequent direction of the adaptive immune response, but also to remove pathogens that have been targeted by the adaptive immune system. Adaptive immunity is delayed by 4-7 days. In this period the innate immunity is crucial in controlling infections.

Phagocytic cells may eliminate a number of microbes thanks to their PAMPs-recognizing surface receptors. After microbial engulfment macrophages and dendritic cells mature into antigen-presenting cells (APC), so called because they can present pieces of the microbial proteins on their surface, for recognition by T-lymphocytes (see details in section 3.2.3, page 24).

T cells receive survival signals from self-molecules on specialized epithelial cells in the thymus during development (see Fig. 2) and from the same or similar molecules expressed by APC in peripheral lymphoid tissues. Lymphocytes must become activated and proliferate to generate sufficient Ag-specific effector cells to overcome an infection (Fig. 2C and D). When a lymphocyte recognizes its specific Ag on an APC it stops migrating and starts to enlarge. The chromatin becomes less dense and the volume of the cytoplasm and nucleus increases. mRNA and proteins are synthesized. The lymphocyte starts to divide two to four times every 24 hours for three to five days and then differentiate into effector cells. After four to five days, the clonal expansion is completed. After eradication of infection, most of the Ag-specific cells undergo apoptosis. Yet, enough of them persist even after clearance of the pathogen to build a memory cell pool. These cells are then ready to rapidly react and eliminate the pathogen in case of reinfection with or reactivation of the same pathogen.

**B cells and T cells:** The adaptive immune response has developed two types of lymphocytes responding to the different lifestyles of the different pathogens. B cells recognize Ag that are present in the extra cellular milieu of the host, as it is the case for most bacteria, fungi, parasites, and extra cellular forms of viruses. In contrast, T cells recognize Ag generated inside of infected host cells, as in productive or persistent viral infections, as well as in some infections due to intracellular bacteria (e.g. *Listeria monocytogenes*) and parasites (e.g. *Plasmodium* spp.).
**Humoral immune response**: B cells recognize and bind soluble Ag via their surface B cell receptor (BCR). Binding of Ag to BCR transmits a signal that leads to B cell activation, clonal expansion, and ultimately Ab production. Ab are identical to the B cell receptor (BCR) except for a small portion of the C-terminus that allows them to be soluble instead of being anchored to the membrane. They have different functions: they neutralize or opsonize pathogens, or they activate the complement system, which then directly destroy bacteria, coat surfaces of pathogens to target them for phagocytes or enhance phagocyte function (Fig. 3). Ab secreted during infection are found in the plasma and interstitial fluids. Some subclasses (called isotypes) are also secreted on mucosal surfaces. However, because Ab are soluble they do not detect intracellular pathogens. Hence, another mechanism is required against such type of microbes.
Figure 3. Different functions of Ab. Antibodies can (a) neutralize toxins and target them for ingestion by macrophages (left panel) (b) opsonize bacteria in the extracellular space or (middle panel) or (c) activate complement system, which then activates phagocytes (right panel). Figure from Immunobiology 6th edition, chapter 1.
Cell-mediated immune response: This second system depends on a direct interaction between T lymphocytes and infected host cells. Cells that are infected display an "infection signal" on their surface. The infection signal is represented by pieces of the invaders' proteins, digested by the cell and presented in the frame of their MHC class I or MHC class II surface receptors (Fig. 4).

This response is ensured by two kinds of T cells bearing different tasks. CD8 T cells recognize Ag displayed on the surface of infected cells and end up killing them (Fig 4A). CD4 T cells, also called helper cells, do not kill the cognate cells, but activate them to kill microbes, and produce additional soluble cytokines and chemokines to trigger local inflammation. One subset of CD4 T cells (T\textsubscript{H1} cells) is important in the control of infections by activating macrophages. Another subset (T\textsubscript{H2} cells) stimulates B cells to secrete Ab (Fig. 4B and C). Both CD4 and CD8 T cells recognize their targets by detecting peptide fragments derived from foreign proteins.

CD8 T cells recognize MHC class I molecules that collect peptides from proteins of the pathogens (i.e. viral proteins) processed in the cytosol of the infected cell. CD4 T cells recognize MHC class II molecules binding to peptides derived from proteins in intracellular vesicles (Fig. 4).

Figure 4. Interaction between T cells and their target cells. (A) Cytotoxic CD8 T cells recognize viral peptides displayed by MHC class I molecules and kill the infected cells. (B) CD4 T\textsubscript{H1} cells recognize bacterial peptides presented by MHC class II molecules at the surface of macrophages and subsequently activate the latter. (C) CD4 helper cells recognize antigenic peptides displayed by B cells and activates them to produce Ab. Figure adapted from Immunobiology 6\textsuperscript{th} edition chapter 1.
3.2. The immune response to viral infection

As mentioned, cell-mediated immunity is the *adaptive immune response* to infections by intracellular microbes. Thus, it is important against viruses. While innate immunity and *humoral immunity* are also implicated in antiviral defenses, the present dissertation principally focuses on cell-mediated antiviral immunity.

Viruses bind to receptors on a wide range of cells, infect these cells and replicate in their cytoplasm. Many viruses cause cytopathic infections, where they kill and sometimes lyse the host cell after replication. On the other hand, some viruses cause latent infections, where the viral DNA persists in the nucleus and may become integrated in the host genome. Viral proteins, but not infectious viral particles, are produced in these infected cells. To handle such infections, cell-mediated immunity must first activate naïve T cells to proliferate and differentiate into effector cells, which in turn will eliminate both the host cell and its intruding virus simultaneously (7).

3.2.1 The surface T cells receptors (CD4 and CD8)

Each T cell bears about 30'000 identical Ag-receptor molecules on its surface. This receptor is referred to as the T-cell receptor (TCR), and is responsible of Ag recognition. It consists of two different polypeptides called α and β chains, respectively, linked via a disulfide bond (left panel of Fig. 5). Some T cells have an alternative γ:δ TCR, the function of which is not fully understood.

Both chains of the TCR have an extracellular amino-terminal variable region (V) with homology to the immunoglobulin (Ig) V region, a constant region (C) with homology to the immunoglobulin C region, and a short hinge region (H) with a cystein residue that forms the interchain disulfide bond. Each chain spans the plasma membrane by a hydrophobic transmembrane domain, followed by a carboxy-terminal hydrophilic intracytoplasmic tail (6). In addition to their TCR, functionally different CD8 and CD4 T lymphocytes express cluster differentiation types 8 and 4 surface molecules. These CD molecules are co-receptors that recognize the MHC class I and II molecules, respectively, on the surface of their cognate target cells. Figure 5 exemplifies the situation with CD8 and MHC class I.
Fig. 5 T cell receptor, CD8 co-receptor and MHC class I molecule. The T cell receptor heterodimer is composed of two transmembrane glycoprotein chains α and β. The extracellular portion consists of a variable and a constant region which both have carbohydrate side chains attached. The short hinge region contains a cystein residue, which forms a disulfide bond to connect the two chains. The CD8 molecule is a heterodimer of an α and a β chain linked by a disulfide bond. MHC class I molecules are expressed on cells of the lymphoid tissue (T cells, B cells, macrophages, other Ag-presenting cells as for example Langerhans’ cells and weakly on epithelial cells of the thymus) and other nucleated cells (neutrophils, weakly on hepatocytes, cells of the kidney and brain). They are heterodimers of a membrane-spanning α chain noncovalently bound to a β2-microglobulin. The α chain folds into three domains: α1, α2 and α3. The folding of the α1 and α2 creates a long cleft which is the site at which peptide Ag bind to the MHC molecules. Figure modified according to chapter 3 Immunobiology 6th edition

T cell precursors are produced in the bone marrow, and migrate to the thymus where they differentiate (for ca. one week) before entering a phase of intense proliferation. In young adult mice, the thymus contains around 10^8 to 2 x 10^8 thymocytes. Every day about 5 x 10^7 new cells are generated but only about 2-5% of these new cells leave the thymus as mature T cells to colonize lymphoid organs. About 95% of the cells die within the thymus by apoptosis.

The development of mature T cells is characterized by changes in the status of T cell receptor genes, in the expression of TCR and by changes in the expression of cell surface proteins such as CD3 complex and the co-receptor proteins CD4 and CD8. The stages of maturation are illustrated in Figure 6. In early development, two distinct lineages of T cells with different TCR (α:β or γ:δ) are produced. Later, α:β T cells develop into the two functional subsets, namely CD4 and CD8 T cells, as mentioned above. Development of α:β T cells proceeds
through stages where both CD4 and CD8 are expressed by the same cell. These double-positive cells first express a pre-TCR (pTα:β). They enlarge and divide. Later they become small resting double-positive cells with low expression of the TCR (α:β). Among these cells, those which carry a TCR able to interact with self-peptide:self-MHC molecular complexes (it has to be mentioned that cells undergo apoptosis when the receptor strongly recognize the MHC-peptide in the thymus a process called negative selection [for review see (8-10)]. This happens when self-peptide is abundant in the thymus and therefore everywhere in the body and with TCR with high affinities) lose either expression of CD4 or CD8 to become single positive thymocytes.

At the same time, they increase their level of TCR expression. These small, resting single-positive thymocytes are then exported from the thymus as mature single-positive CD4 or CD8 T cells.
Double negative thymocyte precursors develop into CD3⁺ cells with different TCR (either γ:δ or α:β). α:β T cells then develop into CD4 and CD8 double positive cells with a precursor TCR. They enlarge and divide. Later they become small and resting again. Most cells die by apoptosis. The surviving cells develop in either CD4 or CD8 single positive thymocytes, which are then exported via blood as mature single positive CD4 or CD8 T cells. Figure modified according to chapter 7 Immunobiology 6th edition.
3.2.2 The Th1 - Th2 decision of CD4 T cells

Upon activation (see Fig. 4, page 19), naïve CD4 T cells can differentiate into either Th1 or Th2 cells. These cells differ in their type of cytokine production and consequently in their function. Their differentiation fate is decided after the first encounter with the Ag. Stimuli that determine whether a proliferating CD4 T cell will differentiate into a Th1 or a Th2 cell are not fully understood. Factors such as cytokines elicited in response to the specific infectious agent (IFN-γ, IL-12 and IL-2), co-stimulators and the nature of peptide:MHC ligand all play a role. Selective production of Th1 cells leads to the production of opsonizing Ab classes (predominantly IgG) and thus is crucial for the activation of macrophages. Th1 cells also boost cell-mediated immunity by producing IL-2 and IFN-γ. IL-2 is required for CD8 T cell proliferation and IFN-γ stimulates amongst others CD8 T cell action by upregulating the expression of the MHC class I molecules of their target cells. In the presence of IL-12 (produced by dendritic cells or macrophages) and IFN-γ (secreted by NK cells or CD8 T cells), CD4 T cells generally develop into Th1. This is partly due to the inhibition of Th2 cell proliferation by IFN-γ.

Th2 cells activate B cells, especially in primary responses. Thus, they favor humoral immunity, and especially the secretion of IgM, IgA and IgE. The presence of IL-4 and especially of IL-6 preferentially targets CD4 cells to the Th2 differentiation. The initial source of IL-4 is not yet entirely clear, but it may be the mast cells. IL-4 or IL-10 either alone or in combination inhibits the generation of Th1 cells.

Hence, although CD4 and CD8 cells apparently guarantee different functions, they are closely linked by a subtle network of cytokines, chemokines and effector cells, the synchronization of which is critical to control both infection, via an inflammatory response, and damper exuberant inflammation, in order to preserve the host from uncontrolled tissue damages.

3.2.3 The special case of CD8 T cells

Because CD8 T cells are implicated in the control of viral infections, they are of special interest in the present thesis. The CD8 co-receptor is a disulfide-linked heterodimer consisting of an α and a β chain (different from the α and β chain of TCR), each consisting of a single immunoglobulin-like domain linked to the membrane by a segment of extended polypeptide chain (see middle panel of Fig. 5, page 21). CD8 binds weakly to an invariant site in the α3 domain of an MHC class I molecule (see right panel in Fig. 5, page 21) and also interacts with...
the $\alpha_2$ domain of this molecule. CD8 binds to the membrane-proximal domain of MHC class I, leaving the rest of the receptor available for interaction with the TCR. CD8 also binds Lck, a molecule of the signaling cascade, and brings it in close proximity to the TCR. On their side, MHC class I molecules present short peptides of 8 to 10 amino acids derived from the infecting microbes (MHC class II molecules bind peptides of at least 13 amino acids) in a specific groove. These peptides, presented in the context of the rest of the MHC molecule, drive recognition by specific cognate T-cells.

3.2.3.1 CD8$^+$ cytolytic T lymphocytes (CTL)

CTL recognize MHC class I-associated peptides on infected cells and kill these cells to eliminate the reservoir of infections. CTL adhere tightly to cells mainly through integrins (e.g. lymphocyte-function-associated Ag-1 [LFA-1]) on their surface, which bind to their ligands (e.g. intracellular adhesion molecule-1 [ICAM-1]) on the infected cell. Cell adherence results in CTL activation, which in turn triggers a signal transduction pathway leading to the exocytosis of the contents of their granules (i.e. granzymes which target cells through perforin pores). Each CTL can kill a target cell, detach, and proceed to kill additional target cells. CTL also secrete cytokines, such as tumor necrosis factor (TNF) and IFN-$\gamma$, which play a role in the inflammatory and antiviral responses (11, 12).

3.2.3.2 CD8 memory T cells

CD8 T cells express numerous additional surface molecules that help identify both differentiation and functional stages. For instance, expression of the chemokine receptor CCR7 controls homing to secondary lymphoid organs. CCR7$^+$ memory cells mediate immediate effector function and home preferentially to inflamed tissues. In contrast, CCR7$^+$ T cells lack immediate effector function and home to secondary lymphoid organs, such as the lymph nodes.

At least four differentiation subsets of CD8 memory T cells were found based on the co-expression of CCR7 and supplementary CD45RA cluster differentiation molecules (Fig. 7) (13, 14). First, CD45RA$^+$CCR7$^+$ cells, which are the precursors for the other subsets, and expand upon Ag re-encountering to ensure continuous replenishment. Second, a subset of CD45RA$^+$CCR7$^+$ CD8 T cells, also called central memory T cells (T_{CM}). Third, a subset of CD45RA$^-$CCR7$^-$ cells, referred to as effector memory cells (T_{EM}). Fourth, a subset of memory
cells that re-express CD45RA after transient downregulation of it, representing the terminally differentiated CD45RA⁺CCR7⁻ cells. These cells are effectors cells that can rapidly intervene following Ag re-encounter. They secrete IFN-γ and show high levels of perforin expression (13).

Additional markers include the CD27 and CD28 co-stimulatory molecules, which also provide information on the CD8 T cell developmental stage (15-18). These co-stimulatory molecules are downregulated with progressive differentiation. CD45RA⁺CD27⁺CD28⁺ T cells represent early memory cells. CD45RA⁺CD27⁻CD28⁺ T cells are at an intermediate stage. CD45RA⁺CD27⁻CD28⁻ T cells are at a late differentiation stage. And CD45RA⁺CD27⁻CD28⁻ cells are effector cells with a potent ex vivo cytolytic activity (15-18).

3.2.3.3 Additional cluster differentiation (CD) markers

Brenchley et al. (19) reported that CD57 was a marker of replicative senescence. They showed that CD8⁺CD57⁺ T cells are unable to proliferate, have shorter telomere length, and have significantly lower numbers of T cell receptor excision circles (TREC) compared to CD57⁻ cells. This means that this subset of CD8 T cells has undergone more rounds of proliferation and might be at the end of the differentiation process. In addition, they could further show that the CD57⁺ subset is more susceptible to apoptosis, which strengthens the hypothesis of being terminally differentiated.

Aandahl et al. (20) used the expression of CD7 as a new marker of T cell differentiation. They found three subsets according to the level of expression of CD7. CD7high, CD7low and CD7neg. The CD7high subset contained both naïve and memory CD8 T cells, whereas the other two subsets were only composed of memory cells. The latter two subsets showed significantly higher levels of cytokine or perforin levels than CD7high cells. Cells secreting cytokines tended not to express perforin and vice versa. Therefore, the authors proposed that these cells can be further divided into cytokine producing effector T cells and cytolytic effector T cells. CD7high cells were able to proliferate in contrast to the other two subsets.
Simone C. Zimmerli 3. Introduction

Figure 7. Lineage differentiation pattern of memory CD8+ T lymphocytes. The model is based on the identification of four subsets of memory CD8 T lymphocytes characterized by the expression of CD8, CD45RA and CCR7. CD8−CD45RA−CCR7+ cells function as precursor cells for the other memory subsets. CD45RA−CCR7− cells are the most differentiated cells and therefore referred to as terminally differentiated memory cells whereas the CD45RA−CCR7+ are the central memory cells and the CD45RA−CCR7− effector memory cells. The capacity of effector function increases with increasing degree of differentiation. Figure from Champagne et al. (2001) *Nature* 410:106-11

3.2.3.4 Cytokine secretion and T cell proliferation

Cytokines are small soluble proteins secreted by one cell that can either alter its own behavior and properties (autocrine effect), or alter the behavior of other cells (paracrine effect). Most cytokines produced by T cells are called interleukin (IL) followed by a number (6). Most of them demonstrate different biological effects.

*Interferon-γ (IFN-γ)*: The main cytokine released by CD8 effector T cells is IFN-γ, which can block viral replication or even lead to the elimination of virus from infected cells without killing them. This includes lowering the intracellular concentration of tryptophan, and hence decreasing synthesis of polypeptides from the pathogen.

Additional effects of IFN-γ are recruiting macrophages to the infection site and activating them into APC, and increasing the expression of MHC class I and other molecules involved in MHC peptide loading for improved recognition of target cells by CD8 effectors. IFN-γ further
activates NK cells. The importance of IFN-γ for intracellular infections was demonstrated in IFN-γ knock-out mice, which had an increased susceptibility to infections by intracellular bacteria such as *Mycobacterium* spp., as well as some viruses (6).

**Interleukin-2 (IL-2):** Another important cytokine is IL-2. It is produced by an array of cells including naïve T cells, CD4 T\(_{H1}\) cells and some CD8 T cells. In B cells, IL-2 stimulates both cell division and J-chain synthesis. In T cells and NK cells, IL-2 stimulates principally cell division.

Resting T cells constitutively express a moderate affinity IL-2 receptor, which allows them to respond to very high concentrations of IL-2. Upon activation they resume rapid division, thus providing the progeny that will differentiate into armed effector cells. The source of IL-2 is multiple (see above) and includes activated T cells themselves. Activation of T cells by specific Ag triggers the expression both of IL-2 and of the IL-2 receptor α chain. The α chain then clusters with the β and γ chains to form a heterotrimer with high affinity for IL-2. This high affinity allows the newly activated T cells to respond to low concentrations of IL-2. This leads T cells to divide two or three times a day, allowing one single cell to give rise to a clone of thousands in a few days, all carrying the same antigen receptor (Fig. 8). In addition of being a stimulator, IL-2 is also a survival factor for these cells. Removal of IL-2 from activated T cells results in their death (6).
Figure 8. IL-2 secretion. IL-2 secreted by activated T cells induces T cell proliferation. Resting T cells express moderate affinity IL-2 receptor consisting of only the $\beta$ and $\gamma$ chains. Once T cells become activated they start to express IL-2 and the IL-2R$\alpha$ chain. IL-2 binds then to this high affinity IL-2 receptor consisting of an $\alpha$, a $\beta$ and a $\gamma$ chain, and thus promotes T cell proliferation in an autocrine fashion. Figure adapted from chapter 8 Immunobiology 6th edition.
3.3. General characteristics of viruses

Viruses are small obligate intracellular parasites. This means that they are strictly dependent on the host cell’s machinery for their replication. The genome of viruses consists either of DNA or RNA, but never both. The nucleic acids may be single stranded (ss) or double stranded (ds) and linear or circular. The genome may be constituted of either one single polynucleotide (in the majority of cases) or by several polynucleotides, as in the Influenzaviruses (Flu) (Table 1). Viruses are classified by the "International Committee on Taxonomy of Viruses" according to their type of nucleic acids, their mode of replication and their shape.

3.3.1 Morphology

Four groups of viruses exist according to their shape: helical, polyhedral, enveloped and complex viruses. Helical viruses have a helicoidal structure (e.g. viruses that cause rabies or Ebola). Polyhedral viruses have complex symmetric shapes, e.g. icosahedron, or polyhedron with 20 triangular faces and 12 corners (e.g. adenovirus, poliovirus). When viruses are enclosed by an envelope, they are called enveloped viruses (e.g. Influenzavirus is a helical enveloped virus; and CMV is a polyhedral enveloped virus). Complex viruses are viruses with a more complicated structure as it is seen in mammalian poxviruses and bacterial bacteriophages Lambda and T4.
### Table 1. Classification of Viruses

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<td>I</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>Rubella virus</td>
<td>SS (+) RNA</td>
<td>10-12</td>
<td>Yes</td>
<td>I</td>
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<tr>
<td>Flaviviridae</td>
<td>Yellow fever virus</td>
<td>SS (+) RNA</td>
<td>10-12</td>
<td>Yes</td>
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<td>SS (+) RNA</td>
<td>20-33</td>
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<td>Rhabdoviridae</td>
<td>Rabies virus</td>
<td>SS (-) RNA</td>
<td>13-16</td>
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<td>Paramyxoviridae</td>
<td>Measles virus</td>
<td>SS (-) RNA</td>
<td>15-16</td>
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<tr>
<td>Filoviridae</td>
<td>Ebola virus</td>
<td>SS (-) RNA</td>
<td>19</td>
<td>Yes</td>
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<td>Arenaviridae</td>
<td>Lymphocytic choriomeningitis virus</td>
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<td>5-7</td>
<td>Yes</td>
<td>H</td>
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<td>Bunyaviridae</td>
<td>California encephalitis virus</td>
<td>3 circular SS RNA segments</td>
<td>10-23</td>
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<td>Orthomyxoviridae</td>
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<td>12-15</td>
<td>Yes</td>
<td>H</td>
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<td>SS (+) or (-) DNA</td>
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<td>No</td>
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</tr>
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<td>JC virus</td>
<td>Circular DS DNA</td>
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<td>I</td>
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<td>I</td>
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<td>I</td>
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<td>Vaccinia virus</td>
<td>Linear DS DNA with covalently closed ends</td>
<td>130-375</td>
<td>Yes</td>
<td>complex</td>
</tr>
</tbody>
</table>

3.4. Viruses used in these studies

3.4.1 Human Immunodeficiency Virus (HIV-1)

3.4.1.1 HIV genes and lifecycle

HIV-1 (Fig. 9) is a complex retrovirus encoding 15 distinct proteins (21). The genome of HIV-1 encodes 9 open reading frames. Three of these reading frames encode the polyproteins Gag, Pol and Env. These polyproteins are subsequently proteolyzed into individual proteins that are common in all retroviruses.

Gag is cleaved into matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins. Env gives rise to surface (SU or gp120) and transmembrane (TM or gp41) proteins. These proteins are the structural components of the retrovirus. They are found in the core and outer membrane.

The Pol polyprotein is proteolyzed into protease (PR), reverse transcriptase (RT) and integrase (IN) which all provide essential enzymatic functions.

Six additional - so-called accessory proteins - are encoded by the HIV-1 genome. Vif, Vpr and Nef are found in the viral particle, Tat and Rev provide essential gene regulatory functions and Vpu indirectly assists in the assembly of the virion.

**Figure 9. Genetic organization and structure of HIV-1 virus.** The HIV-1 genome encodes 9 open reading frames which give rise to the three polyproteins Gag, Pol and Env and six accessory proteins, Vif, Vpr, Vpu, Tat, Rev and Nef. For more details see text. Figure adapted from Frankel *et al.* (1998) *Annu. Rev. Biochem* 67:1-25
The HIV-1 replication cycle can be divided in 15 steps (Fig. 10)(21). It begins with the transcription of the viral genes, which are expressed from the promoter located in the 5’LTR. The rate of transcription is greatly enhanced by Tat (step 1). A set of spliced and genomic length RNA is transported from the nucleus to the cytoplasm (step 2). This is regulated by Rev. The viral mRNA is then translated (step 3). The Gag and Gag-Pol polyproteins are targeted to the cell membrane, whereas Env is targeted to the endoplasmatic reticulum (ER), where it tends to become associated with naturally synthesized CD4. Thereafter the core Gag and Gag-Pol polyproteins (which are later processed into MA, CA, NC, p6, PR, RT and IN), the Vif, Vpr and Nef proteins and the genomic RNA are assembled into an immature virion, which buds at the cell surface (step 4).

To mature Env into plasma membrane protein SU and TM, the Env polyprotein must be released from the complex with CD4. Vpu assists the degradation of CD4 (step 5). The Env polyprotein is then transported to the cell surface (step 6). To prevent further binding of Env to CD4 at the cell surface Nef promotes endocytosis and degradation of surface CD4 (step 7). The particle then buds at the cell surface and is released from the host cell (step 8).

During the budding step, the particle is coated with SU and TM. Once released from the cell, the virion undergoes maturation, which involves morphologic changes such as proteolytic processing of the Gag and Gag-Pol polyproteins by PR and Vif (step 9). The mature virion is now ready to infect the next cell by interaction of SU with CD4 and CC or CXC (step 10). Binding of the virion to the new cell induces conformational changes in TM, which promotes the fusion of the viral membrane and cell membrane, thus allowing entry of the viral core into the cytoplasm (step 11). Once inside, the virion is uncoated and the viral core (MA, RT, IN, Vpr and RNA) becomes exposed (step 12). The nucleoprotein complex is transported into the nucleus (step 13), where the genomic RNA is reverse transcribed by RT into partially duplex linear DNA (step 14). After replication, IN catalyzes the integration of viral DNA into the host chromosome (step 15).
3.4.1.2 HIV-1 and the Immune System

**Humoral immunity**

Neutralizing antibodies arise against both Env and gp41 proteins, which are exposed at the surface of the virus. The first anti-Env response targets the V3 loop of the protein. This neutralizing Ab response is specific for different viral isolates (22). Because this is a variable target, it is not appropriate vaccine development. A second anti-Env neutralizing Ab response targets the Env binding site to the CD4 receptor on CD4 helper cells. This binding site is highly conserved among viral isolates. However, Ab directed against this site are weakly neutralizing. A third target of neutralizing Ab is the transmembrane gp41 (23), but its protective efficacy is not demonstrated. Thus, although a neutralizing Ab-response does exist against HIV-1, humoral immunity does not contribute much to blocking viral replication.
the contrary, it rather selects for viral variants that may pass undetected by existing Abs (23, 24).

**Cell-mediated immunity**

**CD8 T cells:** There are several arguments that point to the importance of specific CD8 T cell response to control HIV-1 infection. First depletion of CD8 T cells by specific anti-CD8 monoclonal antibodies resulted in a failure to control the early peak of viremia in infected animals (25). Second HLA types may significantly influence the rate of HIV-1 disease progression. HLA-B27 and HLA-B57 haplotypes are associated with slow disease progression, whereas HLA-B35 is associated with faster disease progression. This phenomenon could be due to differences in the capacities of HLA molecules to present the Ag (26). Third HIV-specific CD8 T cell responses have been observed in subjects and/or animals that had been exposed to the virus but did not become infected (27-29). In primary HIV-1 infection, a potent CTL response coincides with the peak in viremia and precedes the Ab response (30-32). HIV-1-specific CD8 T cells are found in large numbers in a variety of anatomic compartments, such as peripheral blood, bronchoalveolar spaces, lymph nodes, spleen, skin, cerebrospinal fluid, semen, and vaginal and gastrointestinal mucosal tissues. These CD8 T cells were shown to inhibit viral replication *in vitro* (33). They secrete MIP-1α, MIP-1β and RANTES to lyse HIV-1 infected cells and block viral propagation (34). Moreover, there is an association between the appearance of CTL and a decline in the viral RNA in primary infection (30, 31, 35).

Yet, despite the appearance of a vigorous CTL response during primary HIV-1 infection the immune system is unable to sufficiently control the infection. CD8 T cells show abnormalities at both functional and maturation levels. Appay *et al.* (36) showed a selective defect in the levels of intracellular perforin, and two other studies demonstrated poor lytic activity in freshly isolated HIV-1-specific T cells (37, 38). HIV-1-specific CD8 T cells show a skewed maturation, the memory population of HIV-1-specific CD8 T cells being mainly composed of preterminally differentiated cells. In comparison, CMV-specific CD8 T cells are predominantly terminally differentiated cells (14). Eventually, certain clones of HIV-1-specific CD8 T cells are rapidly deleted during primary infection (39). CD8 T cells expressing Fas receptor – a receptor that renders cells susceptible to apoptosis - are significantly higher in HIV-1 infected than in non-infected individuals and the difference is even more pronounced in patients with a more advanced stage of infection (40). A decreased expression of Bcl-2 – a repressor of apoptosis – was shown *ex vivo* as well in CD8 as CD4 T cells and
there was an association with apoptosis of those cells. Interestingly antiretroviral therapy increased the proportion of Bcl-2 expressing T cells (41).

**CD4 T cells:** Virus-specific CD4 T lymphocytes also have an important role in controlling HIV-1 replication. Indeed, many HIV-1 infected individuals have virus-specific CD4 T cells (42, 43). Rosenberg et al. (44) showed that the magnitude of the proliferation and cytokine secretion in HIV-1-specific CD4 T cells correlated with the clinical status. Several studies have reported a selective defect of HIV-1-specific CD4 helper T cells in progressive HIV-1 infection. In contrast, HIV-1-specific CD4 T cells with effector function (secreting IFN-γ and TNF-α) persist (42, 44, 45).

The decrease of CD4 T cells with the progression of HIV could be shown to be independent of Fas expression (40). As mentioned in the preceding paragraph CD4 T cells expressing low levels of Bcl-2 can be detected. But in apoptotic cells not only Bcl-2 but also CD4 are downregulated and therefore it is likely that the percentage of apoptotic CD4 T cells is underestimated. The same is true for CD8 T cells even though in a smaller extend (41).

In long term non-progressors (LTNP, see next paragraph) the situation is different. Both, HIV-1 specific CD4 helper and effector T cell responses can be found (46).

### 3.4.1.3 Clinical manifestations

The first cases of AIDS were described in the United States in 1981, in homosexual males from New York and San Francisco. These patients presented with unexplained opportunistic infections, such as *Pneumocystis carinii* (recently renamed *Pneumocystis jirovecii*) pneumonia and *Kaposi’s sarcoma* (47-49).

Primary HIV infection is difficult to diagnose, because symptoms such as fever, lethargy, malaise, sore throat, arthralgias, headaches, retroorbital pain, photophobia, lymphadenopathy and *maculopapular rash* are non-specific and can precede the humoral immune response to HIV (50-53). The duration of these symptoms can last from a few days up to more than 10 weeks. However, usually the duration does not go beyond 14 days (54).

Primary infection is followed by a long phase of clinical latency, which lasts in typical progressors between 8-10 years in absence of treatment. A minority of patients, the so-called rapid progressors, develop AIDS within 2-3 years after primary infection (55, 56). Figure 7 shows the natural history of HIV-1 infection in a typical untreated patient. By the end of the disease, the count of CD4 T cells drops radically and is accompanied by sharp increase in
viral load. With the introduction of highly active antiretroviral therapies (HAART), the mortality rate dramatically decreased. In the United States death due to AIDS decreased by 23% in 1996 and by 44% in 1997 (57, 58). A study of the Swiss HIV cohort showed that treatment with HAART lead to a mean reduction of viremia of 1.8 log_{10} copies per milliliter. 63% of the patients reached undetectable viremia. Regarding CD4 counts the course was more variable. After a peak-increase of 40 cells per microliter during the first 6 months, a gradual decrease was observed thereafter (59). Another study performed in Switzerland revealed that the risk of death in patients treated with HAART is equal to that in patients with cured cancer (60).

**Figure 11.** Natural history of HIV infection in absence of therapy (from Fauci AS *et al.* (1996) *Ann Intern Med* 124:654-663)
HIV infection can be divided in different stages. Table 2 shows the 1993 revised classification system. The last stage of HIV-infection is marked by a series of tumors, opportunistic infections and HIV-related neurological diseases (Table 2).

<table>
<thead>
<tr>
<th>CD4 T cell categories</th>
<th>CD4 T cell categories</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 500µl</td>
<td>A1</td>
<td>B1</td>
<td>C1</td>
<td></td>
</tr>
<tr>
<td>200-499µl</td>
<td>A2</td>
<td>B2</td>
<td>C2</td>
<td></td>
</tr>
<tr>
<td>≤ 200µl</td>
<td>A3</td>
<td>B3</td>
<td>C3</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Classification system for HIV-1 infection.** * AIDS-indicator conditions are listed in table 3. From CDC and Prevention 1993 (1992) *MMWR* 41:1-19

About 5% of the HIV-infected patients do not progress to HIV disease and have stable CD4 T cell counts within the normal range for many years (61, 62). This group of patients is called long term non-progressors (LTNP). Low degree of virus trapping is observed in lymph node biopsies of LTNP, and virus-expressing cells are rarely seen (63). The virological parameters (HIV-1 RNA and proviral DNA) are significantly lower (4-20 fold) compared to progressors (64). CD4 T cell counts are constantly in the normal range (>500 cells/µl), whereas CD8 T cell counts were found to be significantly increased (500-2500 cells/µl). The proliferative response to mitogens and alloantigens is conserved and a vigorous HIV-1-specific humoral and cellular immune response can be observed. HIV-1-specific cytotoxicity can be seen *ex vivo* or *in vitro*. On histology, there is a lower degree of reactivity of lymphoid tissues, as compared to progressors (50).
- multiple or recurrent bacterial infections
- Candidiasis of bronchi, trachea or lungs
- esophageal Candidiasis
- invasive, cervical cancer
- disseminated or extrapulmonary Coccidioidomycosis
- extrapulmonary Cryptococcosis
- chronic intestinal Cryptosporidiosis (>1 month duration)
- CMV disease (other than liver, spleen, or nodes)
- CMV retinitis (with loss of vision)
- HIV related encephalopathy
- Herpes simplex, chronic ulcer(s) (> 1 month duration); or bronchitis, pneumonitis, or esophagitis
- disseminated or extrapulmonary Histoplasmosis
- chronic, intestinal Isosporiasis (> 1 month duration)
- Kaposi’s sarcoma
- Lymphoid interstitial pneumonia and/or pulmonary lymphoid hyperplasia
- Burkitt’s lymphoma
- immunoblastic lymphoma
- primary lymphoma of the brain
- disseminated or extrapulmonary *Mycobacterium avium-intracellulare* complex or *Mycobacterium kansaii*
- pulmonary or extrapulmonary (any site) *Mycobacterium tuberculosis*
- disseminated or extrapulmonary *Mycobacterium* (other or unidentified species)
- *Pneumocystis jirovecii* pneumonia
- recurrent pneumonia
- progressive multifocal leukoencephalopathy
- recurrent *Salmonella* septicemia
- Toxoplasmosis of the brain
- wasting syndrome of HIV infection

**Table 3.** AIDS indicator conditions (adapted from Mandell 6th edition)
3.4.1.4 Treatment and prevention

Treatment

HIV-1 infection is treated with antiretroviral therapies (ART). In general a combination of different drugs is indicated due to development of drug resistances. It is now common to administer so-called highly active antiretroviral therapy (HAART). Three classes of substances are used: nucleoside reverse transcriptase inhibitors (NRTI), non-NRTI (NNRTI) and protease inhibitors (PI). NRTI are used in combination with PI and/or NNRI. Combinations of two nucleoside analogs RT inhibitors with a potent protease inhibitor have shown to be effective (65, 66). It is important to underscore that HAART cannot eradicate the virus. AIDS is therefore still not curable.

NRTI target the enzyme reverse transcriptase. They are nucleoside analogues that lack 3’OH groups. Hence, when mistakenly incorporated in polynucleotide strands during transcription of replication, they irreversibly block strand elongation (67). Zidovudine (AZT) and D4T are thymidine analogs, DCC and 3TC are cytosine analogs, DDI is an inosin analog, and abacavir is a guanosin analog. Nucleoside analogs are activated inside the cell by phosphorylation. They are mainly eliminated via the kidney (67).

NNRTIs bind directly to the reverse transcriptase, close to the binding site of nucleosides. Examples for NNRTIs are neverapin, delavirdin and efavirenz. They do not require phosphorylation or intracellular processing to become activated (67).

Protein inhibitors inhibit maturation of the gag-pol polyprotein. This results in non-infectious particles (68). Protein inhibitors bind directly to the active site of the enzyme to inhibit its action. They result in a clear improvement of therapy, but they are associated with several problems, especially in long term treatment. They affect the lipid metabolism and can trigger lipodystrophy and dyslipidemia. Furthermore, they have quite a short half-life and have to be taken three times a day.

Post exposure prophylaxis

This intervention has been proposed since 1990 in case of inadvertent exposure to HIV. If provided within the first 24 hours following exposure, post exposure prophylaxis can attenuate viral replication and prevent systemic HIV infection. It is likely to act by blocking the infection of T cells in the regional lymph nodes. Additionally it might allow the development of a robust HIV-1-specific cellular response (67).
The efficacy of post exposure prophylaxis was demonstrated in animal models. (1) It delayed or completely suppressed viremia, (2) it inhibited viral replication and resulted in a long-lasting, protective cellular immune response, and (3) it provided complete protection (69-74). Importantly, protection appeared closely dependent on both the schedule of drug administration and the window between exposure and treatment start. Tsai et al. (70) showed in a macaque/SIV study that all the macaques treated for 28 days remained uninfected, while 50% of the animals treated only for 10 days and none of those treated for 3 days were protected. Likewise, animals treated within 24 hours remained uninfected, whereas only half of the animal treated within 48 hours and 25% of the animals that started treatment 72 hours after exposure were protected (70). Thus, drug posology and timing of administration is critical.

The perspective of anti-HIV vaccines

Twenty-four years after discovery of the first AIDS cases, HIV infection is still one of the major health problems worldwide. In the year 2004, 39.4 million people living with HIV were counted, and 4.9 million were newly infected with HIV, whereas 3.1 million people died of AIDS (75). Especially in the Sub-Saharan Africa with 25.4 million HIV-infected individuals and no accessibility of HAART AIDS is a major problem (Fig. 12). To break through this pandemic a safe, effective and affordable vaccine would be the only solution. However, developing a vaccine against HIV requires overcoming several challenges:

- Natural infection with HIV does not result in protective immunity.
- HIV is difficult to neutralize with Ab for several reasons, including carbohydrate shielding of gp120 subunit, occlusion of envelope epitopes via oligomerization (76), conformational masking of receptor binding sites (77) and mutational variation of envelope variable loops (23, 24).
- There is both a progressive destruction and impaired regeneration, of CD4 helper cells (50).
- HIV has a high mutation rate and is therefore able to escape from humoral (78) and cellular response (35, 79, 80).
- High levels of HIV genetic diversity might require more than one vaccine for global prevention and prevent HIV superinfections in patients with broad CD8 T cell. (81-83).
Difficulties in generating neutralizing Ab is revealed by the failure of this approach in early vaccination studies (84-87). The first Phase III trial with recombinant monomeric HIV-1 envelope gp 120 vaccine (AIDSVAX; VaxGen Inc, Brisbane, CA, USA) showed no efficacy (84). Likewise, the second phase III trial of VaxGen’s AIDSVAX B/E composed of monomeric recombinant gp120s representing HIV subtypes B and E, did not provide anti-HIV protection either (86).

Because of these failures scientists now focus on eliciting a potent antiviral CD8 T cell response. This type of vaccine will not be able to prevent HIV infection, but might control viral expansion, thus slowing down the progression to AIDS and decreasing the risk of HIV transmission (88-91). Moreover, inducing HIV-specific CD8 T cell response before HIV infection might be favorable in preserving the function of CD4 helper cells, which in turn promote a sustained CD8 T cell response (92).

There are now several candidate vaccines focusing on the CD8 T cells in clinical evaluation. Most of them are still phase I trials, in order to assess safety and generate data to choose the best HIV immunogens and vector systems (92).

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**Figure 12. Geographic distribution of HIV-infections.** Figure from UNAIDS: AIDS epidemics update December 2004
3.4.2 Cytomegalovirus (CMV)

3.4.2.1 Viral structure, genes and lifecycle

CMV is a member of the human herpes virus family (β-Herpes viridae; Table 1). The virion of CMV consists of a 100 nm icosahedral nucleocapsid. It contains a 230 kb double stranded linear DNA genome, surrounded by a protein aqueous layer called the tegument. The tegument itself is enclosed by a lipid bilayer spun by several viral glycoproteins (Fig. 13). The mature virion particle is about 150-200nm in size (93).

![Figure 13. Structure of herpes viruses.](image)

The genome of CMV has a protein-coding capacity of 170-200 open reading frames (94). Predominant structural proteins of the virion are the envelope proteins gB and gH and the matrix proteins pp65/pp150/pp71 and pp28 (95). The life cycle of herpes viruses is depicted in Figure 14. It starts with attachment and penetration of the virus into the cell via cell surface proteoglycans (step 1). As in HIV, the membranes of the virus and the host cell fuse together, leading to the release of the viral particle into the cytoplasm. Inside the cell the viral DNA is uncoated and rapidly translocated to the nucleus (step 2). Sequential transcription (step 3A) of immediate-early, early and late viral genes in the nucleus is followed by their translation in
the cytosol (step 3B). DNA is replicated by the mechanism of rolling circle (step 4). The new viral genome is cleaved, assembled with viral proteins translocated from the cytosol and packed inside the nucleus (step 5). After viral maturation (step 6) the nucleocapsid buds out of the nucleus and acquires thus a primary envelope (step 7). In the cytoplasm the virion further matures through a de-envelopment/re-envelopment process thereby acquiring their tegument (step 8). The tegument capsids then receive their definitive envelope by budding into vesicles of the Golgi apparatus. Finally mature virions are released from the cell via an exocytosis-like pathway (step 9) (96-98). The CMV genome can also be kept latently in the cell. Productive infection is suppressed by histones binding to the genome establishing latency.

Figure 14. Life cycle of herpes viruses. 1. Herpes entry by attachment and penetration. 2. Release of viral DNA 3. Sequential transcription (A) and translation (B) of viral immediate-early, early and late genes 4. DNA replication by rolling circle mechanism 5. Assembly and packaging 6. Maturation and 7. Budding of the nucleocapsid out of the nucleus 8. further maturation 9. release of the virion via exocytosis-like pathway.
3.4.2.2 CMV and the Immune System

CMV is a very potent immunogen, which triggers all arms of the immune system. Humoral immunity is established early in infection. IgG antibodies measurement is the standard assay for determining infection history. However, the cellular immune response is thought to be the major mechanism by which viral replication is controlled.

CD8 T cell response seems to be the most important component of it, even though CD4 T cells and NK cells also play an important role in controlling CMV (99). CD8 T cell response is focused on two CMV proteins, which are the pp65 tegument protein and IE-1 protein (100, 101). The use of a pp65-deleted viral mutant showed that 70-90% of all the CD8 T cell clones isolated by limiting dilution analysis were specific for peptides derived from this protein. Nevertheless, it was recently observed that many other proteins also carry peptides that are epitopes for CD8 T cells (102). Such alternative epitopes are responsible for 1-2% of the total anti-CMV repertoire of CD8 T cells.

3.4.2.3 CMV pathogenesis and clinical manifestations

CMV initially enters the host via the epithelium layers of the upper alimentary tract, respiratory tract, or genitourinary tract (103). Leukocytes and vascular endothelial cells help in disseminating the virus. During acute infection, viral antigens are mainly found in neutrophils and monocytes (103). Virus encoded chemokines can also support the spread of the virus by attracting neutrophils and monocytes (104, 105). The hematogeneous spreading is followed by infection of ductal epithelial cells. CMV-infected cells can be found in the salivary gland, bile duct, bronchial and renal tubular epithelium, islet cells, epithelial cells of the inner ear, the capillary endothelium, astrocytes and neurons (106, 107). CMV resides in the host throughout life without causing any symptoms in healthy, immuocompetent individuals. Depending on the residence country and social status, up to 50-90% of adults are seropositive for the virus (108-110).

Primary infection is in most cases unapparent but can be associated with infectious mononucleosis (IM). IM is caused by EBV, HIV and in 21% of the cases by CMV (111). This can be associated with several complications including interstitial pneumonia specially in bone marrow transplanted (BMT) patients but rarely in immunocompetent hosts (111), hepatitis, mostly with mild or even without symptoms in immunocompetent individuals (67), Guillain-Barré syndrome (67, 112), meningoencephalitis (113), myocarditis, thrombocytopenia and hemolytic anemia as well as skin eruptions (67).
These symptoms are usually mild in immunocompetent patients, but can become dramatic in the immunocompromised host (see below). Primary CMV infection is followed by a prolonged, unapparent infection during which the virus remains alive but usually dormant and resides in cells without causing detectable damage or clinical illness.

Either primary or reactivated infection is frequent in immunocompromised patients. In the case of AIDS, CMV is the cause of the most common opportunistic viral infections (67). It can cause retinitis (114, 115), neurological infections such as encephalitis, polyradiculopathy, or peripheral neuropathy, and gastrointestinal infections such as esophageal ulcer, colitis, acute pancreatitis or cholecystitis (67).

Transplant patients also have a special risk of CMV infection, since the virus is either transmitted by the donor organ (primary infection) or reactivated due to immunosuppressive drugs (reactivation). The most common complication is CMV pneumonia in BMT patients, and a potentially fatal CMV hepatitis following transplantation of a liver, especially if the organ comes from a CMV positive donor. Following kidney transplantation, the generalized CMV syndrome is more frequent than CMV hepatitis or CMV pneumonia (67).

Eventually, a particular form of CMV disease is represented by intrauterine infection of the fetus. Intrauterine infection may occur in 0.5-22% of pregnant women. In the non-immune mothers it may be associated with serious disease of the fetus, including jaundice, hepatosplenomegaly, petechial rush, and multiorgan involvement. Neurologic complications include microcephaly, motor disability, chorioretinitis and cerebral calcifications (67).

### 3.4.2.4 Treatment and prevention

**Therapy**

Therapy includes 3 antiviral drugs inhibiting viral DNA polymerase for treatment of CMV end-organ disease, antisense inhibitor CMV directly injected into intravitreal fluid for treatment of CMV retinitis and antiviral therapy following BMT or solid organ transplantation (67).

In addition to classical antiviral drugs, several studies indicated that prophylactic treatment of patients with CMV-specific CD8 T cells results in a CD8 T cell response that was equivalent to the responses found in healthy seropositive subjects resisting CMV infection (95, 116-118). This was useful in the early period following allogeneic stem cell transplantation. This kind of therapy was also shown to be effective in a patient infected with a multidrug resistant CMV
isolate (119). Thus, adoptive transfer of CMV-specific T cell clones represents a new strategy for restoring protective host immunity and preventing CMV disease in these patients (116).

**Prevention**

CMV disease can be prevented after bone marrow or solid organ transplantation by antiviral therapy. Four independent studies from 1991-95 showed that ganciclovir administrated orally following transplantation was effective in preventing CMV pneumonia or other CMV associated diseases (67).

As for HIV, an effective vaccine against CMV is not yet available. However, several vaccine candidates have been or are being tested in humans. The first candidate is a live attenuated CMV isolate. The second is a chimera between an attenuated CMV and wild-type virus. The third is a non-replicating canarypox vector with either a pp65 core or a gB envelope Ag. The fourth is composed of a recombinant envelope glycoprotein vaccine. The fifth is a mixture of synthetic peptides including a CD4 T helper epitope, known CD8 epitopes and a lipid tail. Other vaccines such as a DNA vaccine and a recombinant vaccine are also proposed (120). All these candidates have proved safe. However, their efficacy in clinical trials has yet to be demonstrated (120).

### 3.4.3. Epstein-Barr virus (EBV)

#### 3.4.3.1 Viral structure, genes and lifecycle

EBV is a γ-herpes virus with a linear double-stranded DNA genome (Table 1). Viral replication involves expression of over 60 proteins involved in the lytic cycle (immediate-early [IE], early [E] and late [L] proteins). BZLF1 and BRLF1 act as transactivators and are IE genes, BMRF1, BALF2, BALF5, BBLF2/3, BBLF4, BSLF1 play a role as replication factors and are E genes. Gp350/220, VCA, Gp35, Gp25, Gp42 are structural proteins and are L genes (121). The BALF5 gene encodes the catalytic subunit of a DNA polymerase (122), BMRF1 the DNA polymerase accessory subunit (123-125) and BALF2 a ssDNA binding protein (126, 127). BBLF4, BSLF1 and BBLF2/3 are thought to form a tight complex, which acts as **helicase, primase** and helicase-primase associated proteins (128). It is suggested that all of them, with the exception of BZLF1, work at the replication fork to synthesize the leading and lagging strands of the **concatemeric** EBV genome (129). BZLF1 acts on viral replication and transcription as oriLyt binding protein and transactivator. It can mediate the switch between latent and lytic forms of the EBV infection, and is
sufficient for the activation of the lytic cascade (130). It further interacts with the CREB-binding protein to activate EBV early gene expression (131). BZLF1 can inhibit host cell proliferation by causing a cell cycle arrest in the G0/G1 phase (132, 133) and it can interact with the activated form of p53 inhibiting its transactivation function, which then prevents downstream signal transduction (134).

Beside the lytic cycle proteins, the EBV genome encodes nine latency-associated proteins. Six are localized in the nucleus and are called EBV nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C and LP, whereas three are membrane proteins and referred to as latent membrane proteins (LMPs) 1, 2A and 2B. EBNA1 links the viral genome to the cellular chromosome, which enables viral replication in dividing host cells as if it was a part of the cellular genome. EBNA2 controls the transcriptional machinery, which is required to block B-cell differentiation at the proliferative stage. EBNA3C allows the activated cells to progress into the G1/S boundary of the cell cycle.

LMP1 is a ligand-independent cell-surface signaling molecule providing a surrogate T-helper cell signal. It interacts with the signaling system of the tumor necrosis factor receptor (TNRF) family. It activates the nuclear factor κB (NFκB), c-Jun N-terminal kinase (JNK) and signal transducers and activators of transcription (STAT) pathways in order to provide both survival and growth signals. LMP2A is also a ligand-independent cell surface signaling molecule. It interacts with members of the Src family of tyrosine kinases through immunoreceptor tyrosine-based activation motifs (ITAMs), which are also found in the α- and β-chains of the B cell receptor (BCR). The signal triggered by LMP2A resembles the signal provided by the intact BCR for positive selection of B cells in the bone marrow and for survival of mature B cells in the periphery in absence of cognate Ag (135).

EBV specifically infects resting B cells via CD21 and HLA class II molecules on the cell surface (136). This infection first induces continuous proliferation, which results in lymphoblastoid cell lines. These cells express a limited number of gene products, such as EBNAs, LMPs, two EBV-encoded small RNAs (EBER1 and EBER2), and transcripts from the BamHI region. This type of cell cycle is called the latent phase. Quiescent B cells are activated to enter the cell cycle, to maintain continuous proliferation and are prevented from apoptosis (121). During the latent cell cycle EBV genomic DNA, which is in form of a closed circular plasmid, behaves like the host chromosomal DNA. It associates with cellular histones, it replicates only once during the S phase and is then distributed to the daughter cells (137-139). Only one viral cis element, the OriP and one viral protein, EBNA1 is required for maintenance (140, 141).
The latent cell cycle can change into a lytic cell cycle, where multiple rounds of replication are initiated within the OriLyt (130). In the lytic cell cycle, the replication has a greater dependence on EBV-encoded proteins (128). The IE lytic genes BZLF1 and BRLF1 are expressed to activate viral and certain cellular promoters and to lead to a cascade of viral gene expression. First early genes which play a role in DNA replication and metabolism are expressed followed by late genes, which code for structural proteins. The EBV genome is amplified 100- to 1000 fold.

3.4.3.2 EBV and the immune system

During primary infection, anti-virus capsid antigen (VCA) IgM and anti-early antigen (EA) IgM are secreted followed by a transient development of anti-EA IgG. This is found in about 80% of the infected individuals. Anti-EBNA IgG are absent during acute infection (142). Early in the acute phase of infection, NK cells limit the number of EBV-infected cells (143). Later a large proportion of CTL that are directed towards EBV emerge. There are two immunodominant EBV lytic cycle epitopes and one immunodominant EBV latent cycle epitope. During primary infection, there is a massive expansion of activated Ag-specific CD8 T cells, which can persist at in relatively high proportion for at least three years after primary infection (144).

During acute infection the cellular response to lytic cell cycle proteins is about ten times stronger than to latent cycle proteins. In follow-up samples at 6 and 37 months after primary infection, the response to lytic proteins decreases, but is still detectable. In contrast, the frequencies of CD8 T cells specific to latent proteins is similar during acute and chronic infection (144).

The EBV glycoprotein gp350 binds to B cells via CD21, whereas gp85 plays an important role in the fusion of the virus to the host cell. Neutralizing Ab are directed against these two glycoproteins, and a CTL response induced against these targets have shown to protect mice against infection with recombinant vaccinia virus expressing those epitopes (145).

Viral escape strategies

EBV has developed several strategies to avoid host defense. It limits gene expression during latency in B cells, produces proteins that inhibit apoptosis and Ag processing, and expresses a cytokine and a soluble cytokine receptor.
There are different viral proteins modulating the immune response. LMP-1 is the homolog of cellular CD40. It binds TNF-receptor-associated factors, activates B cells, stimulates B cell proliferation, up regulates NFκB and kinase and inhibits apoptosis (146-155). BHRF1 is a homolog of bcl-2 and therefore inhibits apoptosis (156). BARF1 a homolog of Colony stimulating factor 1 receptor inhibits proliferation and function of macrophages (157). BCRF1 an IL-10 homolog inhibits IFN-γ and IL-12, promotes B cell growth and inhibits dendritic cells (158, 159) and EBNA-1 is able to inhibit antigen presentation (160).

3.4.3.3 EBV pathogenesis, clinical manifestation and complications

EBV preferentially infects B cells and epithelial cells but is also able to infect NK cells and smooth muscle cells (161, 162). EBV infects B cells through binding of gp350 to CD21 and gp42 to HLA II as co receptor (136). The incubation period lasts between 2 and 7 weeks (163).

Primary infection is often unapparent. The classical clinical manifestation is a glandular fever tonsillitis also called infectious mononucleosis (IM). Rarely a virus-associated haemophagocytic syndrome (HPS) occurs (164, 165). During primary infection, a marked lymphocytosis is common, due to an hyperexpansion of CTL reacting to lytic and latent viral Ag (149). In rare cases, IM can trigger several complications including neutropenia, thrombocytopenia, splenic rupture, airway obstruction by tonsillar hypertrophy, central nervous system involvements and acute liver failure (166).

In the latent phase, several complications can occur such as different types of neoplasia and hematological disorders including X-linked lymphoproliferative disorder (Duncan disease), malignant lymphomas in patients with AIDS or immunosuppressive therapy, gastric cancer and pleural lymphoma (167-170). In some geographical regions, hematological disorders occurs in association with endemic EBV infection (Table 4) (171-177).

<table>
<thead>
<tr>
<th>Africa</th>
<th>Burkitt’s lymphoma</th>
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<tr>
<td>Southern China</td>
<td>Nasopharyngeal carcinoma</td>
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<tr>
<td>Asian and central and south American countries</td>
<td>Nasal-type NK/T cell lymphomas</td>
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<td>Japan</td>
<td>Chronic active EBV infection</td>
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<td>EBV-associated haemophagocytical</td>
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<td></td>
<td>Lymphohistiocytosis (178)</td>
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Table 4. Endemic appearance of EBV associated hematological disorders
3.4.3.4 Treatment

There is no established treatment against EBV infection. Thus supportive therapy is given. More than 95% of the patients recover without specific therapy.

The treatment of lymphoproliferative secondary complications usually consists in decreasing the immune suppression, in patients receiving active immnosuppressive therapy (regression in up to 50% of the cases) or antiviral therapy generally combined with immunoglobulins. However latently EBV infected cells do not express EBV DNA polymerase. Therefore, polymerase inhibitors do not act against latent EBV. In addition, Ab against B cells or cellular immunotherapy, which reconstitutes the cellular immune response, may be tried. The disadvantage of this therapy is the induction graft versus host disease by the infused alloreactive T cells in transplanted patients (67).

3.4.4 Influenzavirus (Flu)

3.4.4.1 Viral structure, genes and lifecycle

The Influenza virus belongs to the family of Orthomyxoviridae (Table 1 and Fig. 15)(179). Its genome is constituted of 8 single-stranded RNA segments, referred to as negative strands because the viral mRNAs are transcribed directly from them. The entire genome is embedded in a helical-shaped nucleocapsid, further enveloped in a lipid bilayer acquired by budding through the membrane of the host cell.

The whole virion has a diameter of about 80-120nm. The Influenza A virion has a layer of about 500 spikes (haemagglutinin [HA] and neuraminidase [NA]) radiating through the outer membrane (179).

The first RNA segment encodes for PB2, which is responsible for Cap binding and endonucleolytic cleavage. Segment 2 encodes for a basic polymerase 1 (PB1), which is an RNA transcriptase. Segment 3 encodes for an acidic polymerase (PA) of unknown function. Segment 4 encodes for haemagglutinin (HA). HA is exposed at the surface of the virus and mediates viral attachment to cellular receptors and subsequent membrane fusion. Segment 5 encodes for the nucleoprotein (NP). NP encapsulates the RNA and plays a role in the regulation of transcription and translation. Segment 6 encodes for neuraminidase (NA). NA is part of the envelope and cleaves sialic acid from the cell surface. It facilitates the release from membranes and prevents aggregation. Segment 7 encodes for the matrix proteins M, which surrounds the viral core and controls nuclear transport and M2. M2 is an ion channel required for uncoating the virus after entry into the cell. Segment 8 encodes for nonstructural proteins.
NS1 and NS2. NS1 is an antagonist of type I interferons, probably involved in regulation of the mRNA transport from the nucleus to the cytoplasm. NS2, also called nuclear export protein (NEP), plays a role in the transport of newly assembled ribonucleoprotein from the nucleus to the cytoplasm (180).

**Figure 15. Structure of Influenzavirus.** The ssRNA genome is associated with nucleoproteins, enveloped in the nucleocapsid and surrounded by a matrix. The surface of the virion is covered with haemagglutinin and neuraminidase molecules. Figure modified according to Fields Virology Chapter 45, Orthomyxoviridae: The Viruses and Their Replication.
The lifecycle of Influenzavirus (Fig. 16) starts with its entry into the host cell by receptor-mediated endocytosis (step 1). Once inside the cell, the virus is uncoated and disassembled in the endosome (step 2). The nucelocapsid consisting of viral RNA and nucleoproteins translocates to the nucleus, where the viral genome is replicated (step 3) and transcribed (step 4). Viral proteins are translated in the cytosol (step 5). Nucleoproteins re-enter the nucleus where they are packed together with the RNA in the ribonucleoprotein core (step 6). Other proteins are further modified in the ER and Golgi-apparatus (step 7) and some are transported to the cell membrane of the host cell (step 8). The new virions leave the cell by budding (step 9). At the end the viruses are released and spread (step 10) (180).

**Figure 16. Life cycle of Influenza virus.** Step 1, entrance of virus into the cell by receptor mediated endocytosis. Step 2, uncoating of the viral particle. Step 3, replication of viral RNA . Step 4, transcription of viral genome. Step 5, translation of viral proteins. Step 6, packaging of the ribonucleoprotein core and translocation out of the nucleus. Step 7, maturation of viral proteins in the endoplasmatic reticulum and Golgi apparatus. Step 8, budding. Step 9, release and spread of virus.
3.4.4.2 Influenza and the immune system

**Humoral immunity**

Antibodies (IgM, IgA and IgG) generated during the primary response are completely protective against a secondary challenge with the same virus (181-183). However, they are generated late in primary response and do not play a critical role in clearance of primary infection unless the viral load is very high (184-186). Moreover, they are not protective against challenge with serologically different viruses (181-183).

Only antibodies to HA are neutralizing. Antibodies to NA are not neutralizing, but may reduce efficient release from infected cells. Antibodies to M or NP do not play a role in protective immunity (67).

**Cell-mediated immunity**

During primary infection, CD8 T cell response is essential to clear Influenzavirus from the lungs of infected mice (184, 187-189). Dendritic cells and macrophages carry the antigen from the lungs to the lymph nodes (190), where they prime naïve CD8 T cells. The activated CD8 T cells then migrate to the lungs to clear the infection (191). Under normal circumstances Flu-specific CD8 T cells appear in the lung at day7 and peak at day 9 to 10. There is a correlation between the appearance of virus-specific CD8 T cells and the clearance of primary infection, which is either performed via perforin or the Fas mechanism (192, 193).

In contrast to humoral immunity, cellular response to cross-reactive epitopes can provide substantial protection against serologically different viruses. Even though they do not prevent re-infection, they are able to reduce the viral load and clear the virus faster (194, 195).

Flu-specific CD8 T cells primed during primary infection persist and enable a rapid and vigorous response to secondary challenges. Some of the virus-specific CD8 T cells persist in the lungs. These cells were shown to be able to secrete IFN-γ directly *ex vivo*. Yet, they did not exhibit cytolytic function directly *ex vivo*, although they could be induced to proliferate and acquire cytolytic function after re-stimulation *in vitro* (196). CD4 T cells also contribute to protection both by promoting the humoral antiviral response (188, 197), and by the secretion of IFN-γ (184, 198, 199).

**Viral escape strategies**

Several Influenza epidemics have been recorded during the 19th and 20th century, but the first pandemic was not accurately recorded until 1889-92. A second pandemic occurred in 1918-19, known as the Spanish Influenza, which was lethal for 20-25 Mio people, especially young
adults. There were further pandemics in 1957 (Asian Flu) and 1968 (Hong Kong Flu). Every year, almost identical epidemics occur in most of the countries. However extensive pandemics occur only every 10 to 12 years.

**Antigenic shift:** Viruses recovered from different pandemics show a wide variation, which means that pandemics occur because of appearance of new Influenza A subtypes, against which the population has no immunity (antigenic shift). The appearance of new subtypes parallels the disappearance of old subtypes. HA is always involved in the antigenic shift since HA is a binding site for Ab.

There are three different theories for the appearance of antigenic shift. The first implies a mechanism of reassortment. Reassorted viruses result from double infections with human and animal or bird viruses, where the 8 RNA segments of each strain reassort to form a new virus. This is possible because Influenza A is capable of crossing species barriers. The second possibility is a gradual adaptation of animal viruses to human. The third possibility is the recirculation of existing subtypes. This implicates that several Influenza A subtypes exist in the human population and that they are recycled. However, the evidence supporting this hypothesis is not very strong (180).

**Antigenic drift:** In addition to large pandemics smaller epidemics occur regularly. Viruses causing these epidemics show some strain differences since they do not completely cross-react with preexisting antibodies (antigenic drift). This antigenic drift results from the accumulation of spontaneous mutations, which are responsible for amino acid substitutions at antigenic sites. This evolution is likely to be driven by the pressure of neutralizing Ab in the population. In case of antigenic drift, prior immunization yields only partial immunity (180).

3.4.4.3 *Pathogenesis, clinical manifestations and complications*

Infection of cells with Influenzavirus causes cell death by *necrosis* and apoptosis. Especially bronchiolar epithelial cells and alveolar cells are concerned. The virus is released from the cell before the latter dies and infects nearby or adjacent cells. Infection of polymorphic nuclear cells, lymphocytes and monocytes is nonproductive but causes dysfunctions of those cells such as defective chemotaxis and phagocytosis (200) or decreased proliferation and co-stimulation (201, 202).

Influenzavirus is spread via respiratory droplets. The virus binds to cells of the respiratory tract, which are rich in viral receptors. The neuraminidase in the envelope helps the virus to
be spread by releasing virus particles, which have been bound by mucous present on the surface of epithelial cells.

The incubation period lasts between 18 and 72 hours, before an abrupt onset of symptoms such as fever, headache, photophobia, shivering, dry cough, malaise, myalgia and a dry tickling throat occurs. Several complications can arise in association with Influenza infection. Pulmonary complications include primary Influenza viral pneumonia, as well as secondary bacterial pneumonia mostly caused by Streptococcus pneumoniae, Haemophilus influenzae or Staphylococcus aureus. Further pulmonary complications are Croup and acute exacerbation of chronic bronchitis.

Examples for nonpulmonary complications are myositis, cardiac complications such as myocarditis and pericarditis (rarely) and central nervous complications such as Guillain-Barré syndrome and Reye’s syndrome (67).

3.4.4.4 Treatment and prevention

**Therapy**

Flu normally is a self-limiting virus, which means that the virus is cleared without treatment. Nevertheless in some cases treatment is indicated. There are two kinds of approaches. First M2 inhibitors inhibiting the M2 ion channel activity and thus inhibiting virus uncoating (203, 204). Second, NA inhibitors, which prevent detachment of the virus from host cells and curb the local and distant spread of the virus (67).

**Prevention**

Vaccines have been available for 50 years, but their efficacy is only partial, i.e. in approximately 65% (205, 206). Moreover, their ability to prevent epidemics could not be proven. Antibodies against HA reduce the severity of infection and decrease virus spreading in infected persons. Antibodies against NA also contribute to protection.

There are different types of vaccines:

- Whole virus vaccine, which was the first Influenza vaccine to be produced. The virus was inoculated into embryonated eggs, harvested 2-3 days later and inactivated. This confers 60-90% of protection during 1-5 years. The protection is less effective if the infecting virus undergoes progressive Ag drift.

- Split virus vaccines, where inactivated particles are disrupted with detergents. This type of vaccine induces fewer side effects than whole virus vaccines, but has the same immunogenicity.
• Subunit virus vaccines. These contain only HA and NA antigens. They are used in aqueous suspension or absorbed to carriers such as alhydrogel. Aqueous suspensions demonstrate fewer side effects than whole virus vaccines or vaccines absorbed to carriers.

• Life attenuated vaccines. These induce a stronger immunity than inactivated vaccines. The problem is that normal methods for attenuation such as repeated passages and temperature adaptation require long periods of development and manufacturing. Hence an attenuated vaccine against a given strain may already be obsolete at the time of application. One possibility is to mix already attenuated strains with wild type (wt) virus to produce recombinants with appropriate RNA fragments. However, even with modern technologies the purification and safety procedures require ca. 2 years for production. Much too long considering the yearly turnover of new viruses.
4. Results

4.1. Publication #1

HIV-1-Specific IFN-γ/IL-2 Secreting CD8 T Cells Support CD4-Independent Proliferation of HIV-1-Specific CD8 T Cells

Simone C. Zimmerli, Alexandre Harari, Cristina Cellera, Florence Valletian, Pierre-Alexandre Bart and Giuseppe Pantaleo


Summary

Previous studies in monkeys and humans have indicated the importance of CD8 T cells in the initial control of HIV-1 infection. The absence of CD8 T cells was correlated with high viral load and rapid progression to AIDS. However, the different conditions defining the type of CD8 T cell response are not yet established. Important criteria could be the Ag load (dose) on the one hand, or the duration of persistence (time) on the other hand.

In this work, functional and phenotypic characterization of virus-specific CD8 T cells against Cytomegalovirus (CMV), Epstein Barr virus (EBV), Influenzavirus (Flu) and HIV-1 were performed on the basis of the ability of CD8 T cells to secrete IFN-γ and IL-2, and their capacity to proliferate and to express CD45RA and CCR7. The different viruses served as models of distinct Ag persistence and dose. Flu reflects a situation of pathogen clearance, CMV, EBV and HIV-1 in LTNP of repetitive Ag exposure at low Ag dose, whereas HIV-1 is a model for Ag persistence at high Ag dose.

We identified two functional distinct populations of CD8 T cells, namely dual IFN-γ/IL-2 and single IFN-γ secreting cells. Virus-specific IFN-γ/IL-2 secreting CD8 T cells were CD45RA⁻CCR7⁻. In contrast, single IFN-γ CD8 T cells were either CD45RA⁺CCR7⁻ or CD45RA⁺CCR7⁺. We found a good correlation (p<0.02) between the proportion of virus-specific IFN-γ/IL-2 secreting CD8 T cells on the one hand, and the specific proliferation capacity of CD8 T cells on the other hand. The loss of HIV-1-specific CD8 T cells, capable to secrete IL-2, was associated with the failure of HIV-1-specific CD8 T cells to proliferate. Even in CD4 T cell-depleted populations, or after stimulation with MHC class I tetramer-
peptide complexes, a substantial proliferation of virus-specific CD8 T cells could be observed. IL-2 was the factor responsible for the CD4-independent CD8 T cell proliferation. Taken together, our results indicate: a) a wide heterogeneity of antiviral CD8 T cell immune responses under different conditions of virus persistence, b) a combined loss of virus-specific IFN-γ/IL-2 secreting and proliferating CD8 T cells in progressive HIV-1 infection, c) a typical phenotype of effector cells, i.e. CD45RA−CCR7−, for the IFN-γ/IL-2 secreting CD8 T cells, d) a correlation between the proportion of virus-specific IL-2 secreting and proliferating CD8 T cells, and e) the occurrence of Ag-specific CD8 T cell proliferation also in experimental conditions excluding the involvement of Ag-specific helper CD4 T cells.
HIV-1-specific IFN-γ/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells

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Functional and phenotypic characterization of virus-specific CD8 T cells against cytomegalovirus, Epstein–Barr virus, influenza (flu), and HIV-1 were performed on the basis of the ability of CD8 T cells to secrete IFN-γ and IL-2, to proliferate, and to express CD45RA and CCR7. Two functional distinct populations of CD8 T cells were identified: (i) dual IFN-γ/IL-2-secreting cells and (ii) single IFN-γ-secreting cells. Virus-specific IFN-γ/IL-2-secreting CD8 T cells were CD45RA−CCR7−, whereas single IFN-γ CD8 T cells were either CD45RA−CCR7+ or CD45RA+CCR7+. The proportion of virus-specific IFN-γ/IL-2-secreting CD8 T cells correlated with that of proliferating CD8 T cells, and the loss of HIV-1-specific IL-2-secreting CD8 T cells was associated with that of HIV-1-specific CD8 T cell proliferation. Substantial proliferation of virus-specific CD8 T cells (including HIV-1-specific CD8 T cells) was also observed in CD4 T cell-depleted populations or after stimulation with MHC class I tetramer–peptide complexes. IL-2 was the factor responsible for the CD4-independent CD8 T cell proliferation. These results indicate that IFN-γ/IL-2-secreting CD8 T cells may promote antigen-specific proliferation of CD8 T cells even in the absence of helper CD4 T cells.

CD8 T cells play a critical role in the control of viral infections (reviewed in ref. 1). Several studies have shown a wide heterogeneity of memory CD8 and CD4 T cells with multiple phenotypes and functions in response to virus infections (2–7). Functionally distinct populations of CD8 T cells can be defined by the expression of CD45RA and CCR7 (8) and are able to proliferate and/or to secrete cytokines such as IL-2, IFN-γ, and TNF-α after antigen (Ag)-specific stimulation (9–11). The determination of quantitative and qualitative changes of virus-specific CD8 T cells in rapidly controlled acute, more slowly controlled or uncontrolled chronic infections showed that high load of lymphocytic choriomeningitis virus resulted in the progressive diminution of the ability of CD8 T cells to secrete IL-2, TNF-α, and IFN-γ (9). Of interest, the capacity to secrete cytokines could be restored if the viral load was brought under control (9).

IL-2 production from virus-specific CD8 T cells has been the object of few studies in humans. Recent studies have shown that a variable percentage of cytomegalovirus (CMV)- and Epstein–Barr virus (EBV)-specific CD8 T cells were able to secrete IL-2 (10, 11), whereas IL-2 was not produced by melanoma-1-specific CD8 T cells obtained from patients with stage IV melanoma (10). With regard to HIV-1 infection, no studies have investigated the ability of HIV-1-specific CD8 T cells to secrete IL-2. However, it has been shown that HIV-1-specific CD8 T cells of HIV-1-infected subjects with nonprogressive disease, i.e., long-term nonprogressors (LTNPs), had greater proliferation capacity as compared with HIV-1-specific CD8 T cells from progressors (12), and this finding was associated with a better ability to control virus replication (12). A recent study has shown that the loss of HIV-1-specific CD8 T cell proliferation was associated with the loss of HIV-1-specific helper CD4 T cells and has proposed a critical role of HIV-1-specific helper CD4 T cells in sustaining Ag-specific CD8 T cell proliferation (13).

Recent studies (14–16) investigating antiviral memory CD4 T cell responses have shown that the combined assessment of IL-2 and IFN-γ is instrumental to distinguish functionally distinct populations of memory CD4 T cells and patterns of antiviral immune responses associated with different conditions of virus persistence and control.

In the present study, we have performed functional and phenotypic characterization of antiviral CD8 T cell responses specific for HIV-1, CMV, EBV and influenza (flu) on the basis of their ability to proliferate, to secrete IL-2 and IFN-γ, and to express CD45RA and CCR7. Our results indicate: (i) a wide heterogeneity of antiviral CD8 T cell immune responses under different conditions of virus persistence; (ii) a combined loss of virus-specific IFN-γ/IL-2-secreting and -proliferating CD8 T cells in progressive HIV-1 infection; (iii) a typical phenotype of effector cells, i.e., CD45RA−CCR7−, for the IFN-γ/IL-2-secreting CD8 T cells; (iv) a correlation between the proportion of virus-specific IL-2-secreting and -proliferating CD8 T cells; and (v) the occurrence of Ag-specific CD8 T cell proliferation also in experimental conditions, excluding the involvement of Ag-specific helper CD4 T cells.

Materials and Methods

Study Groups. The 21 subjects with progressive chronic HIV-1 infection enrolled in this study were naive to antiviral therapy, with CD4 T cell counts of >250 cells per microliter (mean ± SE: 810 ± 39) and plasma viremia counts of ~5,000 HIV-1 RNA copies per ml (mean ± SD: 41,854 ± 12,339). Five HIV-1-infected patients with nonprogressive disease, i.e., LTNPs, as defined by documented HIV-1 infection for >14 years, stable CD4 T cell counts of >500 cells per microliter (mean ± SE: 912 ± 125) and plasma viremia of <1,000 HIV-1 RNA copies per ml (mean ± SE: 97 ± 38) were also included. Patient 1010 has a documented HIV-1 infection since March 1999. He was treated with antiviral therapy at the time of primary infection and remained on antiviral therapy for 18 months. He interrupted therapy spontaneously in December 2000. During the last 4 years, he constantly had levels of viremia of <50 HIV-1 RNA copies per ml and CD4 T cell count in the range of 1,400 cells per microliter. In addition, blood from 28 HIV-negative subjects was obtained from the local blood bank or from laboratory coworkers. The studies were approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois.

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Abbreviations: EBV, Epstein–Barr virus; CMV, cytomegalovirus; Ag, antigen; LTNPs, long-term nonprogressors; CFSE, carboxyfluorescein succinimidyl ester; SEB, staphylococcal enterotoxin B.

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4. Results


For tetramer stimulations, A2- and B7-restricted class I peptide tetramers were produced as described (25, 26).

Detection of IFN-γ and IL-2 Secretion. Cell stimulations were performed as described (14). For stimulation of CD8 T cells, individual peptides (5 µg/ml) or peptide pools (1 µg/ml for each peptide) were used. Cells were then stained with CD8-PerCP-Cy5.5, CD69-FITC, IFN-γ-APC and IL-2-PE (Becton Dickinson). For phenotypic analysis, the following Abs were used in combination: Rat anti-human CCR7 (Becton Dickinson) followed by goat anti-rat IgG(H +L)-APC (Caltag, Burlingame, CA), CD8-Pacific blue (DAKO, Glostrup, Denmark), CD45RA-Biotin followed by anti-Streptavidin-PercP, anti-CD69-APC-Cy7, anti-IL-2-PE, and anti-IFN-γ-FITC (Becton Dickinson). Data were acquired on a FACScalibur or an LSR II and analyzed by using CELLQUEST and DIVA software (Becton Dickinson). The number of nongated events ranged between 10^5 and 10^6 events.

Ex Vivo Proliferation Assay. After an overnight rest, cells were washed twice, resuspended at 1 × 10^6/ml in PBS, and incubated for 7 min at 37°C with 0.25 µM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes). The reaction was quenched with 1 volume of FCS, and cells were washed and cultured in the presence of anti-CD28 Ab (0.5 µg/ml) (Becton Dickinson). Cells were either stimulated with HIV-1 peptide pools (1 µg/ml of each peptide), individual peptides (5 µg/ml), or tetramers (0.31 µg/ml). Staphylococcal enterotoxin B (SEB) stimulation (200 ng/ml) served as positive control. Where indicated, 10% exogenous IL-2 (Roche, Basel) was added 48 h after peptide stimulation. For neutralization experiments, anti-IL-2-neutralizing Ab or isotype control Ab (Becton Dickinson) were added at 10 µg/ml. At day 5, cells were harvested and stained with CD4-PE-Cy5 (Becton Dickinson) and CD8-APC (Becton Dickinson). Cells were fixed with CellFix (Becton Dickinson) and acquired (1 × 10^6 nongated events) on a FACScalibur (Becton Dickinson).

CD4 T Cell Depletion. CFSE-labeled cells were stained with CD4-APC and sorted by using a FACS Vantage (Becton Dickinson). The purity of the CD4-depleted cell populations was 99%.

Statistical Analysis. Statistical significance (P values) of the results was calculated by using a two-tailed Student t test. A two-tailed P value of <0.05 was considered significant. The correlations among variables were tested by simple regression analysis.

Results

Distinct Cytokine Secreting Populations of Virus-Specific CD8 T Cells. We used different models of virus-specific CD8 T cell responses, including HIV-1-, CMV-, EBV-, and flu-specific CD8 T cell responses. Based on the observation that functionally distinct Ag-specific CD4 T cell populations are defined by the secretion of IL-2 and IFN-γ (14–16), we performed functional characterization of virus-specific CD8 T cell responses by simultaneous assessment of IFN-γ and IL-2 secretion after Ag-specific stimulation. Representative examples obtained from the analysis of 21 HIV-1-infected progressors and 28 HIV-negative blood donors in whom CMV-, EBV-, or flu-specific CD8 responses were detected are shown in Fig. 1A. The dual IFN-γ/IL-2-secreting T cells were absent in HIV-1-specific CD8 T cells, whereas they were found within CMV-, EBV-, and flu-specific CD8 T cells as shown in Fig. 1A. These observations were confirmed by the analysis of a larger number of subjects. A significant difference was found between the percentage of HIV-1-specific IFN-γ/IL-2-secreting cells in progressive HIV-1 infection and that found in the virus-specific IFN-γ/IL-2-secreting CD8 T cells (P < 0.05) of the other virus infections (Fig. 1B). We also evaluated the proportion of IL-2-secreting cells within IFN-γ-secreting CD8 T cells. Cumulative data of this analysis are shown in Fig. 1C. The proportion of CMV-specific (12.7 ± 1.8%, n = 11) and EBV-specific (19.2 ± 3.2%, n = 10) IL-2-secreting CD8 T cells was significantly higher (P < 0.05) compared with that of HIV-1-specific IL-2-secreting CD8 T cells (2.3 ± 0.6%, n = 21) (Fig. 1C). The proportion (25.6 ± 3.6%, n = 7) of flu-specific IL-2-secreting CD8 T cells was significantly higher (P < 0.05) compared with that...
of HIV-1- and CMV-specific but not with that of EBV-specific IL-2-secreting CD8 T cells (Fig. 1C). Finally, the proportion of EBV-specific IL-2-secreting cells was also significantly higher compared with that of CMV-specific IL-2-secreting CD8 T cells ($P < 0.05$) (Fig. 1C). CMV-, EBV-, and flu-specific CD8 T cell responses were also studied in HIV-1-infected individuals either by using peptides specific to CMV and EBV ($n = 7$) and flu ($n = 6$) or a pool of 21 CMV-, EBV-, and flu-derived peptides in 30 HIV-1-infected subjects. The proportion of CMV-, EBV-, or flu-specific IL-2-secreting CD8 T cells in HIV-1-infected subjects was not significantly different from that observed in HIV-negative subjects ($P > 0.05$).

To exclude the possibility that the lack of detection of HIV-1-specific IFN-$\gamma$/IL-2-secreting CD8 T cells was specific of the response to certain peptides, we performed stimulation with peptide pools spanning gag, pol, and nef proteins of HIV-1. A representative flow cytometry profile of one (of 21) HIV-1-infected subjects with progressive disease (progressors) is shown in Fig. 2A. Despite the presence of HIV-1-specific IFN-$\gamma$-secreting CD8 T cells after stimulation with different HIV-1 peptide pools, IL-2-secreting CD8 T cells were not detected (Fig. 2A).

Previous studies (12) have shown that HIV-1-specific CD8 T cells of LTNPs, but not of progressors, proliferated in response to Ag-specific stimulation (12). The evaluation of the presence of HIV-1-specific IFN-$\gamma$/IL-2-secreting CD8 T cells in three of five representative LTNPs showed variable intensities of the response to the different peptide pools (Fig. 2B). HIV-1-specific IFN-$\gamma$-secreting CD8 T cells were detected consistently after stimulation with different peptide pools (Fig. 2B), and a substantial percentage of dual IFN-$\gamma$/IL-2-secreting cells was also found after stimulation with peptide pools 1 and 2 (Fig. 2B). The percentage ($0.13 \pm 0.04, n = 5$) of IFN-$\gamma$/IL-2-secreting cells in LTNPs was significantly different ($P = 0.0003$) compared with progressors ($0.01 \pm 0.002, n = 21$).

**Phenotypic Analysis of Cytokine-Secreting Virus-Specific CD8 T Cells.** Previous studies in humans and mice have shown that IL-2-secreting CD8 T cells were contained within the CCR7$^+$ central memory CD8 T cell population, whereas the IFN-$\gamma$-secreting CD8 T cells were contained within the CCR7$^+$ effector CD8 T cells (8, 27). Enriched mononuclear cells of LTNPs and HIV-negative donors with known HIV-1, flu, or CMV CD8 T cell responses were stimulated with the appropriate virus-derived peptides, and cells were stained with CD8, CD45RA, CCR7, IL-2, IFN-$\gamma$, and CD69 Abs. The results obtained indicated that the virus-specific IFN-$\gamma$/IL-2 CD8 T cells were contained within the CD45RA$^+$CCR7$^+$ effector cell population and the IFN-$\gamma$-secreting CD8 T cells within the CD45RA$^-$CCR7$^+$ and CD45RA$^+$CCR7$^+$ effector cell populations (Fig. 3). These results were representative of the analysis of two LTNPs and seven HIV-negative subjects.

**Proliferation Capacity of Virus-Specific CD8 T Cells.** Recent studies (12, 13) have shown the loss of proliferation capacity of HIV-1-specific CD8 T cells of subjects with progressive disease, whereas HIV-1-specific CD8 T cell proliferation was retained in CD8 T cells of LTNPs. Based on these observations, it has been proposed that Ag-specific CD8 T cell proliferation represents a characteristic of effective and protective immune response (12). Furthermore, it has been proposed that the loss of HIV-1-specific CD8 T cell proliferation depends on the loss of HIV-1-specific CD8 T cell memory CD8 T cell population, whereas the IFN-$\gamma$-secreting CD8 T cells of subjects with progressive disease (12, 13) have shown the loss of proliferation capacity of HIV-1-specific CD8 T cells. A substantial proportion of CD8 T cells of subject 248 proliferated after stimulation with CMV- and Flu-derived peptides (Fig. 4A). Similarly, CD8 T cells of subject 359 proliferated after stimulation with two different EBV-derived peptides (Fig. 4A). We then determined the proliferation of HIV-1-specific CD8 T cells after stimulation with HIV-1-derived peptide pools in progressors ($n = 9$) and LTNPs ($n = 5$). HIV-1-specific CD8 T cell proliferation was barely detected or was absent in these two representative progressors [two of nine patients each tested with one to three pools (16 responses were tested in total)] (Fig. 4B). However, CD8 T cells of progressors were able to proliferate after SEB stimulation (Fig. 4B), thus indicating a selective loss of HIV-1-specific proliferation. Consistent with results previously shown by Migueles et al. (12), vigorous HIV-1-specific CD8 T cell proliferation was observed in two of five representative LTNPs (Fig. 4C). The mean $\pm SE$ percentage of HIV-1-specific CD8 T cell proliferation in progressors was $0.45 \pm 0.16$ compared with $6.88 \pm 1.69$ in LTNPs ($P < 0.0001$).

We then determined the correlation between the proportion of Ag-specific proliferating CD8 T cells and the proportion of IL-2-secreting CD8 T cells within IFN-$\gamma$-secreting cells. This analysis was performed by pooling together 32 individual determinations from 21 subjects of Ag-specific CD8 T cell proliferating and IL-2-secreting CD8 T cells. We found a significant correlation between
the proportion of Ag-specific IL-2-secreting and -proliferating CD8 T cells (Fig. 4D). The correlation was even stronger when only HIV-1-specific CD8 T cell responses were analyzed ($R = 0.53, P < 0.01, n = 24$).

Having demonstrated a correlation between the ability to secrete IL-2 and the proliferation capacity of CD8 T cells, we further investigated the mechanism responsible for Ag-specific CD8 T cell proliferation. Firstly, we assessed Ag-specific CD8 T cell proliferation under experimental conditions excluding the involvement of CD4 T cells. For this purpose, Ag-specific CD8 T cell proliferation was determined by using either MHC class I tetramer–peptide complexes as stimuli or CD4 T cell-depleted populations in the absence of exogenous IL-2. HLA-A2 tetramer complexed with flu- and CMV-derived peptides induced vigorous Ag-specific proliferation of CD8 T cells of subjects 172 and 180 (Fig. 5A). It is important to underscore that no CD4 T cell proliferation was observed (Fig. 5A), thus indicating that Ag-specific CD8 T cell proliferation was not associated with the stimulation of Ag-specific helper CD4 T cells. Consistent with the observations previously reported (12, 13), HIV-1-specific CD8 T cell proliferation was barely detected in progressors after stimulation with the HLA-A2 tetramer complexed with an HIV-1 pol ILKEPVHGV-derived peptide (20) (Fig. 5B). Of interest, in agreement with the work of Lichterfeld et al. (13), HIV-1-specific CD8 T cell proliferation was recovered in the presence of exogenous IL-2 (Fig. 5B). No proliferation was observed in CD4 T cells after MHC class I tetramer–peptide complex

Fig. 3. IFN-γ- and IL-2-secreting CD8 T cells in different populations defined by CD45RA and CCR7. Shown is the distribution of IFN-γ- and IL-2-secreting CD8 T cells in different populations defined by CD45RA and CCR7. (A) Cells of LTNP 2073 were stimulated with different peptide pools spanning gag, pol, and nef proteins. (B) Cells of subjects 205 and 35 were stimulated with CMV or flu peptides, respectively.

Fig. 4. Virus-specific CD8 T cell proliferation after stimulation with single peptides or peptide pools. (A) CFSE-labeled cells of HIV-negative donors 248 and 359 were stimulated with CMV-, flu-, or EBV-derived peptides. Profiles of proliferating cells, i.e., CFSE low cells, are gated on CD8 T cells. (B) HIV-1-specific CD8 T cell proliferation in HIV-1 progressors after stimulation with different HIV-1 peptide pools or SEB. (C) HIV-1-specific CD8 T cell proliferation in LTNP after stimulation with different HIV-1 peptide pools. (D) Correlation between the proportion of IL-2-secreting and -proliferating virus-specific CD8 T cells.
stimulation (Fig. 5B). To further confirm the hypothesis that HIV-1-specific CD8 T cell proliferation was independent of CD4 helper T cells, we compared the HIV-1-specific CD8 T cell proliferation in response to the p24-derived GPGHKARVL peptide that has been previously characterized as a CD8 epitope (17) restricted by HLA-B7. Unfractionated blood mononuclear cells or CD4 T cell-depleted populations of patient 1010 with chronic HIV-1 infection were stimulated with the peptide GPGHKARVL. As reported in Materials and Methods, patient 1010 had constantly controlled viremia since 4 years after interruption of antiviral therapy. A large percentage (59%) of HIV-1-specific CD8 T cells proliferated after stimulation of unfractionated cell populations with the p24 peptide (Fig. 6A). Substantial HIV-1-specific CD8 T cell proliferation (32.7%) occurred also in the CD4 T cell-depleted populations although it was reduced (45% reduction) compared with the cell cultures containing CD4 T cells (Fig. 6A). It is important to underscore the fact that the CD8 T cell proliferation in the CD4-depleted cell populations was not due to contaminating CD4 T cells because CD4 T cells were almost absent (0.6%) in the CD4-depleted cell populations at day 5 (Fig. 6A). The experiments shown in Fig. 6A were performed in the absence of exogenous IL-2.

Secondly, Ag-specific CD8 T cell proliferation was assessed in the presence of anti-IL-2 Ab. The substantial proliferation of CD8 T cells from subject 180 observed after stimulation with the CMV tetramer NLVPMVATV was completely abolished (95% inhibition of proliferation) in the presence of anti-IL-2 Ab (Fig. 6B). Therefore, virus-specific CD8 T cell proliferation, including HIV-1-specific proliferation, depends on IL-2 and on the presence of the IFN-γ/IL-2-secreting CD8 T cells, and may occur in the absence of helper CD4 T cells. The finding that CD8 T cell proliferation was independent of CD4 T cell help and dependent on the presence of IFN-γ/IL-2-secreting CD8 T cells was also confirmed for CMV- and EBV-specific CD8 T cell-mediated proliferation in three HIV-negative subjects (data not shown).

Discussion

In the present study, we have investigated the function and phenotype of memory CD8 T cells in different models of virus-specific T cell responses, including HIV-1, CMV, EBV, and Influenza A. HIV-1-specific CD8 T cell responses were studied in subjects with progressive and nonprogressive infection who were naïve to therapy. The other virus-specific CD8 T cell responses were analyzed in HIV-negative donors. Functional characterization was performed by the measurement of the ability of CD8 T cells to proliferate and to secrete IFN-γ and IL-2 after Ag-specific stimulation.

**Fig. 5.** Virus-specific CD8 T cell proliferation after stimulation with HLA class I tetramers. (A) Blood mononuclear cells of HIV-negative donors 172 and 180 were stimulated with A2-flu or -CMV tetramers, respectively. Flow cytometry profiles of proliferating CD8 (Left) and CD4 (Right) T cells are shown. (B) Blood mononuclear cells of progressor 2056 were stimulated with an A2-pol tetramer and cultured in the absence or presence of 10% of exogenous IL-2.

**Fig. 6.** Virus-specific CD8 T cell proliferation in CD4-depleted cells or after neutralization of IL-2. (A) CD8 T cell proliferation was evaluated in CD4 T cell-depleted populations stimulated with HIV-1-derived peptide. The purity of the sorted CD4+ T cell populations was 99%. (B) Inhibition of virus-specific CD8 T cell proliferation with anti-IL-2 Ab. Cells of subject 180 were stimulated with an A2-restricted CMV tetramer and cultured in the presence of anti-IL-2 or isotype control Abs.
Most studies performed on CD8 T cells in different models of antiviral responses in both mice and humans were predominantly focused on the characterization of effector functions such as perforin and granzyme expression or secretion of IFN-γ and TNF-α (9−11). Recently, a series of studies have shown the importance of investigating other functions such as the ability to proliferate and to secrete IL-2 (14−16) that have generally been the object of extensive investigation in CD4 T cells. With regard to CD8 T cells, it has been shown that the preservation of the proliferation capacity and the ability to secrete IL-2 were generally associated with an apparently effective immune response because virus replication was controlled in both mouse and human models of virus infection (12, 28). In addition, a recent study has shown a paralleled loss of HIV-1-specific helper CD4 T cells and HIV-1-specific CD8 T cell proliferation, and concluded that HIV-1-specific helper CD4 T cells are critical for the maintenance of HIV-1-specific proliferating CD8 T cells (13).

This is the first study, to our knowledge, investigating IL-2 secretion in HIV-1-specific CD8 T cells. In addition, it compares the function of HIV-1-specific CD8 T cells with that of CMV-, EBV-, and flu-specific CD8 T cells that are able to keep either on check (CMV and EBV) or clear (flu) the virus. The rationale for studying antiviral CD8 T cell responses in different models of virus persistence resides on recent studies (28) performed in mice, demonstrating that the function of CD8 T cells was modulated by different conditions of Ag levels and/or persistence. HIV-1 infection in subjects with progressive disease corresponded to the model of immune failure with Ag persistence and high Ag levels. CMV, EBV, and HIV-1 infection in subjects with nonprogressive disease corresponded to the model of immune control with protracted virus persistence and low Ag levels and flux to the model of Ag clearance. Our results demonstrated the presence of an Ag-specific IFN-γ/IL-2-secreting CD8 T cell population in the models of virus infections associated with resolved virus infection or with virus control, i.e., CMV, EBV, and nonprogressive HIV-1 infection or virus clearance, i.e., flu. This cell population was absent in progressive HIV-1 infection. Therefore, we provided evidence for (i) a loss of IFN-γ/IL-2-secreting CD8 T cells in progressive HIV-1 infection and (ii) a skewed representation of functionally distinct memory HIV-1-specific CD8 T cells in progressive HIV-1 infection. The present results showed that the same pathogen, i.e., HIV-1, can be associated with substantially different CD8 T cell responses in progressive and nonprogressive infection where the major difference between these two conditions was indeed represented by Ag levels. Therefore, along with the observation from the lymphocytic choriomeningitis virus model (28), our results rather supported the hypothesis that also in humans the functional heterogeneity of virus-specific CD8 T cell responses was influenced by Ag persistence and Ag levels.

In agreement with previous studies (12, 13), HIV-1-specific CD8 T cell proliferation was lost in progressive HIV-1 infection. Of interest, we have provided evidence for the combined loss of HIV-1-specific IFN-γ/IL-2-secreting and -proliferating CD8 T cells in progressive HIV-1 infection. This association raised the issue on the role of IFN-γ/IL-2-secreting CD8 T cells in Ag-specific CD8 T cell proliferation. To address this issue, we evaluated the virus-specific CD8 T cell proliferation under experimental conditions excluding any involvement of helper CD4 T cells. These latter have been proposed to be critical for sustaining HIV-1-specific CD8 T cell proliferation (13). Virus-specific CD8 T cell proliferation, including HIV-1-specific, occurred in CD4 T cell-depleted populations or after stimulation with MHC class I tetramer–peptide complexes. Under these experimental conditions, virus-specific CD8 T cell proliferation was found in the HIV-1-, CMV-, EBV- and flu-specific immune responses, and a significant correlation between the proportion of IL-2-secreting and -proliferating CD8 T cells was observed.

These results demonstrated that the persistence of virus-specific IFN-γ/IL-2-secreting CD8 T cells was associated with the persistence of CD8 T cell proliferation. Virus-specific CD8 T cell proliferation was supported by IL-2 because it was completely abolished in the presence of the anti-IL-2 Ab. Therefore, taken together, they indicate that IFN-γ/IL-2-secreting CD8 T cells are able to promote CD8 T cell proliferation through the secretion of IL-2 even in the absence Ag-specific helper CD4 T cells. Despite the demonstration in vitro of a CD4-independent CD8 T cell proliferation, it is important to underscore that Ag-specific helper CD4 T cells are crucial in vivo for the maintenance and for preventing impairment of optimal CD8 T cell function (29). Of interest, this CD4-independent proliferation capacity was present in the effector, i.e., CD45RA+CCR7+ cell population. The importance in vivo of this CD4-independent proliferation capacity of effector CD8 T cells during the expansion phase of the immune response remains to be determined.

These results represent a further step in the understanding of the functional characterization of virus-specific CD8 T cell responses and in the understanding of the impairment of CD8 T cell functions in progressive HIV-1 infection.

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4.2. Publication #2

Attenuated poxviruses expressing a synthetic HIV protein stimulate HLA-A2-restricted cytotoxic T cell responses

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Summary

The aim of this study was to test the immunogenicity of modified vaccinia ankara (MVA) and poxvirus (NYVAC) containing a construct harboring a fusion of the HIV-1 IIIB gag, pol and nef genes (gpn) in HLA-A2 transgenic mice. Poxviruses are useful for vaccination since they can accommodate large amounts of foreign genes, infect mammalian cells and express foreign proteins in the latter. MVA was developed towards the end of the campaign for the eradication of smallpox. Passages on chicken embryo for over 500 times lead to the loss of about 15% of the genetic material and the ability to grow in human and most other mammalian cells. MVA shows similar levels of recombinant gene expression as replication-competent vaccinia viruses in human cells. NYVAC is a highly attenuated strain of vaccinia, in which 18 open reading frames from the viral genome have been deleted.

The gagpolnef polygene was generated by back-translation of HIV-1 IIIB amino-acid sequence and optimized by elimination of splice-donor/acceptor sites and RNA destabilizing sequences. The resulting gagpolnef fusion protein is composed of Gag fused and in frame to Pol and Nef, which replaces the active site of RT. The RT sequence overlapping the active site was translocated to the 3’ end. The terminal Glycine was substituted by Alanine to prevent myristoylation of the N-terminus and a point mutation was introduced to the active site of the protease domain to damage its enzymatic activity. Gagpolnef was then cloned into a vaccinia virus insertion vector and MVA or NYVAC were transfected with this construct. To assess if Gpn is immunogenic mice were immunized intraperitoneally or subcutaneously with peptides, recombinant MVA or NYVAC and splenocytes of immunized mice were tested.
on their ability to secrete IFN-γ and to perform cytotoxic activity. Next it was checked if Gpn expressed by MVAgpn was recognized by human MHC class I molecules. Transgenic mice exclusively displaying a chimeric human HLA-A2.1 MHC class I molecule were immunized with six HLA-A2 restricted peptides, MVAgpn or MVA expressing luciferase (MVA-luc) as control. Again IFN-γ and CTL response was tested following immunization.

My task in this work was to investigate whether MVAgpn could stimulate human pol- or gag-specific CD8 T cells. For this purpose, human PBMC containing pol- and gag-specific CD8 T cells (as verified by tetramer staining at baseline) were stimulated with MVAgpn at increasing multiplicity (m.o.i) of infection or MVA-luc as negative control. At day 10 following stimulation, the frequency of pol- or gag-specific CD8 T cells, respectively, was measured using HLA-A2 restricted pol- or gag-tetramers. To ensure that the stimulation is specific PBMC of an HIV-negative subjects were stimulated in parallel.

We found that Gpn delivered by recombinant poxvirus vectors induced a robust HIV-specific CTL response. The HIV-specific CD8 T cell epitopes were immunogenic in transgenic mice displaying human MHC class I molecules. Furthermore MVAgpn was able to trigger expansion of human pol- and gag-specific CD8 T cells proportional to the m.o.i.
Attenuated poxviruses expressing a synthetic HIV protein stimulate HLA-A2-restricted cytotoxic T-cell responses

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Abstract

Efficient HIV vaccines have to trigger cell-mediated immunity directed against various viral antigens. However little is known about the breadth of the response induced by vaccines carrying multiple proteins. Here, we report on the immunogenicity of a construct harbouring a fusion of the HIV-1 IIIB gag, pol and nef genes (gpn) designed for optimal safety and equimolar expression of the HIV proteins. The attenuated poxviruses, MVA and NYVAC, harbouring the gpn construct, induced potent immune responses in conventional mice characterised by stimulation of Gpn-specific IFN-γ/H9253-producing cells and cytotoxic T cells. In HLA-A2 transgenic mice, recombinant MVA elicited cytotoxic responses against epitopes recognised in most HLA-A2+ HIV-1-infected individuals. We also found that the MVA vaccine triggered the in vitro expansion of peripheral blood cells isolated from a HIV-1-seropositive patient and with similar specificity as found in immunised HLA-A2 transgenic mice. In conclusion, the synthetic HIV polyantigen Gpn delivered by MVA is immunogenic, efficiently processed and presented by human MHC class I molecules.

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Keywords: HIV vaccine; HLA-A2; Recombinant poxvirus

1. Introduction

Many HIV candidate vaccines that target both cellular and humoral immunity have been tested for a decade in mice and non-human primates [1,2]. Successful HIV vaccination, however, has been hampered by the complexity of HIV infection and the absence of a defined correlate of protection. More recently, protective immunity against simian immunodeficiency (SIV) challenges in macaques has been reported [3–5]. These data indicate that induction of cytotoxic T cells (CTLs) prevents the spread of the viral infection [6]. Although induction of neutralising antibodies at mucosal surfaces is probably a prerequisite to induce sterile protection, a robust CTL response may lower viral load and, therefore, limit transmission [7].

Among the vectors used to carry HIV antigens, Modified Vaccinia Ankara (MVA), an attenuated vaccinia virus, was shown to induce HIV-specific CTL responses [3,8,9]. Upon MVA infection of mammalian cells, early and late protein synthesis is restricted to the first 24–48 h post-infection [10,11]. MVA is safe for humans [12] and is currently used in several HIV vaccine trials [13,14]. Within the family of attenuated poxviruses, NYVAC harbouring multiple SIV/HIV antigens has also been used in monkeys [15,16]. NYVAC is a highly attenuated strain of vaccinia virus generated by precise deletion of 18 open reading frames from the viral genome, which affects non essential genes for virus growth in some cell lines but are important for virulence in animal models [17]. MVA has been generated by passage on
chicken embryo fibroblast cells more than 500 times, loosing about 15% of genetic information and ability to grow in human and most other mammalian cells [18]. Both NY-VAC and MVA share common deleted genes, like the serpins (B13R and B14R) and host range (K1L), but most other of the deleted genes are distinct between the two virus strains. A careful evaluation of the differences in MVA and NYVAC biology remains to be done [19].

Peptides corresponding to CTL epitopes administered with adjuvant or via recombinant vectors are immunogenic but their use is limited due to HLA variability and high mutation rate of HIV. A strategy that targets as many viral proteins as possible and that is not restricted to a few HLA molecules is more likely to be successful. So far, few candidate vaccines have used fusion proteins that contain multiple HLA determinants and little is known about the breadth and the magnitude of responses against such vaccines. This information is critical as immunodominance may favor the induction of restricted sets of specificities in vaccinated individuals. In order to develop strategies overcoming immunodominance, it is important to validate mouse model, such as transgenic mice carrying human MHC class I to predict human CD8+ T cell responses [20]. In particular, the HHD mouse, that is H2-D6−/− β2-microglobulin−/− and transgenic for a chimeric HLA-A2 molecule, was shown to be a good model for the prediction of HLA-A2-restricted responses [21].

Within the frame of EuroVacc (http://www.eurovacc.net), a European effort to bring HIV vaccines into clinical trials, candidate vaccines harbouring a fusion of the HIV-1 gag, pol and nef genes (gpn) have been constructed. Before entering clinical trials in humans, we sought to verify that immunisation with Gpn induced CTLs with similar specificities than found in natural infections. We show here that MVA and NYVAC expressing Gpn induce strong CTL and IFN-γ-producing T cell responses in conventional mice. HLA-A2 transgenic mice immunised with MVA expressing Gpn developed HLA-A2-restricted responses against two CTL epitopes known to be immunogenic in HIV infected individuals. In addition, the candidate vaccine specifically triggers the in vitro expansion of human HIV-1-specific CD8+ T cells. Therefore, we provide evidence that these vaccines tested for safety in mice [22], are immunogenic and elicit CTL responses against HLA-A2-restricted epitopes recognised by HIV-infected patients.

2. Material and methods

2.1. Construction of the chimeric gpn

The synthetic read-through gagpolnef polygene was synthesized and provided by GENEART GmbH (Regensburg, Germany). Gagpolnef was generated by back- translating the amino-acid sequence of the HIV-1IM (BH10, GenBank Accession no. M15654) using a matrix for the most frequently occurring codons in mammalian cells. By optimising the gagpolnef sequence, cryptic splice donor/acceptor sites and RNA destabilising sequence motifs were eliminated. The synthetic gagpolnef DNA was designed in fragments of 300–800 nucleotides, assembled via unique restriction sites and cloned into pCRscript giving rise to pCRscript/gpn. The gagpolnef fusion protein comprises the group-specific antigen Gag including p7 (aa 1–432; GI: 326388) fused and in frame to Pol (aa 1–793; GI: 326385) lacking the integrase domain. Furthermore, the active site of the reverse transcriptase (RT) was replaced by a scrambled nef gene (aa 1–74 fused to aa 75–123; GI: 326393) resulting in an artificial budding defective 1326 aa read-through gagpolnef fusion protein. The RT sequence overlapping the active site was translocated in frame to the 3′ end (aa 296–396; GI: 326385) of the gagpolnef polygene. Accordingly to prevent myristoylation, the N-terminal glycine was substituted by alanine (G to A). A point mutation was also introduced into the active site of the protease domain (aa 93; GI: 326385) to impair its enzymatic activity.

2.2. Construction of recombinant poxvirus vectors

After digestion with EcoRI and SacI, the DNA fragment containing gagpolnef sequence was isolated from pCRscript/gpn and cloned into the Vaccinia virus (VV) insertion vector pH101. In the resulting plasmid, the expression of Gpn and the selection marker β-glucuronidase is regulated by the VV synthetic early/late promoter e/l [23] and promoter p7.5, respectively. The synthetic e/l promoter contains 40bp which largely overlap early and late regulatory elements [23]. This construct was further checked by sequencing and inserted into the VV haemagglutinin gene. The selection of recombinant MVA viruses (MVAgpn) was performed as in [11]. The purity of the recombinant virus was confirmed by PCR analysis. The construction of MVA delivering the luciferase protein is described elsewhere (MVALuc [11]). MVA recombinants were grown in CEF, purified by sucrose-cushion and titrated by immunostaining in CEF [11]. Recombinant NYVAC expressing Gpn (NYVACgpn) was provided by Dr. Frachette (Aventis Pasteur, Lyon). NYVACgpn was grown in CEF and titrated by immunostaining in CEF or by plaque assay in BSC-40 cells. Both MVAgpn and NYVACgpn recombinants represent a homogenous virus population, as established after PCR analysis. In NYVACgpn, Gpn expression is regulated by the same synthetic e/l promoter as in MVAgpn except that the expression cassette is inserted into the thymine kinase gene. To generate WRgp, the sequences encoding natural HIV Gag and Pol proteins as well as the selection marker β-galactosidase were inserted in the thymine kinase gene. The expression of HIV antigens was assessed in BHK-21 cells infected at a multiplicity of infection (m.o.i.) of five with either sucrose-purified MVAgpn, NYVACgpn or WRgp. Cells were collected at various times after infection and extracts (12 μg) were run on 10% SDS-PAGE.
Gpn was visualized after Western blotting using rabbit polyclonal anti-gag p24 serum (ARP 432, NIBSC, Centralised Facility for AIDS reagents, UK). Alternatively, the analysis of Gpn intracellular location was performed at 24 hpi by immunofluorescence and confocal microscopy on permeabilized BHK-21 cells that were incubated with the antibody ARP 432 and the nuclei staining reagent To-Pro (Molecular Probes).

2.3. Mice immunisation

C57BL/6 mice were purchased from Harlan. Transgenic HHD mice kindly provided by Dr. Lemonnier (Pasteur Institute, France) are double-knockout for H-2D<sup>b</sup> and β2-microglobulin and transgenic for a chimeric HLA-A2 molecule, the only MHC class I, therefore, expressed [24].

For peptide immunisation, 25 μl of each peptide were mixed with 50 μg of tetanus toxoid universal helper peptide P30 in 200 μl PBS to allow expression of the viral antigens and used in complete medium for 6 days once or twice. For C57BL/6 effectors, Gpn-specific stimulation was achieved by adding 5 × 10<sup>5</sup> EL4gpn and 5 × 10<sup>5</sup> irradiated naïve C57BL/6 splenocytes. For HHD effectors, incubation was performed with 5 × 10<sup>5</sup> LPS blasts derived from HHD mice and independently loaded with each peptide [26]. Target cells for CTL assays were EL4gpn effector cells and RMA-S/HHD loaded with individual HLA-A2-restricted peptide, respectively for C57BL/6 and HHD-stimulated effector cells [24].

2.5. Stimulation of HIV-1-specific human CD8<sup>+</sup> T cells with MVAgpn

The expansion of Pol2- and Gag-specific CD8<sup>+</sup> T cells induced by MVAgpn was studied as described in [27] using cryo-preserved peripheral blood mononuclear cells (PBMC) from an HIV-1-infected patient. Patient CNA 2099 is part of a cohort of HIV-1-infected patients with progressive disease enrolled in therapeutic clinical trials with anti-retroviral regimens [28,29]. These studies were approved by the local Institutional Review Board and the subject gave written informed consent. PBMCs from an HIV-seronegative subject (LDH 197) were included in the study as negative control.

PBMCs were cultured at 1 × 10<sup>6</sup> cells per ml with a sonicated preparation of MVAgpn or MVA<sub>lac</sub> at the indicated m.o.i. Fresh culture medium supplemented with recombinant human IL-2 (0.02 U/ml, Roche Diagnostics) was added every 2–3 days to the PBMC cultures. On day 10, the expansion of Pol2- and Gag-specific CD8<sup>+</sup> T cells was monitored by staining with APC-conjugated anti-CD8 antibodies (clone SK1) and PE-labelled tetramers. Class I-peptide tetramers were produced as previously described [30] and visualised after Western blotting using rabbit polyclonal anti-gag p24 serum (ARP 432, NIBSC, Centralised Facility for AIDS reagents, UK). Alternatively, the analysis of Gpn intracellular location was performed at 24 hpi by immunofluorescence and confocal microscopy on permeabilized BHK-21 cells that were incubated with the antibody ARP 432 and the nuclei staining reagent To-Pro (Molecular Probes).

3. Results

3.1. Construction of the attenuated poxviruses candidate vaccines

3.1.1. Characteristics of the chimeric Gagpolnef

Gagpolnef is a fusion protein of 1326 amino acids composed of Gag, Pol and Nef from the HIV-1 clade B clone IIIB [31] that has been modified to enhance its immunogenicity and for safety reasons (Fig. 1). The gag sequence was fused in frame to the polnef part by creating a 1 frame shift in the natural slippery sequence. A glycine to alanine
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3.1.2. Expression of Gagpolnef

The synthetic gagpolnef gene was introduced into the MVA or NYVAC genome, referred to as MVA-gpol and NYVACgp, respectively. After infection of permissive BHK21 cells with MVA-gpol, a band corresponding to the full length Gagpolnef protein (∼150kDa) was revealed on immunoblot with anti-Gag polyclonal antibodies (Fig. 2A). Pol- and Nef-specific antibodies recognised the same band (data not shown). BHK-21 infection by WR harbouring wild type HIV IIIB gag and pol genes (WRgp) resulted in PR-mediated Gag processing as shown by the presence of p55 and p24. In contrast, native HIV proteins resulting from PR activity were not observed in cells infected with MVA-gpol. Therefore, according to the construct design, MVA-gpol-infected cells produce a Gagpolnef fusion protein that cannot be processed by PR into native and potentially harmful HIV proteins. As compared to MVA-gpol, the full length Gpn fusion protein was produced in NYVACgp infected cells but at different levels depending on the time point after infection (Fig. 2B). In these cells, expression of Gpn was higher at 6h post infection (hpi) in NYVAC-gpn when compared to MVA-gpol-infected cells. We have consistently found that in permissive (CEF, BHK-21) and non-permissive (TK-143, HeLa) cells infected with NYVACgp, the levels of Gpn at 6hpi are somewhat higher or similar to those found in cells infected with MVA-gpol depending on the cell lines (Fig. 2B and data not shown). This observation rules out the possibility that putative mutations in the early/late promoter of NYVACgp influences expression. With time (18 and 24hpi), the Gpn expression level decreases in NYVACgp versus MVA-gpol-infected cells. This results from differences of cytopathic effect and inhibition of virus-induced protein synthesis between both poxviruses (Najera et al, manuscript in preparation).
3.2. Recombinant MVA and NYVAC induce Gagpolnef-specific CTL response

To assess the immunogenicity of Gpn, C57BL/6 mice (H-2b) were immunised with MVAgpn and NYVACgpn and the response against the whole fusion protein was assessed using EL4 cells (H-2b) expressing Gpn (EL4gpn). As shown in Fig. 3A, a significant CTL activity against EL4gpn was detected in mice immunised with MVAgpn but not in animals treated with MVAuc. These results indicate that the fusion protein delivered by MVAgpn is efficiently processed and presented in a MHC class I-restricted pathway to CD8+ T cells.

To allow a direct comparison between immunisation protocols, Gpn-specific T-cell response was assessed using EL4 cells (H-2b) expressing Gpn (EL4gpn) and EL4 cells (H-2b) expressing luciferase (MV Aluc). Data are representative of three experiments. (B) C57BL/6 mice (n = 3) were injected i.p. or s.c. with MVAgpn or NYVACgpn. Ten days later, splenocytes from immunised animals were stimulated in vitro with LPS blasts loaded with each peptide. The magnitude of the response varied among the peptides, Gag, Pol2 and Pol3 being more immunogenic than Prot and Pol1. These results show that the selected HIV-specific CD8 epitopes found to be immunodominant in humans are immunogenic in HHD mice.

3.3. MVAgpn induces HIV-specific HLA-A2-restricted CTL in HHD mice

3.3.1. Immunogenicity of selected HLA-A2-restricted peptides

To test whether Gagpolnef expressed by MVAgpn was recognised by human MHC class I molecules, we immunised transgenic HHD mice that exclusively display a chimeric human HLA-A2.1 as MHC class I molecule [24]. Six HLA-A2-restricted peptides recognised by CTLs from HIV-infected individuals were selected throughout the fusion protein (Fig. 4A) and tested for their intrinsic immunogenicity in HHD mice. Seven days after immunisation with a mixture of the peptides, splenocytes were stimulated in vitro with LPS blasts loaded with each peptide. All peptides except Pol4 induced an HLA-A2-restricted IFN-γ-producing and CTL (Fig. 4C) responses against RMAS-HHD cells loaded with each peptide. The magnitude of the response varied among the peptides, Gag, Pol2 and Pol3 being more immunogenic than Prot and Pol1. These results show that the selected HIV-specific CD8 epitopes found to be immunodominant in humans are immunogenic in HHD mice.

3.3.2. MVAgpn triggers CTLs specific for peptides immunogenic in HLA-A2+ HIV-infected individuals

To assess whether HHD mice could respond to MVAgpn immunisation, an IFN-γ ELISPOT assay specific for MVA antigens was first performed using naive splenocytes infected with MVAuc as MVA-specific antigen presenting cells (Fig. 5A). The frequency of IFN-γ-producing cells was lower in HHD mice than in C57BL/6 animals, which correlates with the lower proportion of total splenic CD8+ T cells in HHD mice (on average 3% of total splenocytes). The response against the different HLA-A2 restricted epitopes spanning Gpn was next investigated after i.p. injection of MVAgpn. After 6 days in vitro expansion with each
Fig. 4. HLA-A2-restricted peptides from Gagpolnef stimulate CTL responses in HHD mice. (A) The position on Gagpolnef of six HLA-A2-restricted peptides (Gag, Prot, Pol1, Pol2, Pol3 and Pol4) recognised by HIV infected individuals is depicted. (B, C) HHD mice (n = 5) were immunised with a mixture of the HLA-A2 peptides with helper peptide P30 in IFA. One week later, splenocytes of immunised animals were collected and stimulated in vitro for 1 (B) or 2 (C) weeks with LPS blasts loaded independently with each peptide. An IFN-γ/ELISPOT assay (B) and a chromium release assay (C) were then performed using RMAS-HHD loaded with each peptide as target cells. Values obtained with non-loaded RMAS-HHD as target cells were subtracted from values obtained with peptide-loaded RMAS-HHD. This experiment was reproduced twice. SFC, asterisk and E:T mean spot forming cells, no detectable response, and effector/target, respectively.

3.4. MVAgpn stimulates human Pol2-specific CD8+ T cells

To define the relevance of our observations in a human model, we investigated whether MVAgpn could stimulate Pol2-specific human CD8+ T cells. To this aim, we used the in vitro assay developed by Dorrell and co-workers to show that recombinant MVA efficiently stimulates human CTLs [27]. PBMCs from an HLA-A2+ HIV-1-infected patient (CNA 2099) were found to contain Pol2-specific CD8+ T cells as shown by staining with Pol2 HLA-A2 tetramer (Fig. 6). The cells were then stimulated with MVAgpn at increasing m.o.i. or with MVAuc as control. Pol2-specific CD8+ T cells were specifically expanded in the presence of MVAgpn but not in the presence of MVAuc. Moreover, the expansion of Pol2-specific CD8+ cells, ranging from a 5.2- to 16.7-fold increase in cell number, was proportional to the m.o.i. No Pol2-specific T cells were detected in PBMCs from seronegative donors (LHD 197) stimulated with MVAgpn. Thus, these data show that the Pol2 epitope is generated in MVAgpn-infected human cells, loaded onto HLA-A2 and presented to human CD8+ T cells, confirming the results obtained in the HHD mice. Gag-specific CD8+ T cells were also specifically expanded upon stimulation with MVAgpn (13.5-fold increase compared to baseline, at a m.o.i. of 1). This observation suggests that some epitopes found to be cryptic upon immunisation of the HHD mice are however generated in human cells.

4. Discussion

Here we report the construction of a novel antigen containing multiple HIV proteins and its immunogenicity when expressed in poxvirus vectors. The antigen reported in this study has several advantages. First, it is constituted of the major proteins targeted by CTLs in a natural infection and gathered in a single expression cassette. In particular, the Pol protein is often seen by the immune system but its expression level is low during a natural infection. Here, the removal of the frameshift between gag and pol results in similar expression level of Gag and Pol proteins, i.e. Gag- and Pol-specific epitopes. Second, it has been designed for optimal safety as it does not produce functional proteins and, therefore, no potential virus-like particles (Fig. 1). Third, it has been engineered for human codon usage, which is known to increase both antigen expression and immunogenicity [34–36]. Gpn has been inserted into several vac-
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Splenocytes were also stimulated independently with each peptide. An IFN-γ/H9253 spot forming cells, no detectable response, and effector/target, respectively.

Pol2 and Prot1 epitopes was not significant. SFC, asterisks and E:T mean RMAS-HHD. The difference in the magnitude of the response against the effectors, respectively. Dashed line is background lysis of non loaded Pol2- and Prot1-loaded RMAS-HHD cells by Pol2- and Prot1-stimulated target cells. In panel C, squares and triangles represent specific lysis of chromium release assay (C) were then performed using RMAS-HHD as vaccination.

combinant poxviruses is a suitable antigen to use in human genetic when expressed either from NYVAC or MVAgpn using two different routes of inoculation. This also indicates that the different insertion sites for the heterologous antigen (TK or HA) have no apparent effect on the strength of the immune response.

Mice transgenic for human HLA molecules are important models to study the immunogenicity of vaccines dedicated to human trials. Our results support the use of HHD mice to study HIV-specific HLA-A2-restricted responses since selected HIV-specific CD8 epitopes found to be immunodominant in humans are immunogenic in HHD mice. Moreover, we provide strong evidence that an immunodominant epitope found after immunisation in the HHD mice is similarly processed in humans cells, thereby reinforcing the relevance of this mouse model in the prediction of vaccine trials.

When the peptides were tested individually for their intrinsic immunogenicity, the magnitude of the response was found to be different between peptides: Gag, Pol2 and Pol3 were more immunogenic than Prot and Pol1. This observation could reflect a variation in HLA-A2 binding affinity within these epitopes. Pol4 for instance is not immunogenic in HHD mice and has indeed a poor binding affinity within these epitopes. Pol4 is not a control MV A stimulated pre-existing Pol2-specific hu-

doing proteolytic processing and stabilising capacity for HLA-A2[38]. Gagpolnef fusion protein delivered upon infection of mammalian cells with MVAgpn is processed into HLA-A2-restricted epitopes. Among the selected epitopes, Prot and Pol2 are immunodominant. The response against Pol2, also called I9V, is particularly interesting since this peptide has been reported in most HLA-A2 infected patients and is well conserved among HIV isolates, in particular B and C clades (http://hiv-web.lanl.gov/immunology/index.html). This indicates that the CTL response induced by MVAgpn could be cross-reactive in humans. The magnitude of the CTL response observed against Pol2 and Pol was weaker than the level of the Gagpolnef-specific response in conventional mice. The difference could be due to the immunological limitation of the HHD mice, i.e. low CD8+ T cell number and limited CD8+ T-cell repertoire [39]. In addition, the response in conventional mice is assumed to be directed against the three antigenic determinants including Gag, Pol and Nef and to be polyclonal, i.e. specific for various epitopes on each determinant. The strong Gagpolnef-specific response in C57BL/6 supports this hypothesis. Therefore, many HLA-A2-restricted epitopes different from those selected in this study are likely to stimulate CTL responses in the HHD mice.

The Gag peptide (known also as S9L) combined to an adjuvant elicit strong CD8 responses in the HHD mice whereas it is cryptic in the context of the fusion protein delivered recently.

Recently, Belyakov and colleagues have reported that MVA and NYVAC induce similar levels of protection against virulent Vaccinia virus challenge in mice [37]. However, no studies have thoroughly compared the immunogenicity of both vectors when carrying an heterologous antigen. Our study indicates that Gpn-specific immune responses are similar when induced by NYVAC and MVAgpn using two different routes of inoculation. This also indicates that the different insertion sites for the heterologous antigen (TK or HA) have no apparent effect on the strength of the immune response.

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Fig. 6. MV Agpn specifically stimulates Pol2-specific human CD8+ T cells. Diagrams represent flow cytometry analysis of PBMCs from HIV-seropositive (CNA 2099) and seronegative (LHD 197) patients stimulated or not with recombinant MV A for 10 days at indicated m.o.i. The number of Pol2-specific CD8+ T cells was evaluated by staining with anti-human CD8 antibodies and Pol2-specific tetramer before (baseline) and after stimulation. Diagrams represent cells gated on lymphocytes and numbers indicate the percentage of Pol2-specific cells among total lymphocytes.

...by MVA. Therefore, the lack of Gag-specific response upon immunisation with MV Agpn can not be explained by the absence of specific naive cells in the HHD mice. In humans, the Gag epitope emerges only after chronic HIV infection and has been, therefore, referred as subdominant [40]. However, we found that Gag-specific human CD8+ T cells are stimulated by MV Agpn in vitro. The difference between human and mouse could mirror some differences in the respective processing machineries. Overlapping peptide pools spanning the entire Gp120 molecule should be useful to address this point. This observation also means that human vaccination with MV Agpn could induce CTL specificities even broader than anticipated using the HHD model.

The induction of a restricted set of CTLs might favour the emergence of viral escape mutants, accelerating disease progression [41]. It is therefore crucial for a successful vaccine to induce the greatest breadth of CTL responses. As it was also shown for hepatitis B DNA vaccination [42], we demonstrate here that a novel complex multi-determinant HIV antigen is efficiently processed and is likely to induce multiepitopic T-cell response in humans. As antibodies may also play an important role in neutralising free viruses, the Gp120 membrane protein will be included in the vaccine to be used in clinical trials. The use of mouse model could be valuable in comparing various vaccines and vaccination protocols. The clinical trials that will be conducted by the EuroVacc consortium are going to establish whether CTL responses in HHD mice are predictive of those that the vaccines will elicit in humans.

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4.3. Publication #3

Cytomegalovirus (CMV)-Specific Cellular Immune Responses

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Cytomegalovirus (CMV)-Specific Cellular Immune Responses

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ABSTRACT: A large percentage of healthy individuals (50–90%) is chronically infected with Cytomegalovirus (CMV). Over the past few years, several techniques were developed in order to monitor CMV-specific T-cell responses. In addition to the identification of antigen-specific T cells with peptide-loaded MHC complexes, most of the current strategies to identify CMV-specific T cells are centered on the assessment of the functions of memory T cells including their ability to mediate effector function, to proliferate or to secrete cytokines following antigen-specific stimulation. The investigation of these functions has allowed the characterization of the CMV-specific T-cell responses that are present during different phases of the infection. Furthermore, it has also been shown that the combination of virus-specific CD4 and CD8 T-cell responses are critical components of the immune response in the control of virus replication. Human Immunology 65, 500–506 (2004). © American Society for Histocompatibility and Immunogenetics, 2004. Published by Elsevier Inc.

KEYWORDS: CMV; immune response; T cells; HIV; monitoring

ABBREVIATIONS
BMT bone marrow transplantation
CMV cytomegalovirus
CTL cytotoxic T lymphocytes
ER endoplasmatic reticulum
HIV human immunodeficiency virus
HLA human leukocyte antigen
IE immediate early
IFN interferon
IL interleukin
MHC major histocompatibility complex
PBMC peripheral blood mononuclear cells
TNF tumor necrosis factor
US unique short

INTRODUCTION
Cytomegalovirus (CMV) is a member of the human herpesvirus family, which includes herpes simplex virus types 1 and 2, Epstein-Barr virus, varicella-zoster virus, and human herpesvirus 5–8. It is a human double-strand DNA 230kb β-herpesvirus. More than 200 proteins are produced in three overlapping phases (immediate early (IE), early, and late). The predominant proteins critical for virion production are envelope proteins gB, gH, gM, and gL and the matrix proteins pp65/pp150/pp71 and pp28 [1].

CMV resides in the host throughout life without causing any symptoms in healthy, immunocompetent individuals, and 50–90% of the population have become seropositive by adulthood [2].

Primary CMV infection is always followed by a prolonged, inapparent infection during which the virus remains alive but usually dormant and resides in cells without causing detectable damage or clinical illness. The occurrence of CMV diseases is almost exclusively restricted to immunocompromised hosts. In acquired immunodeficiency syndrome (AIDS) patients, CMV causes retinitis or enteritis [2]. CMV syndrome is characterized by fever, leukopenia, hepatosplenomegaly, myalgias and occasionally pneumonitis in organ transplant recipients. Interstitial pneumonitis and rarely retinitis may complicate bone marrow transplantation (BMT) [3]. In some cases CMV is also a cause of mononucleosis-like illness [1].

CMV cellular reservoirs are leukocytes, epithelial cells of salivary glands, and cervix. Infectious CMV may be
shed in body fluids of infected persons, and may be detected in urine, saliva, blood, tears, semen, and breast milk. Examination of organ tissues and of peripheral blood obtained from patients with CMV disease has suggested that peripheral blood mononuclear cells (PBMC) are also a viral reservoir, and further analyses of PBMC revealed monocytes as the predominant infected cell type [2]. On allogeneic stimulation of PBMC, latent PBMC revealed monocytes as the predominant infected (PBMC) are also a viral reservoir, and further analyses of peripheral blood mononuclear cells blood obtained from patients with CMV disease has shed in body fluids of infected persons, and may be detected in urine, saliva, blood, tears, semen, and breast milk. Examination of organ tissues and of peripheral blood obtained from patients with CMV disease has suggested that peripheral blood mononuclear cells (PBMC) are also a viral reservoir, and further analyses of PBMC revealed monocytes as the predominant infected cell type [2]. On allogeneic stimulation of PBMC, latent CMV can be reactivated to produce infectious virus. CMV is thought to be controlled by antigen-specific antiviral CD8 T cells. However, reactivation of CMV occurs often in certain high-risk groups such as immunocompromised subjects, but also in asymptptomatically healthy individuals. A series of mechanisms have been proposed to be responsible for CMV reactivation. These include: (1) stress (through catecholamine using the cAMP system), (2) inflammation (through tumor necrosis factor [TNF]-α using nuclear factor κB or through prostaglandins using the cAMP pathway), and (3) some cAMP-elevating drugs (e.g., pentoxifylline). The ultimate result is the activation of the CMV immediate early (IE) enhancer/promoter, which is responsible for initiation of virus replication [4]. The high frequency of CMV-specific effector CD8 T cells found in healthy individuals indicates that CMV is more frequently reactivated than previously expected [4] but reactivation remains unnoticed and asymptomatic. In contrast, most of the situations of reactivation associated with clinical CMV diseases occur after transplantation or in immunocompromised subjects.

Reactivation of CMV from latency results in serious morbidity and mortality in immunocompromised transplant recipients or immunodeficient individuals and has both direct and indirect effects. Actually, CMV is the leading cause of death in allogenic BMT recipients and CMV-associated disease affects 40% of AIDS patients [5]. In AIDS patients, CMV reactivation is associated with the loss of CMV-specific proliferative responses. Interestingly, CMV infection is reactivated during bacterial sepsis. Four days after the onset of sepsis 32.4% of patients with sepsis show signs of active CMV infection [5]. This percentage is equally high in renal transplantation patients [5].

The loss of immune control of CMV that is evidenced by the detection of antigenemia is closely associated with an impaired function of CMV-specific CD8 T cells. In fact, it is the reduced cytokine production rather than a lower frequency or absolute number of CMV-specific CD4 or CD8 T cells that is thought to be responsible for the loss of immune control. Reduced numbers of cytokine-producing CMV-specific CD8 T cells were found in individuals with a higher risk of CMV reactivation. The highest frequencies and absolute numbers of CMV-specific CD8 T cells were noted in those subjects who experienced early or late CMV reactivation. CMV-specific cytotoxic T lymphocyte (CTL) responses are generally lost in subject undergoing allogeneic BMT and restoration of those responses requires an extended period [6].

A series of genes are directly involved in these mechanisms of immune evasion [7]. The primary target of the proteins encoded by these genes is the class I antigen processing pathway: the unique short (US) region 3, which is expressed in the IE phase binds to and retains major histocompatibility complex (MHC) class I molecules in the endoplasmatic reticulum (ER). US2 and US11, both early gene products, cause the translocation of MHC class I to the cytosol where it is degraded. US6 blocks the transport of antigen-peptide into the ER. CMV protein US2 contributes to the degradation of human leukocyte antigen (HLA)-Dr and HLA-Dm through the inhibition of class II transactivator. The inhibitory effects on T-cell antigen recognition is active when viral or self-antigens are synthesized within the cells, but not if the specific epitope is given as a peptide [7]. These strategies may explain the immunodominance of CTL directed against viral antigens that can be presented before expression of the US genes. In CMV-infected cells, the expression of the viral phosphoprotein pp65 inhibits the generation of CMV-specific T-cell epitopes [7].

**Monitoring**

The monitoring of CMV replication is critical after transplantation. Among the laboratory tests that have been used, the CMV DNA amplification assay in plasma has shown limited sensitivity as compared with the detection of pp65 antigen in leukocytes. Recently, Kaiser et al. [8] developed an ultrasensitive plasma DNA polymerase chain reaction assay with a limit of detection of 20 CMV DNA copies/ml of plasma. By using this latter technique, the occurrence of CMV reactivation in stem cell transplantation patients and renal transplant recipients is detected on average 4 and 12 days earlier, respectively, compared with the pp65-positive test [8].

Until recently, chromium release assays and limiting-dilution analyses were the most common techniques used to measure specific T-cell responses for research purposes. However, these techniques are time-consuming and not very sensitive [9]. An additional strategy to evaluate antigen-specific T-cell responses is the proliferation assay, which is based on the ability of cells (mostly CD4 T cells) to proliferate after antigen stimulation. The major advantage of this technique is that it requires only very few cells, but this strategy does not allow precise quantification of the actual frequencies of helper CD4 T cells and presents a series of technical constraints that have made difficult its standardization.
A novel methodology has been recently developed to determine cell division and proliferation in mononuclear cells after antigen-specific stimulation. This method is based on the use of a dye, carboxyfluorescein succinimidyl ester (CFSE), which allows to visualize proliferating cells by flow cytometry (Figure 1A). After cell division, CFSE equally distributes within the two daughter cells and, after each cell division, fluorescence intensity is reduced of 50%. The advantage of this technique is the possibility to combine multiple surface markers to identify and characterize the type of dividing cells.

The enzyme-linked immunospot assay (Elispot) allows the delineation of the functional properties of T cells and, particularly, their ability to secrete cytokines after antigen stimulation [9]. T cells (either PBMC or purified CD4 or CD8 T cells) and antigens are mixed in anticytokine antibodies precoated plates (Figure 1B). A second chromogenic substrate coupled to anticytokine antibodies is added and the secreted cytokine molecules form spots. The advantage of this technique is the high degree of sensitivity (i.e., at least 30–100 times more sensitive than the chromium release assay [9]). Furthermore, it is the most efficient assay available for the quantitative evaluation of antigen-specific immune responses [9].

Intracellular cytokine staining is another methodology available for the functional characterization and quantification of antigen-specific T cells. The advantage of using this technique resides in the possibility of assessing simultaneously the phenotype, the function, the replicative history, and the number of antigen-specific T cells. The limitations are mostly associated with the technical difficulties in the standardization of flow cytometry procedures (Figure 1C).

The development of MHC class I tetramer complexes has substantially advanced the characterization of antigen-specific CD8 T-cell responses. Along the same line, MHC class II tetramers were developed to study CD4 T-cell responses. However, the development of MHC class II tetramers is still in a preliminary phase. Fluorescent multimers of MHC-peptide complexes are used to quantify, to perform functional and phenotypic characterization, and to isolate peptide-specific T cells by flow cytometry (Figure 2).

**Acute Infection**

Primary CMV infection is usually asymptomatic but can also cause a mononucleosis-like illness, with leukopenia, fever, and hepatitis. Acute CMV infection is usually localized within the salivary gland epithelium [1].

After antigenic stimulation, naive T cells follow a program of proliferation and differentiation that leads to the generation of effector cells and, ultimately, to the generation of memory cells. The different models of effector and memory differentiation have been extensively described [10].

The major expansion of CMV-specific CD8 T cells during primary CMV infection is well-documented but until recently little was known about CD4 T-cell responses. In this regard, it has recently been shown, in a study analyzing CD4 T-cell responses during primary and chronic CMV and human immunodeficiency virus (HIV)-1 infections, that a major expansion of virus-specific interferon (IFN)-γ secreting CD4 T cells is associated with primary CMV but not with HIV-1 infection [11] (Figure 3). In contrast to HIV-1 infection, the magnitude of primary CMV-specific CD4 T-cell response was indeed significantly different from that observed during chronic infection. It is also worth noting that the large majority (>90%) of the expanded IFN-γ secreting virus-specific CD4 T cells was contained within the cell population lacking CCR7 and therefore belonged to the effector memory cell population [11] (Figure 3).

Of interest, the kinetics and characteristics of CMV-specific CD4 and CD8 T cells were recently investigated in the course of primary CMV infection in asymptomatic and symptomatic recipients of renal transplants [12]. It has been shown that in the case of an asymptomatic primary CMV infection, the peak of IFN-γ secreting CD4 T cells appears 10 days after CMV DNA is detectable and is followed, 7 days later, by the appearance of immunoglobulin M and immunoglobulin G and 14 days later by the appearance of CMV-specific CD8 T cells. Interestingly, in the few symptomatic patients, there were no significant differences in the kinetic or magnitude of the antibody or CD8 T-cell responses but there was a delay in the CD4 T-cell response [12].

In contrast to CD4 T-cell response during primary infection that was only analyzed recently [10], the clonal expansion of virus-specific CD8 T cells during primary infection is a hallmark of the adaptive immune response. Recently, Gamadia et al. [12] have performed an analysis of the CMV-specific CD8 T-cell response during primary infection. They have demonstrated that these cells had a phenotype of typical effector cells (i.e., CCR7 CD27 CD45RA). They also demonstrated that, in contrast to the previous experiments performed in mice, functional CD8 T cells were not sufficient to control viral replication and that CMV-specific IFN-γ secreting CD4 T cells were necessary for recovery from the infection [12].

**Chronic Infection**

In the immunocompetent host, the virus remains efficiently controlled and several components of the immune system are shown to play a role. In mice it has been demonstrated that both T and B cells play a role in the control of CMV [2, 13].
FIGURE 1  (A) Representative flow cytometry profiles of carboxyfluorescein succinimidyl ester (CFSE) labeled CD4 T cells. After 6 days of culture, staphylococcal enterotoxin B (SEB)–stimulated cells (right panel) show three peaks of cell division as compared with the unstimulated cells (left panel). (B) Representative enzyme-linked immunospot (ELISPOT) wells after different stimulations. Each dark spot correspond to one cytokine-secreting cell. (C) Flow cytometry profiles of cytomegalovirus (CMV)-specific interferon (IFN)-γ secreting CD4 and CD8 T cells of one representative subject. CD4 T cells were stimulated with CMV lysate, whereas CD8 T cells were stimulated with a pool of pp65 peptides and then stained with anti-CD4 or CD8 PerCP Cy5.5, anti-CD69 FITC and anti-IFN-γ-APC, and analyzed by flow cytometry. The cluster of events shown in red corresponds to the responder T cells (i.e., co-expressing CD69 and IFN-γ), whereas the cluster of events in blue correspond to the nonresponder T cells. Data are expressed as the percentage of cells coexpressing IFN-γ and CD69 within CD4 or CD8 T cells.
In HIV and CMV coinfected patients, elevated and very stable CD4 and CD8 T-cell responses to CMV were observed [14, 15]. The majority of CMV-specific CD8 T cells in peripheral blood were able to produce a range of antiviral factors after stimulation with specific antigens (IFN-γ, macrophage inflammatory protein-1β, TNF-α) [13].

With regard to CD4 T cells, CMV-specific lymphoproliferation and interleukin (IL)-2 secreting CD4 T-cell responses were positive in healthy subjects [14] but deficient CD4 T-cell responses were reported in BMT recipients [2]. The majority of CMV-specific IFN-γ secreting CD4 T cells belonged to the CCR7 - compartment whereas interleukin-2 – secreting CD4 T cells represented the dominant virus-specific population within CCR7 + cells [14].

Our group has previously reported that CMV-specific memory CD8 T cells were composed by 50% of terminally differentiated cells (CD45RA -CCR7 -), 40% of preterminally differentiated cells (CD45RA–CCR7 -), and 10% of CCR7 + cells in both blood and lymph nodes [15]. A phenotype of differentiated cells was also confirmed by others using different immunologic markers, such as CD27 and CD28 or CD7 [13, 16]. In this regard, Aandahl et al. [16] have also recently shown a model in which CD7 identifies three populations of CD8 T cells. CD7 high CD8 T cells include naive and memory cells and retained proliferative capacity and in contrast CD7 low and CD7 neg CD8 T cells discriminate between two subsets of effector cells (i.e., cytokine-secreting cells and cells with high lytic potential respectively [16]).

The comparison between HIV- and CMV- specific CD8 T cells has also allowed the identification of a weak level of perforin in HIV-specific CD8 T cells as compared to CMV-specific CD8 T cells. This was related to a significantly higher cell lysis capacity for a given effector/target ratio [13]. However, conflicting results have been reported regarding the ability of CMV-specific CD8 T cells to express perforin.

Most of the CMV-specific CD8 T-cell responses are specific to pp65 but, in a number of cases, the response to the identified HLA-A2–restricted IE-1 peptides was shown to exceed that of A2-pp65. IE-1–specific cells have high CD57, low CD28 expression, absence of CCR7, and high levels of intracellular perforin, which is typical of CMV-specific memory T cells [17].

Even HIV-1–infected individuals can maintain high and stable frequencies of CMV-specific CD4 and CD8 T cells. In contrast, there is a decrease in the frequency of CD4 T cells (as assessed by the proportion of CMV-specific TNF-α secreting CD4 T cells) that leads to the inability to sustain CD8 T cells in patients with CMV-associated end-organ disease [2].

**Tissue Distribution**

As shown in mice, memory T cells with effector function accumulate in the target organ of the pathogen away from the lymphoid tissue [18]. The anatomic distribution (e.g., blood and lymph nodes) of CMV-specific as compared with HIV-1–specific T cells was recently investigated. Although HIV-1–specific IFN-γ CD4 T cells

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**FIGURE 2** (A) Major histocompatibility complex (MHC) class I heavy chain molecules lacking the transmembrane domain are refolded with β2-microglobulin together with a peptide of interest. Bacterial BirA enzyme is used to attach a biotin molecule to the specific BirA-recognition sequence that has been incorporated into the C-terminus of the MHC molecule. Tetramers are finally formed by incubation with fluorescence labeled streptavidin molecules. (B) Example of a tetramer staining of blood mononuclear cells (PSMC) from an human leukocyte antigen (HLA)–matched cytomegalovirus (CMV)-infected subject.
were equally distributed in the blood and lymph node compartments, CMV-specific IFN-γ CD4 T cells predominantly accumulated in the blood [14]. Similar observations were obtained for CD8 T cells where memory CMV-specific CD8 T cells were accumulated mostly (10-fold higher frequency) in the blood as compared with the lymph nodes, whereas HIV-1–specific CD8 T cells were equally distributed [15]. Therefore, the results obtained are compatible with the different target organs of HIV-1 (i.e., the lymphoid tissue) and of CMV (i.e., lung, cervix, salivary glands, the retina) [19].

**Clonality, Repertoire, and Immunodominance**

CMV matrix protein pp65 has been identified as a significant target antigen for CMV-specific CTL. CMV-specific CTL recognition of pp65 on target cells occurs immediately after infection of a cell, before the onset of viral gene expression, and persists throughout the duration of the replicative cycle [1].

There is a high degree of clonal focusing among memory CTL–specific for a given CMV pp65 peptide-MHC complex. CTL from different donors that recognize the same peptide-MHC complex use only one or two Vβ gene segments. A given β-chain is always associated with the same α-chain. Memory CTL response to individual CMV pp65 epitopes contains individual clones that have undergone extensive expansion in vivo [20]. In some individuals, IE-1 (72kDa major IE protein), which is a non-structural protein found very early and throughout the replicative cycle, may be of the same importance as pp65. In healthy subjects, the percentage of individuals with pp65-specific CD8 T-cell response seems clearly larger than the percentage of those with IE-1–specific CD8 T-cell response. CD8 T cells specific to pp65 are found in a similar proportion of healthy subjects and renal transplant patients (85% vs 83%), whereas CD8 T cells reactive to IE-1 occurred in a higher percentage of renal transplant patients than of healthy subjects (75% vs 48%) [21]. The ubiquity of pp65-specific CTL in natural CMV infection suggests that pp65 epitopes can be presented by a wide variety of HLA alleles. Kondo *et al.* [17] recently identified 14 novel CTL epitopes derived from

**FIGURE 3** (A) Analysis of human immunodeficiency virus (HIV)-1–specific and cytomegalovirus (CMV)-specific CD4 T cells within different populations of memory cells defined by the expression of CCR7. Patients 3 and 4 had primary HIV-1 and CMV coinfection, and patient 8 had primary HIV-1 infection and chronic CMV infection. Blood mononuclear cells were stimulated with p55 gag (i.e., an HIV-1 protein) and CMV lysates and analyzed for the expression of CD4, CCR7, CD69, and interferon (IFN)-γ (intracellular expression). The data show the expression of CD69 and IFN-γ within CD4⁺CCR7⁻ and CD4⁺CCR7⁺ T-cell populations. Negative control: unstimulated blood mononuclear cells. After in vitro antigen-specific stimulation, blood mononuclear cells were stained with anti–IFN-γ APC, anti-CD69 FITC, anti-CCR7 PE, and anti-CD4 Cyochrome. Adapted from Harari *et al.*, Blood 2002 [11]. (B) Summary of the magnitude of the response of HIV-1–specific and CMV-specific primary and chronic infection.

MHC complex. CTL from different donors that recognize the same peptide-MHC complex use only one or two Vβ gene segments. A given β-chain is always associated with the same α-chain. Memory CTL response to individual CMV pp65 epitopes contains individual clones that have undergone extensive expansion *in vivo* [20]. In some individuals, IE-1 (72kDa major IE protein), which is a non-structural protein found very early and throughout the replicative cycle, may be of the same importance as pp65. In healthy subjects, the percentage of individuals with pp65–specific CD8 T-cell response seems clearly larger than the percentage of those with IE-1–specific CD8 T-cell response. CD8 T cells specific to pp65 are found in a similar proportion of healthy subjects and renal transplant patients (85% vs 83%), whereas CD8 T cells reactive to IE-1 occurred in a higher percentage of renal transplant patients than of healthy subjects (75% vs 48%) [21]. The ubiquity of pp65–specific CTL in natural CMV infection suggests that pp65 epitopes can be presented by a wide variety of HLA alleles. Kondo *et al.* [17] recently identified 14 novel CTL epitopes derived from
CMV pp65 antigen, restricted to several HLA-A, HLA-B, and HLA-C alleles.

For CMV-specific CD4 T cells, stable clonotypic hierarchy could be shown in which 1–3 clonotypes accounted for 10–50% of the overall CMV response and comprised 0.3–4.0% of peripheral blood CD4 T cells [22]. The CMV-specific CD4 T-cell memory repertoire in normal subjects is characterized by striking clonotypic dominance suggesting that primary responsibility for immunosurveillance against CMV reactivation rests with a handful of clones recognizing a limited array of CMV determinants [22].

Conclusion
A large percentage (50–90%) of healthy individuals is chronically infected with CMV. Of interest, CMV infection is successfully controlled by the host immune system and the infection goes generally unnoticed with the exception of immune-compromised hosts. The ability to control CMV infection is mediated by the combination of both virus-specific CD4 and CD8 T-cell responses.

REFERENCES
5. Conclusions and Outlook

The aim of my thesis was to investigate memory CD8 T cells in different models of virus-specific T cell responses. In particular, CD8 T cell responses to HIV-1, CMV, EBV and Influenza virus (Flu). HIV-1-specific CD8 T cell responses were studied in cohorts of subjects with progressive infection and compared to individuals with non-progresssive infection who where naïve to antiretroviral therapy. Knowledge about the keypoints of an effective CD8 T cell response would be helpful developing efficient therapeutic vaccines against HIV-1 infection.

Several studies highlight the importance of CD8 T cells in the rhesus monkey model:
1. Depletion of CD8 T cells by monoclonal Ab results in enhanced viral replication (25, 207, 208).
2. CTL are able to exert selective pressure on the viral genome (209, 210).
3. Vaccine strategies that are able to elicit virus-specific CD8 T cell response are also capable of controlling viral replication and prevent the onset of disease (211-213).

The importance of CD8 T cells has also been proven in human studies:
1. CTL are able to control viral replication in vitro (33).
2. The appearance of HIV-1-specific CTL occurs simultaneously with the control of a burst of viral replication (31).
3. HIV-1 infected individuals with low viral loads show a potent virus-specific CTL response (63, 214-217).
4. Loss of HIV-1-specific CD8 T cells is linked to a rapid progression to AIDS (217).
5. Similar as in monkeys, CTL exert a potent selective pressure on AIDS (35, 79, 80).

In preliminary experiments, we performed a phenotypic characterization of the virus-specific CD8 T cell responses by staining PBMC with MHC class I tetramers loaded with virus-specific peptides and CD45RA and CCR7 markers. Confirming previous studies from our and other groups (14, 36, 218), we found a phenotypic heterogeneity within the different models of virus-specific CD8 T cell responses. HIV-1-specific CD8 T cells in subjects with progressive disease were almost exclusively CD45RA-CCR7- TEM while CMV- and EBV-
specific CD8 T cells were mostly CD45RA^CCR7^- (14, 219, 220). The latter phenotype is therefore typical of a CD8 T cell response associated with an immune control of the pathogen. In support of this hypothesis, a recent study has shown a correlation between the proportion of HIV-1-specific CD45RA^CCR7^- CD8 T cells and control of viral replication (221). Thus, the CD45RA^CCR7^- phenotype seems to be a marker of immune control in virus infections, where the pathogen is able to establish chronic infection. However, the same phenotype could not be found in the pool of Flu-specific CD8 T cells, despite the fact that the virus is efficiently cleared by the immune response. Thus, it is likely that the CD45RA^CCR7^- subpopulations are only generated under conditions of protracted Ag stimulation which is not the case in Flu infection. The CD45RA^CCR7^- phenotype which is typical for T_{EM} CD8 T cells, was found both under conditions of high Ag load (progressive HIV-1 infection) and of Ag clearance (Flu infection), therefore reflecting situations of ongoing or pre-existing effector responses. Finally, consistently with what has been shown in the LCMV model (222-224), the highest proportion of CD45RA^CCR7^- T_{CM} were found under conditions of complete Ag clearance, i.e. Flu-specific CD8 T cell response.

The results of functional characterization of virus-specific CD8 T cell responses have demonstrated a selective defect of IL-2 secreting cells in the settings of progressive HIV-1 infection accompanied by an inability of HIV-1-specific CD8 T cells to proliferate in response of specific stimulation. Virus-specific IL-2 secreting cells were consistently detected in CMV, EBV, non-progressive HIV-1 and Flu infections. In all these conditions, there is an effective immune control, where the Ag level is either low or even cleared. Previous studies have hypothesized that phenotypic and functional heterogeneity of virus-specific CD8 T cell responses was pathogen-specific (36). However, the present results show that the same pathogen (i.e. HIV-1) is associated with substantially different CD8 T cell responses in progressive as compared to non-progressive HIV-1 infection. The major difference between those two settings is the level of Ag. Therefore, consistent with the observations in the LCMV model (223), our results rather support the hypothesis that the phenotypic and functional heterogeneity of virus-specific CD8 T cells is influenced by Ag persistence and Ag load. The fact that IFN-γ/IL-2-secreting cells are lacking in progressive HIV-infection, but not in LTNPs indicate that IL-2 secretion by CD8 T cells might be associated with control of HIV-1 infection or viral infections in general. Interestingly therapies administrating exogenous IL-2 have only an effect on CD4 T cells but not CD8 T cells (225-228). Another study demonstrated that the capacity of IL-2 to increase CD8 T cell counts and the expression of perforin and granzyme B is limited to the time of cytokine administration. Prolonged
observations indicated rather a tendency of CD8 T cells to decline over time (229). In view of the results of our studies, this might suggest that autocrine secretion of IL-2 by virus-specific CD8 T cells, but not exogenous IL-2 exposure is crucial for the maintenance of their own proliferation.

The factors responsible for the capacity of a CD8 T cell to secrete IL-2 still remain to be investigated. From our studies it is not clear whether the high viral load exhausts IL-2 secretion, or whether IL-2 secretion is responsible for the low viral load. Since our results demonstrate that only the CD45RA/CCR7 memory subset secretes IL-2, the presence of different memory subsets in the various virus infections might be the key. However, this subset is also predominant in individuals with progressive HIV-1 infection. Thus, the mechanism of total Ag clearance must have an alternative explanation. It may be interesting to look at a molecular level. The strength of signal (peptide affinity, avidity), influenced by the higher viral load might influence the decision if IL-2 or IFN-γ is secreted or which receptor is expressed or downregulated.

To evaluate whether the Ag-load is responsible for the absence of IL-2 secreting CD8 T cells, different manipulations of the in vivo Ag level and exposure might be performed. Therefore the capacity of CD8 T cells to secrete IL-2 or to proliferate should be investigated in HIV-1 infected individuals under ART. This would be equal to the model of protracted viral infection with controlled virus replication. Under these conditions, we would expect to find IL-2 secreting HIV-specific CD8 T cells that are able to proliferate as it could be shown in LTNP. To prove that the absence of IL-2 secreting cells is not restricted to HIV-infection, cytokine secretion should be addressed in individuals with acute CMV or EBV infection. Furthermore, it would be interesting to compare the viral immune response in the same individual before ART, during ART and after treatment interruption. Last but not least, individuals with cleared viral infections should be re-exposed to the same virus by re-immunization. We tried to perform this in individuals getting vaccinated against Influenza. Unfortunately we were not able to detect Flu-specific CD8 T cells following immunization possibly because the vaccine didn’t contain the same peptides as used for our experiments. To avoid this, we should probably screen Flu-specific CD8 T cells by stimulating PBMCs with the vaccine, but it is not sure whether a 12h-stimulation would be long enough for peptide-processing and presentation to CD8 T cells.

Previous studies performed in our lab (46, 230, 231) have addressed these issues in Ag-specific CD4 T cells. As a model of Ag clearance, tetanus toxoid (TT) was used, CMV, EBV and HIV-1 from LTNP were used as models of Ag-persistence and protracted Ag exposure
with controlled viral replication. Progressive HIV-1 infection represented a situation of Ag persistence with uncontrolled virus replication. In addition, primary CMV and HIV infection was analyzed as fourth model of acute Ag exposure and high viral Ag load. As shown for CD8 T cells, different functional subsets could be defined by the secretion of IFN-γ and IL-2. However, in CD4 T cells there is an additional subset i.e. single IL-2 secreting cells which is the dominant response to TT, and which is also present for CMV, EBV and HIV in non-progressive infection. The results are quite similar for CD4 and CD8 T cells. In both T cell subsets, the absence of IL-2 secreting cells is associated with a situation of high Ag levels. In vivo manipulations of Ag levels and exposure confirmed that the Ag level is critical for the appearance of the different functional memory subsets. In the case of CD4 T cells, it was possible to re-immunize subjects having exclusively TT-specific single IL-2 secreting cells. A substantial increase in the percentage of IL-2 secreting cells could be observed with a peak at day 11 but more interestingly, the appearance of dual IL-2/IFN-γ and single IFN-γ could be demonstrated.

In contrast, manipulation of the conditions with virus persistence with controlled virus replication and low Ag exposure (ART, HIV-treatment interruption) showed a change from a polyfunctional profile (i.e. presence of single IL-2, dual IL-2/IFN-γ and single INF-γ subsets) to a single IFN-γ secreting CD4 T cell response. Since there seems to be an analogy of the CD4 and CD8 T cell response to virus infection, we would expect similar results in CD8 T cells. Along the same line, the phenotype with regard to CD45RA and CCR7 of Ag-specific T cells is always similar between CD4 and CD8 T cells. Three different memory subsets can be found in CD4 T cells as well as in CD8 T cells (14, 219, 231). In both cell types, the CD45RA-CCR7 subset dominates in HIV-1 infection, whereas CMV- or EBV-specific T cells are predominantly CD45RA⁺CCR7⁺.

While in CD4 T cells it could clearly be demonstrated that the Ag is dictating the functional and phenotypic profile of the response, this remains to be done in CD8 T cells.
6. References


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7. Glossary

**adaptive immunity**

antigen-specific defense mechanisms that take several days to become protective and are designed to remove a specific antigen. This immunity persists throughout life. There are two major branches of the adaptive immune response: humoral and cell-mediated immunity.

**antibodies**

Y-shaped protein of the surface of B cells that is secreted into blood or lymph in response to an antigenic stimulus, such as a bacterium, virus, parasite, or transplanted organ, and that neutralizes the antigen by binding specifically to it. Also called immunoglobulin.

**antigen**

any molecule that can bind specifically to an antibody. Their name arises from their ability to generate antibodies. However some antigens do not, by themselves, elicit antibody production; those antigens that can induce antibody productions are called immunogens.

**antigenic drift**

refers to mutations in the Influenza virus over time. Such mutations occur almost yearly in the Influenza virus. Mutations happen frequently because the virus is highly unstable and has no way of proofreading.

**antigenic shift**

process by which two different strains of Influenza combine to form a new subtype which is characterized by a mixture of the surface antigens of the two original strains.

**apoptosis**

programmed cell death; form of cell death in which the cell activates an internal death program. It is characterized by nuclear degradation, nuclear degeneration and condensation, and the phagocytosis of cell remains. Proliferating cells frequently undergo apoptosis, which is a natural process in the development and during immune responses. Apoptosis contrasts with necrosis, death caused by external factors, which occurs in situations such as poisoning or anoxia.

**arthralgias**

Pain in the joint

**attenuation**

a dilution, thinning or weakening of a substance, especially a reduction
in the virulence of a pathogen through repeated inoculation, growth in a different culture medium, or exposure to heat, light, air or other weakening agents

**autocrine** mode of hormone action in which a hormone binds to receptors on and effects the function of the cell type that produced it.

**Burkitt lymphoma** form of undifferentiated malignant lymphoma usually found in central Africa, but also in other parts of the world; commonly manifested as a large osteolytic lesion in the jaw or as an abdominal mass. B cell Ag are expressed on the immature cells that make up the tumor in virtually all cases of Burkitt lymphoma.

**Cap** 7-methyl guanosin added to the 5' end of a pre-mRNA molecules in eukaryotes. This cap is necessary for recognition by the ribosom, for export and also serves as protection against phosphatases and nuclease. (capping is specific for transcripts produced by RNA polymerase II)

**CD3 complex** complex of α:β or γ:δ T cell receptor chains with the invariant subunits CD3γ, δ and ε, and the dimeric ζ chains.

**CFSE** 5- (and -6)-Carboxyfluorescein diacetate succinimidyl ester is a membrane-permanent, fluorescein-based dye that can be used to follow proliferation of cells. The two acetate side chains render the molecule highly membrane permanent. Once inside the cells, the acetate groups are removed by intracellular esterases and the carboxyfluorescein exits from cells at much slower rate. CFSE couples covalently to intracellular molecules by reaction with intracellular amine groups forming a highly stable amide bond. When cells divide, CFSE labeling is equally distributed between the daughter cells, which are therefore half as fluorescent as the parent cells. As a result each successive generation in a population of proliferating cells is marked by a halving of cellular fluorescence intensity.

**chemokines** small chemoattractant proteins that stimulate the migration and activation of cells, especially phagocytic cells and lymphocytes. The
have a central role in inflammatory responses.

**chronic active EBV infection**
lymphoproliferative disorder characterized by abnormally high titers of anti-EBV Ab and increased levels of EBV-DNA in PBMC and plasma; 50% of patients die of complications such as hepatic failure, gastrointestinal bleeding, HLH and malignant lymphomas several years after onset of the disease (232-234)

**cis element**
regulatory sequence in DNA that can control a gene only on the same chromosome. In bacteria cis-acting elements are adjacent or proximal to the gene(s) they control, whereas in eukaryotes they may also be far away. (trans-acting elements → DNA sequences encoding diffusible proteins [e.g. transcription activators or repressors] that control genes on the same or different chromosome)

**coccidioidomycosis**
infection caused by inhalation the microscopic spores of the fungus Coccidioides immitis; acute form produces flu-like symptoms whereas the chronic form can develop as late as 20 years after initial infection with purulent lung disease

**concatemer**
a DNA segment composed of repeated sequences linked end to end

**croup**
condition characterized by resonant barking cough, hoarseness and persistent stridor and caused by allergy, foreign body or infection. It mainly occurs in infants and children.

**cytokines**
proteins made by cells that affect the behavior of other cells. Cytokines made by lymphocyte are often called lymphokines or interleukins (IL), but the generic term cytokine is used in this book and in most of the literature. Cytokines act via specific cytokine receptors on the cells that they affect.

**endemic infection**
a disease that is constantly present to a greater or lesser degree in a defined population.

**endocytosis**
process of cellular ingestion by which the plasma membrane folds
inward to bring substance into the cell.

epidemic infection  outbreak of a contagious disease that spreads rapidly and widely

epitope  site on an antigen recognized by an antibody or an antigen receptor; epitopes are also called antigenic determinants. A T cell epitope is a short peptide derived from a protein antigen. It binds to an MHC molecule and is recognized by a particular T cell.

exocytosis  process of cellular secretion or excretion in which substances contained in vesicles are discharged from the cell by fusion of the vesicular membrane with the outer cell membrane.

FACS  fluorescence activated cell sorter. Measures cell size, granularity and fluorescence due to bound fluorescent antibodies as single cells pass in a stream past photodetectors. The analysis of single cells this way is called flow cytometry and the instruments that carry out the measurement and/or sort cells are called flow cytometers.

Gianotti-Crosti-syndrome  synonym: papular acrodermatitis; discrete papules on the cheeks, dorsal surfaces of the hands and buttocks and the extensor aspects of the arms and thighs of infants.

Guillain-Barré syndrome  temporary inflammation of the nerves, causing pain, weakness, and paralysis in the extremities and often progressing to the chest and face. Typically occurs after recovery from a viral infection or, in rare cases, following immunization against influenza.

haemophagocytic syndrome  synonym: hemophagocytic lymphohistiocytosis (HLH); unusual syndrome characterized by fever, splenomegaly, jaundice, pancytopenia, disseminated intravascular coagulation and features of haemophagocytosis of erythrocytes, leucocytes and platelets by macrophages in the bone marrow and other tissues.

helicase  protein/enzyme that unwinds DNA molecules at the replication fork
histoplasmosis: disease caused by the fungus *Histoplasma capsulatum*; infects lungs, skin, mucous membranes, bone, skin and eyes

HIV encephalopathy: any disorder or disease of the brain caused by HIV

HIV wasting syndrome: involuntary weight loss > 10% associated with intermittent or constant fever and chronic diarrhea or fatigue for more than 30 days in the absence of a defined cause other than HIV infection. A constant feature is major muscle wasting with scattered myofiber degeneration. A variety of etiologies, which vary among patients, contributes to this syndrome.

Hodgkin’s lymphoma: synonym: Hodgkin’s disease; malignant progressive, sometimes fatal disease, marked by enlargement of the lymph nodes, spleen and liver.

Humoral immunity: the component of the immune system involving antibodies that are secreted by B cells and circulate as soluble proteins in blood plasma and lymph.

Hydroa vacciniforme: photosensitive dermatitis of childhood, mediated by the infiltration of EBV-infected T cells and reactive cytotoxic T cells (235). Resolves in most patients spontaneously with age.

Kaposi’s sarcoma: vascular proliferation characterized by the presence of spindle cells and vascular channels mixed with cellular infiltrates.

Lymphadenopathy: chronic, abnormal enlargement of the lymph nodes, usually associated with disease.

Macropinocytic: Macropinocytosis is a form of regulated endocytosis that involves the formation of large endocytic vesicles after the closure of cell-surface membrane ruffles.

Maculopapular rash: maculopapular describes a rash that contains both macules (flat discolored area of the skin) and papules (small raised bump). Usually manifested as large red area with small confluent bumps.
**meningoencephalitis**  
Inflammatory process involving the brain and meninges, most often produced by pathogenic organisms which invade the central nervous system, and occasionally by toxins, autoimmune disorders and other conditions.

**MHC**  
major histocompatibility complex; cluster of genes of human chromosome 6 or mouse chromosome 17. It encodes a set of membrane glycoproteins called the MHC molecules. The MHC class I molecules present peptides generated in the cytosol to CD8 T cells, and the MHC class II presents peptides degraded in intracellular vesicles to CD4 T cells. The MHC also encodes proteins involved in antigen processing and other aspects of host defense. The MHC is the most polymorphic gene cluster in the human genome, having large numbers of alleles at several different loci. Because this polymorphism is usually detected by using antibodies or specific T cells, the MHC molecules are often called major histocompatibility antigens.

**mononeuritis multiplex**  
disorder characterized by simultaneous or sequential damage to more than one nerve group.

**multifocal**  
leukoencephalopathy  
demyelinating disease that predominantly affects immunocompromised hosts.

**myalgia**  
muscular pain or tenderness, especially when diffuse and nonspecific.

**myocarditis**  
inflammation of the heart muscle

**myositis**  
inflammation of a muscle, especially a voluntary muscle, characterized by pain, tenderness, and sometimes spasm in the affected area.

**nasopharyngeal carcinoma**  
malignant transformation of EBV-infected epithelial cells; subset of gastric adenocarcinomas and certain salivarygland carcinomas

**necrosis**  
death of cells due to chemical or physical injury, as opposed to apoptosis; leaves extensive cellular debris that needs to be removed by phagocytes.
neutralization inhibition of the infectivity of a virus or the toxicity of a toxin molecule by antibodies (neutralizing antibodies)

opsonization alteration of the surface of a pathogen or other particle so that it can be ingested by phagocytes. Antibody and complement opsonize extracellular bacteria for destruction by neutrophils and macrophages.

oral hairy leukoplakia refers to a white patch – or white patches – that can develop in the mouth; patches usually occur along the sides of the tongue, although they can sometimes develop on the top and underside of the tongue or along the inside of the cheek; it is caused by Epstein-Barr virus

pandemic epidemic over a wide geographic area and affecting a large proportion of the population.

pericarditis inflammation of the lining sac (pericardium), which surrounds the heart. Can be associated with a collection of fluid in the space between the heart and the pericardium.

phagocytosis Phagocytosis is the term describing the ingestion of micro-organisms, cells, and foreign particles by phagocytes, eg phagocytic macrophages.

polyradiculopathy inflammation of multiple spinal nerve roots

primase enzyme that creates an RNA primer for the initiation of DNA replication.

pyomyositis primary acute bacterial infection of skeletal muscles, usually caused by Staphylococcus aureus

RANTES a chemokine that is a chemoattractant for eosinophils, monocytes, and lymphocytes. It is a potent and selective eosinophil chemotaxin that is stored in and released from platelets and activated T-cells. RANTES affects HIV activity and is believed to act in conjunction with 2 other chemokines, MIP-1α and MIP-1β.
retroorbital behind the eye (orbita)

Reye’s syndrome acute encephalopathy characterized by fever, vomiting, fatty infiltration of the liver, disorientation, and coma, occurring mainly in children and usually following a viral infection, such as chicken pox or influenza.

rolling circle replication a model of DNA replication that accounts for a circular DNA molecule producing linear daughter double helices. A long dsDNA strand is built consisting of many copies of the originally circular DNA. This copies are joined, which is called concatemere DNA, and are later cut in single copies. One of the DNA cleaved and nucleotides are joined to the 3’OH end to push away the 5’ end. Subsequently okazaki fragments are bound to the 5’ end. These are then polymerized to build a complementary strand. The strang is continuously elongated and can possess many copies.

tetramers soluble versions of the MHC class I molecules are synthesized in E. coli bacteria. The molecules achieve an appropriate conformation following addition of β2 microglobulin and a synthetic peptide that represents the epitope that is recognized by the TCR of interest. In addition the enzyme BirA is used to attach a biotin molecule to the specific BirA-recognition sequence, which has been incorporated into the C terminus of the MHC molecule. Four MHC-biotin complexes are linked to a single fluorochrome “tagged” streptavidin molecule. Tetramers can be used to detect antigen-specific T cells by flow cytometry.

transcription process whereby one strand of a DNA molecule is used as a template for synthesis of a complementary RNA by RNA polymerase.

translation the ribosome-mediated production of a polypeptide whose amino acid sequence is specified by the nucleotide sequence in an mRNA.

TREC T cell receptor excision circles, also called T cell receptor rearrangement circles, are DNA circles resulting from gene rearrangement. They are used as makers of T cells of thymic origin. TREC’s are stable and cannot replicate. They are maintained in T cells after exit from the thymus, but
are diluted out by cell division as the T cells get older.

**virion**

A complete virus particle that exists outside of a host cell; a mature infectious virus particle existing outside a cell; The mature virus. The name of the virus once it is out of a cell; a complete viral particle; nucleic acid and capsid (and a lipid envelope in some viruses).

**virus**

Ultramicrscopic infectious agent that replicates itself only within cells of living hosts; many are pathogenic; a piece of nucleic acid (DNA or RNA) wrapped in a thin coat of protein.