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Dedicated to my mother

Barbara Veronique

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General Introduction

Historical Perspective

The interest of mankind in the phenomenon of immunity to infectious diseases probably dates back to the first infection itself. Although we have just begun to identify the components of the immune system and to understand some of the mechanisms that are essential for the mediation of immunity, concepts of immunological memory, in terms of protective immunity against diseases, have been proposed for more than 2000 years. In 430 B.C., the Greek historian Thucydides recorded first reports of immunity to viral infections. He recognized during the Plague of Athens (this "plague" was most probably not due to *Yersinia pestis*, but rather to a virus) that a person, who had recovered from disease, was never taken ill a second time (Silverstein, 1999). Thus, this might be the first report, which unknowingly describes the phenomenon of immunity to homotypic viral infection. Many centuries later, in 1846, the Danish physician Ludwig Panum made another key observation illustrating viral immunity: during a new outbreak of measles on the remote Danish Faeroe islands, not one of the many aged people that were still living on the Faeroes and had suffered from measles during the first reported epidemic on these islands in 1781, was attacked a second time (Panum, 1847). This was a particularly valuable observation on protective immunity. The remote location of the Faeroe Islands and the fact that more than 65 years had passed since those people had been exposed to measles in a previous outbreak, almost ruled out that intermittent exposure had provided these people with florid immunity. More recently, similar observations have been made during yellow fever endemics in Virginia, USA, (Sawyer, 1931) as well as during polio outbreaks among Alaskan Eskimos (Paul *et al.*, 1951).

Based on similar observations during the smallpox epidemic of the late 18th century in Europe, the English physician Edward Jenner was the first who performed a successful manipulation of the immune system. In 1796, he discovered that injection of material from cowpox pustules into smallpox-inexperienced individuals could prevent disease. Furthermore, even subsequent intentional inoculation of smallpox-material did not cause smallpox disease in these individuals. This experimental approach made him to become the founder of vaccination immunology.

Together, these (and many more) historic events demonstrated that natural and experimental exposure to viruses provides long-lived (or even life-long) immunity to subsequent homotypic viral infections. This phenomenon indeed represents the basis for many of the currently employed strategies in the prevention of viral diseases. Vaccination with attenuated or killed strains of infectious agents such as polio, smallpox, measles, mumps, rubella, and yellow fever successfully prevents illness caused by these viruses. In addition, the concept of immunological memory remains central to the com-

prehension and development of vaccination strategies for many current major public health concerns including many acute (yellow fever and ebola virus) and chronic viral infections caused by agents such as human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV, HCV), as well as members of the herpes virus family (herpes simplex viruses (HSV), varizella zoster virus (VZV), Ebstein-Barr-virus (EBV)). These viruses have developed a multitude of strategies for evading or overwhelming the immune system. Therefore, understanding the mechanisms that generate and maintain immune responses and induce immunological memory during acute and chronic viral infections, remains crucial to the prevention and cure of these diseases (Ahmed and Biron, 1999).

The Immune System

The immune system provides protection to the host against viral infections and infections caused by a great variety of other infectious microbes such as bacteria, fungi, protozoa and multicellular parasites. It is principally comprised of two distinct but cooperating arms: the innate (unspecific) immunity and the adaptive (specific) immunity. Every immune response against a pathogen has different requirements and involves both, appropriate recognition of foreign structures and mounting of an adequate reaction. Thus, both arms of the immune system pursue diverse but complementary defensive strategies and therefore contribute in their own way to successfully resolve the infection. Each arm of the immune response plays a critical role at distinct times in the control of viral infection. The innate response starts almost immediately. This early non-specific response is critical in controlling the overall extent of viral replication, dissemination and spread. Innate responses can usually not eliminate the virus on their own but provide a crucial first line of defense, especially during primary infection. The specific response is essential for clearing the virus. It develops less rapidly over days and constitutes the most effective defense mechanism the immune system can provide. Moreover, *in sui generis* it confers immunity to the host against subsequent homotypic infections by the generation of immunological memory (Ahmed and Biron, 1999; Ahmed and Gray, 1996).

Innate Immunity

Innate immunity is the first line of defense against infections. The mechanisms of innate immunity are preexistent and preformed to the encounter with any microbes. They are rapidly activated by generic molecular patterns of the pathogen, such as structural surface carbohydrates and have long been appreciated for their role in defense at early times during primary infections (Aderem and Underhill, 1999; Ahmed and Biron, 1999). Therefore, the innate immune response is not restricted to specific antigens and is thus called not specific. The innate immune system consists of epithelial barriers (mucous membranes) as well as circulating cells and proteins that recognize the pathogens or microbial substances (for example toxins) produced during infection. The principal effector cells of innate immunity are neutrophils, eosinophils, mononuclear phagocytes like macrophages and dendritic cells, γδ T cells, and natural killer cells (NK cells). These cells attack the microbes that have breached the epithelial barriers or passed the mucous membranes and entered tissues or the circulation. Additionally, macrophages, γδ T cells, and NK cells secrete cytokines that cause inflammation, activate phagocytes and stimulate cellular reactions of the innate response. Later in the response these cytokines stimulate

cells of the specific immunity and enhance the elimination of the infectious agent.

Beside cellular effectors, various plasma proteins combat pathogens that have entered the circulation, too. The major circulating proteins of innate immunity are secreted cytokines, proteins of the complement system and of the coagulation system (Abbas *et al.*, 2000). Some components can mediate protection against viruses by blocking their initial uptake into the host cells (opsonization). Others, particularly cytokines, induce conditions that inhibit viral replication within already infected cells, and/or directly eliminate the virus-infected cells.

Adaptive Immunity

The adaptive immune system is basically composed of three distinct but interacting and cooperating populations: B lymphocytes (B cells), which produce virus-neutralizing antibodies; $CD4^+$ T lymphocytes (CD4 T cells), which produce cytokines that can directly inhibit viral replication as well as aid in the activation of other populations of the adaptive immune response; and $CD8^+$ cytotoxic T lymphocytes (CD8 CTL, CD8 T cells), which produce antiviral cytokines and directly kill virally infected cells (Abbas *et al.*, 2000; Janeway *et al.*, 2001; Paul, 1999) (Table 1.1).

A specific immune response is initiated by the presentation of viral antigens to B and T cells. Traditionally, B cells recognize conformational epitopes within surface glycoproteins or outer capsid proteins of a virus. For the recognition of these structures, B cells employ membrane-bound immunoglobulins, called B cell receptors (BCR). Upon activation aided by CD4 T cells (DeFranco, 1999), they differentiate into effector B cells and start to secrete these immunoglobulins (Reth, 1992). The produced antibodies are specific for the activating antigen. The secretion of antibodies by B cells is referred to as the humoral response of specific immunity and targets free virus and surface-bound viral antigen on infected cells, whereas the CD8 and CD4 T cell-mediated activities represent the cellular response. In contrast to antibodies, T cells only recognize short viral peptides that are bound to cellular major histocompatibility complex (MHC) molecules (Townsend *et al.*, 1985). Consequently, T cells are not capable of detecting free virus particles and therefore, their antiviral activities are confined to infected cells. Thus, the task of T cells is to survey and search the organism for infected cells. T cells employ a clonally distributed antigen receptor, called T cell receptor (TCR), for peptide–MHC complexes. The most common form of the TCR is composed of a disulfide-linked heterodimer of various α- and β-chains. To ensure binding to certain peptide–MHC complexes, the TCR is highly diverse. Diversity is the result of somatic rearrangements in variable, joining, and diversity segments in the genes of the α- and β-chains and their further combination with different constant gene regions during T cell development and

maturation (Davis and Bjorkman, 1988; Wilson *et al.*, 1988). Successful (highly specific) engagement of TCR leads to activation and subsequent T cell-mediated cytotoxicity and inflammation, predominately mediated by αβ CD8 and αβ CD4 T cells, respectively. CD8 T cells recognize short viral peptides (eight to ten amino acids) (Engelhard, 1994) in association with MHC class I molecules that are present on most nucleated cells. Class I molecules consist of two non-covalently linked polypeptide chains, an α-chain and a non-MHC-encoded subunit, called β2-microglobulin (β2m). Conversely, CD4 T cells recognize viral peptides of various length (up to 30 amino acids) (Engelhard, 1994) bound to MHC class II molecules, which are restricted largely to professional antigen-presenting cells (APC). Class II molecules are composed of two non-covalently associated polypeptide chains, an α-chain and a β-chain (both MHC-encoded). Peptide fragments for both MHC classes can be derived from any viral protein, structural (surface or internal) or nonstructural, but they are processed differently (Germain, 1999). Thus, all viral proteins are potential targets for T cell recognition. The limiting factors for obtaining immunogenic epitopes capable of eliciting a T cell response are intracellular processing of the viral proteins and the capability of the generated peptides to bind to MHC molecules (Falk *et al.*, 1991; Rammensee *et al.*, 1997).

Antigen Processing and Presentation

In an infected cell, newly synthesized cytosolic viral proteins are degraded into short peptides through proteolysis by proteasomes. Proteasomes are large multi-protein enzyme complexes with a broad range of proteolytic activity. Peptides derived from this cytosolic digestion are translocated into the endoplasmatic reticulum (ER) by a specialized transporter. There, newly synthesized MHC class I molecules are available to bind the peptides. Peptide–MHC class I complexes then are exported in vesicles from the ER to the surface, resulting in the presentation of the bound peptide to CD8 T cells (York and Rock, 1996). This way of antigen presentation is referred to as the endogenous pathway (Figure 1.1). Viral components that are captured and internalized by specialized APC, end up in endosome–lysosome complexes (phagosomes) (Aderem and Underhill, 1999). There, internalized viral proteins are degraded by lysosomal proteases to generate peptides that are capable to bind class II MHC molecules. MHC class II molecules are synthesized in the ER and are transported in vesicles to the endosome–lysosome complexes. After fusion of the vesicles, stabilizing components of the MHC molecule are cleaved off and peptides can bind. Stable peptide–MHC class II complexes are delivered to the cell surface of the APC, where they are displayed for the recognition by CD4 T cells. This way of presentation is called the exogenous pathway. Some peptides manage to escape the endosome–lysosome-complexes and therefore enter the endogenous pathway, resulting in presentation on MHC class I molecules (Watts, 1997; Watts and Powis, 1999).

Figure 1.1

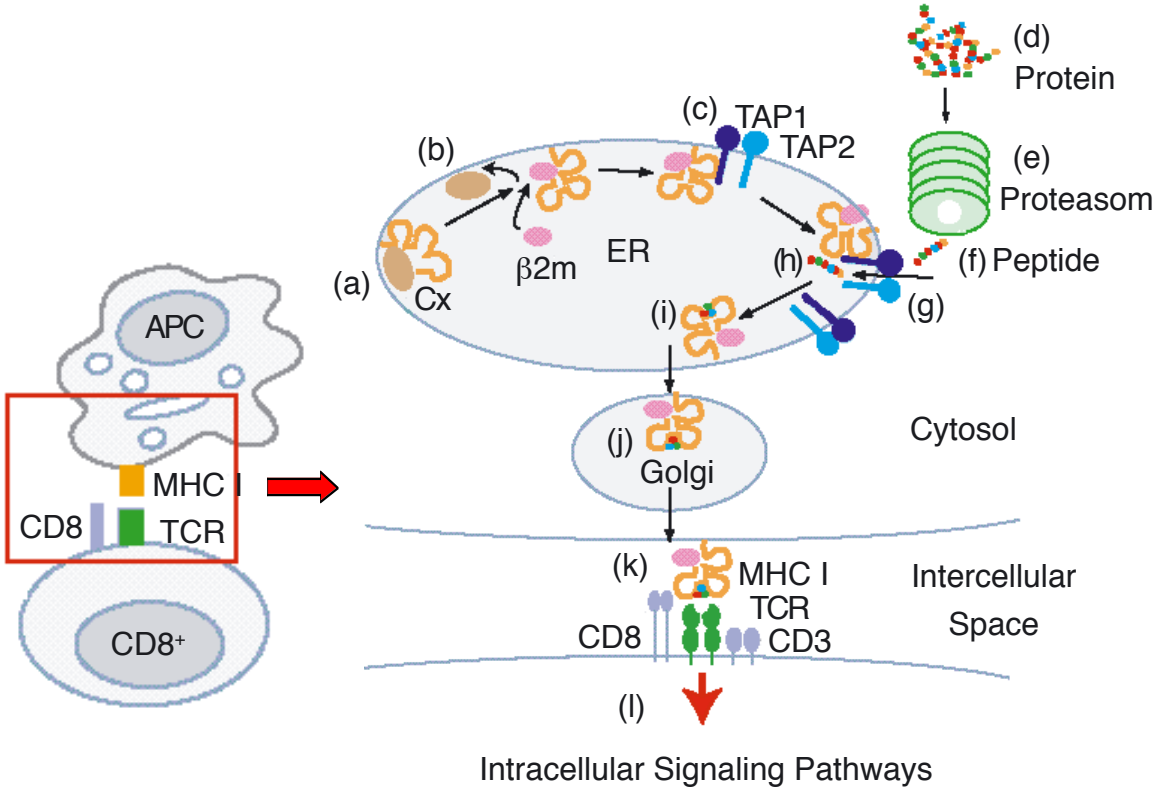


Figure 1.1: Degradation and Transport of Antigens that bind Major Histocompatibility Complex Class I (MHC I) Molecules.

(a) In an antigen-presenting cell (APC), newly synthesized MHC class I molecules bind to calnexin (Cx), which retains them in a partially folded state in the endoplasmic reticulum (ER). (b) Binding of MHC class I molecules to β2-microglobulin (β2m) displaces Cx and allows binding of chaperonin proteins (calreticulin and tapasin; not shown). (c) The MHC class I–β2m complex binds to the TAP complex (TAP1–TAP2), which awaits the delivery of peptides. (d) Peptides (e.g. from viruses) are formed from the degradation of cytosolic proteins (self-, pathogen- and tumor-derived proteins in the cytoplasm). (e) These are degraded by proteasomes into (f) short peptides. (g) Peptides are transported into the ER by the TAPs where association with the MHC class I–β2m complex can take place (h). Binding of the peptide into the antigenic groove of the MHC stabilizes the structure of the MHC class I molecule and (i) releases the TAP complex. (j) The fully folded MHC class I molecule with its bound peptide is transported to the cell surface via the Golgi apparatus. (k) Recognition of the MHC class I–peptide complex by the T cell receptor (TCR) of an antigen-specific T lymphocyte (CD8 T cell) takes place and (l) a signal transduction event can activate effector functions in the MHC-class-I-restricted T cell.

Modified from (Man, 1998).

Table 1.1: Antiviral T Cell and B Cell Immunity

Effector System	Recognition Molecule	Mechanism of Viral Control
Antibody	Surface glycoproteins or outer capsid proteins of virus particle	Neutralization of virus Opsonization of virus particles
	Viral glycoproteins expressed on membrane of infected cells	Antibody-complement-mediated and antibody-dependent cell-mediated cytotoxicity of virus-infected cells
CD4 T cells	Viral peptides (10-20mers) presented by MHC class II molecules: This could be any viral protein (surface, internal or non-structural). Peptides presented by MHC class II molecules usually are derived from exogenous proteins.	Release of antiviral cytokines (IFN-γ, TNF) Activation/recruitment of macrophages Help for antiviral antibody production Help for CD8 responses Killing of virus-infected cells?
CD8 T cells	Viral peptides (8-10mers) presented by MHC class I molecules: This could be any viral protein. Peptides presented by MHC class I molecules are usually derived from endogenous proteins, but the exogenous pathway is also quite efficient in loading MHC class I molecules.	Killing of virus-infected cells Release of antiviral cytokines (IFN-γ, TNF) Activation/recruitment of macrophages

Adapted from (Ahmed and Biron, 1999).

Viral Infection

Viruses are obligatory intracellular microorganisms. Therefore, their successful propagation depends on the ability to infect and to replicate within cells of a susceptible host and to spread to a new host. A short appreciation of the different sequential events that are needed for a virus to successfully infect a host and to guarantee its propagation *in vivo* (Figure 1.2) is necessary to understand the types of immune responses that are elicited by different viruses and the mechanisms by which viral infections are controlled.

Virus Propagation

Successful propagation of a virus requires the following sequence of events *in vivo*:

(a) Entry into a susceptible host

(b) Replication and spread within the infected host

(c) Shedding to the exterior environment

(d) Transmission to a new host

(a) Viral Entry

The first step of infection is the entry into the host. This basically occurs through absorption of the virus particle to any mucous membrane (i.e. urogenital, respiratory, or gastrointestinal tract, and conjunctivae). The skin, despite acting as outer frontier and representing the largest organ of the body, is unlikely to be the initial site of viral entry. However, its physical and chemical barrier mechanisms can be bypassed by trauma like injection, animal or insect bites, minor cuts and sores, or chemical irritation. The mucous membranes do not constitute a major physical barrier but provide some protection by mucociliary activity, mucus production, and secretion of fluids containing protective components like immunoglobulins, proteolytic enzymes, and organic acids. However, this is usually not sufficient to prevent primary infection (Ahmed and Biron, 1999).

(b) Viral Spread

Following entry into the host, viral infections can either remain confined to the site at which the virus entered (local infection), or infections can spread systemically to other organ systems (systemic infection). After initial replication at the point of entry, most viruses spread locally by cell-to-cell transmission. In addition, free virus or virus particles ingested by phagocytic cells are transported through afferent lymphatic drainage from the site of initial infection to regional lymph nodes. Having reached the first draining lymph station, a specific immune response is initiated by presentation of viral antigen on infected APC to specific precursor cells. As for some viruses (for example LCMV, see

later), an important determinant of spread is the ability of the virus to replicate and/or survive in macrophages. Viruses that replicate well in macrophages tend to spread more efficiently, probably through interference with antigen presentation (Ahmed and Biron, 1999).

Initial subepithelial invasion and lymphatic transportation lead to local spread and amplification of the virus, but the most effective mechanism of viral spread is dissemination via the blood circulation, which can transport the virus to any organ of the body. A virus that gains access to the blood from its initial site of infection and/or via the draining lymph nodes causes so called primary viremia. In the blood stream, viral particles are transported either free within the plasma or travel cell-associated. The cell-associated way provides a disadvantage for the host because the virus potentially escapes from neutralizing antibodies. Additionally, this means that the carrier cell is bearing appropriate receptors and/or coreceptors for the virus and therefore is very likely to be particularly susceptible to infection. During dissemination, substantial amplification of the virus occurs within these circulating blood cells and theoretically in all tissues that become infected through the blood. After replication in infected tissues, new virus particles can enter the blood stream again, causing secondary viremia. Within the circulation the reticuloendothelial system (RES, mainly within liver and spleen) is very effective in removing viral particles from the blood. This means viremia can only be maintained by continuous virus production within cells that are in contact with blood (circulating blood cells, organs with extensive sinusoids like liver and spleen).

(c) Viral Shedding and
(d) Transmission

The last stage of the *in vivo* viral life cycle is shedding and transmission. The biologic imperative for any virus is transmission, because its survival depends on continual subsequent infection of susceptible hosts. Some viruses are limited to a single species, whereas other viruses can circulate in more than one species. Shedding occurs via one of the body surfaces involved in the entry of the virus (mainly mucous membranes). Principally, viral particles can be shed through every secreted liquid and fluid of the body. Particularly contagious is the transmission of virus-contaminated blood.

Types of Viral Infection

Viral infections can be divided into distinct categories, based on levels of infectious virus that is detectable in different tissues (viral load) and the duration of the infection. Basically, three different courses of viral infection can be observed (Figure 1.3):

Acute Infection followed by Viral Clearance:

This type of infection is the consequence of a successful host immune response. Additionally, some acute viral infections are even self-limiting. After a short time, infectious particles are resolved from all tissues; this means virus is not detectable. Many common viruses belong to this category (polio, influenza, rota, mumps, yellow fever virus).

Acute Infection followed by Latent Infection:

This type is defined by persistence of a virus in a non-infectious form. The non-infectious virus may intermittently reactivate, exert its pathogenic properties and cause shedding of infectious particles. Viruses that establish latent infections with periodic reactivation need to have the capability of undergoing a productive infection in certain cells or under certain conditions that results in virus propagation with cell lysis, dissemination and shedding. On the other hand, it is essential for those viruses as well to undergo a non-permissive infection in other cells, where they are veiled to escape the detection by immunocompetent cells. Classic examples of viruses that establish such a type of infection are the herpes viruses (HSV, VZV, EBV).

Acute Infection followed by Persistent Infection (Chronic Infection):

In this condition, viral replication continues after the acute phase has subsided. This means that virus – infectious or not infectious – is present and detectable in tissues throughout time. Such infections are established when the host immune response fails to completely eliminate the virus. Productive infection of host cells during the acute stage may be followed by spread to cells that are less permissive, or by evolution/alteration of an immune response that only dampens viral replication but cannot completely clear the virus (for example HIV, HBV, HCV infection). Some viruses may develop escape mechanisms to prevent immune recognition and thus are capable to persist. For other instances, the immune system may permanently be confronted with viral antigen due to unsuccessful elimination resulting in immune exhaustion.

Sometimes the course of a viral infection does not fit into those idealized patterns and forms of infection. Different patterns of infection in different tissues or cell types are observed. Some viruses show combinations of these general patterns, so that their infection type does not fit clearly into those definitions. For example, EBV causes a latent infection in B cells but a productive infection in pharyngeal epithelial cells (Fields, 1996; Nathenson, 1996).

Figure 1.2

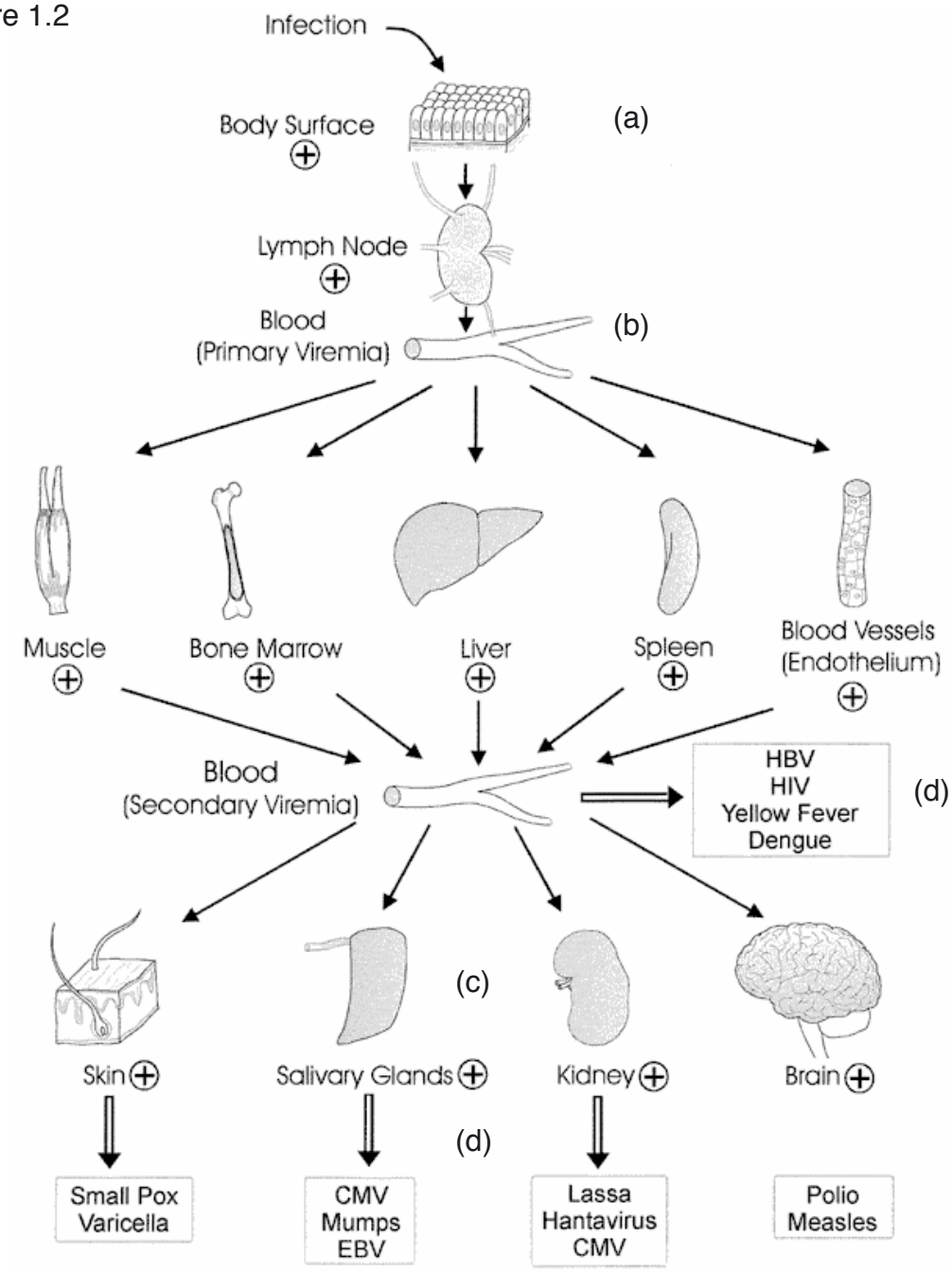


Figure 1.2: The Virus Life Cycle *in vivo*,

showing entry (a), spread (b), shedding (c) and transmission (d). The symbol (+) indicates possible sites of viral replication, single arrows indicate movement of virus and double arrows indicate sites of shedding. Only a few examples of viruses are listed. Transfer from blood occurs by transfusion (HBV and HIV) and by insect bites (Yellow Fever and Dengue). Shedding and transmission via the mucous membranes is not shown. This figure does not show neural spread. In addition to hematogenous spread, viruses can also spread to the central nervous system (CNS) via peripheral nerves.

Adapted from (Mims and White, 1984).

Figure 1.3

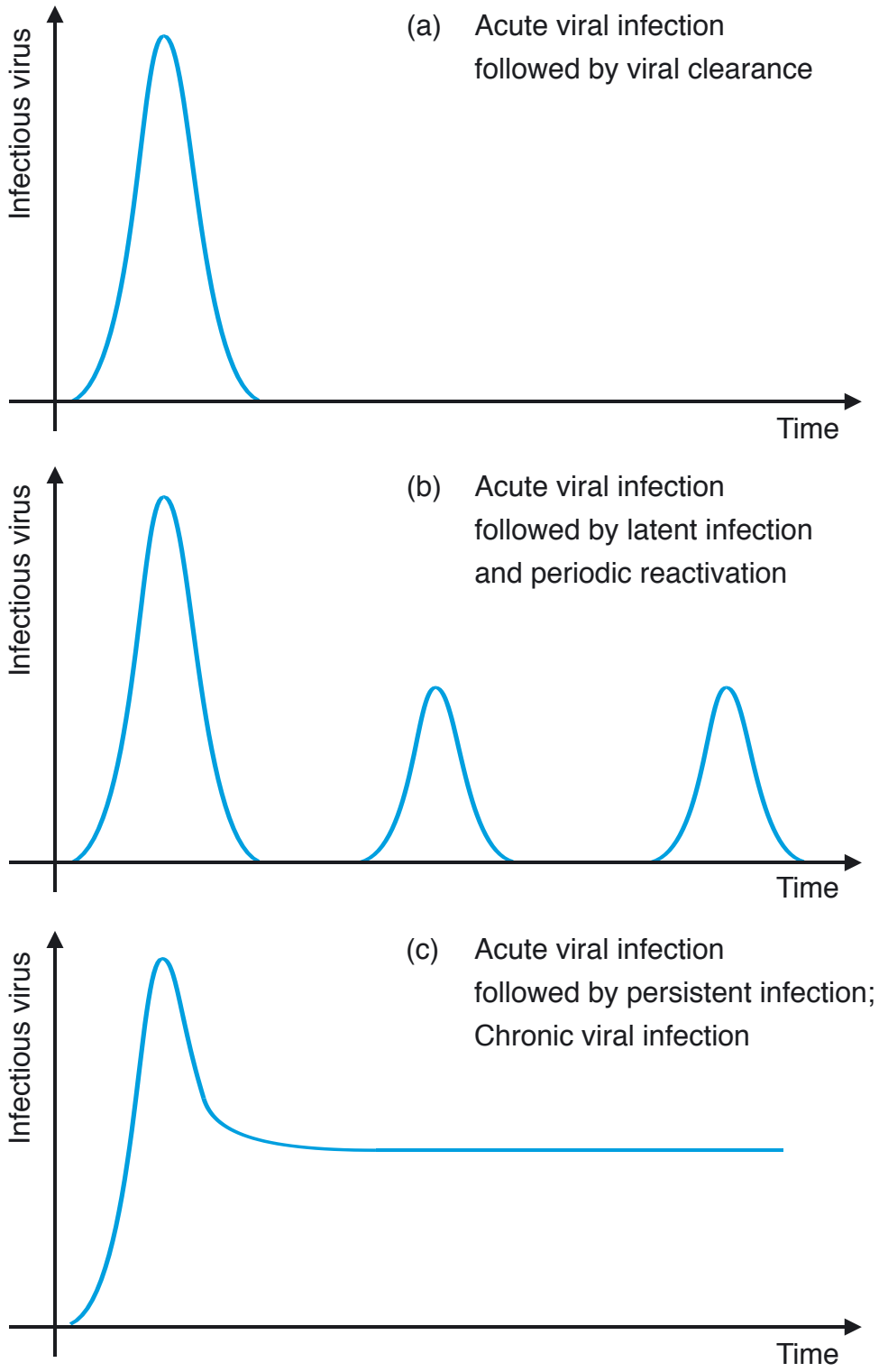


Figure 1.3: Types of Viral Infection.

Panels (a), (b), and (c) are showing idealized ways of virus–host interactions. Infection patterns may vary for each virus and are influenced by the host's immune status.

Immunity to Viruses I – The Virus-specific Immune Response

Historically, there has been great interest in determining the relative importance of T and B cell immunity in controlling viral infections. Much effort has been made to assess the role of T and B cell responses in viral elimination and in protection from reinfection (Ahmed and Gray, 1996; Doherty *et al*., 1992; Zinkernagel *et al*., 1996). Addressing those questions might be very difficult since antibodies and T cells have evolved to perform entirely different functions. In a variety of experimental and clinical systems it has been shown that cytotoxic CD8 T cells play a pivotal role in the prevention of and in the recovery from viral infections (Ahmed *et al*., 1987; Callan *et al*., 1998; Kägi and Hengartner, 1996; Riddell *et al*., 1992). Additionally, it has been established that the cooperation between CD8 and CD4 T cells is crucial, particularly under conditions of chronic viral infections (Appay *et al*., 2002; Blattman, 2001; Cardin *et al*., 1996; Imami and Gotch, 2002; Lieberman *et al*., 2002; Matloubian *et al*., 1994; Zajac *et al*., 1998).

B Cell Response

Naïve B cells that bind viral proteins specifically via their BCR complexes internalize and process these proteins to peptides through the exogenous pathway. The result is the presentation of peptide–MHC class II complexes on the cell surface (acting as APC). Antigen-specific CD4 T helper cells that recognize the presented peptide can now provide help signals to the B cells. These CD4 signals induce B cells to proliferate and terminally differentiate into antibody-secreting cells (Abbas *et al*., 2000; DeFranco, 1999; Janeway *et al*., 2001).

Activated B cells either differentiate into short-lived antibody-secreting cells (ASC) or germinal center (GC) B cells, which further differentiate into memory B cells and long-lived plasma cells (Manz *et al*., 1997; Slifka *et al*., 1995). ASC die pretty quickly (after around one week) but provide some level of protection rapidly during primary viral infection. They secrete almost exclusively type M immunoglobulins (IgM), indicating that affinity maturation has not occurred in these cells (Berek, 1999). In contrast, GC B cells undergo isotype switching (Snapper and Finkelman, 1999) and affinity maturation that results in IgG of higher affinity. Consequently, memory B cells and plasma cells are conferred with higher affinities for the viral antigen than are ASC. ASC are important for the control and limitation of viral spread during primary infection, whereas plasma cells are responsible for the prevention of subsequent reinfections through continuous secretion of large quantities of specific antibodies. Antibodies can control infection by neutralizing virus particles and by killing infected cells through complement- or cell-mediated mechanisms (antibody-dependent cellular cytotoxicity, ADCC). If neutralizing anti-

body is present at sufficiently high concentrations at the site of viral entry (IgA at mucous membranes, IgG in the blood), so called "sterile immunity" can result. Antibodies that prevent binding of the virus to cellular receptors and/or prevent penetration and uncoating of the virus do not only limit dissemination but are able to block infection (Ahmed and Biron, 1999; Parren *et al.*, 2001). If present at no sufficient concentrations, antibodies in the serum still can limit viral spread and prevent disease after virus has disseminated into the blood.

Complement can work synergistically with antibody to enhance virus neutralization. Binding of complement to virus–antibody complexes can result in enhanced uptake and subsequent degradation of virus particles by phagocytic cells. In addition, antibody together with complement can directly lyse enveloped viruses. Paradoxically, in some instances, antibodies can actually enhance virus infectivity (antibody dependent enhancement of viral infection, ADE) (Hober *et al.*, 2001; Sullivan, 2001).

Protection against viral infection often relies on the level of preexisting antibody in the serum or at mucosal surfaces. Therefore, the number and specificity of preexisting plasma cells are critical components of protective immunity (Ahmed and Biron, 1999; Ahmed and Gray, 1996).

T Cell Responses

Virus-specific T cell responses are initiated during a complex T cell–APC interaction, based on the recognition of MHC-bound viral antigen by the TCR (Zinkernagel and Doherty, 1974). A large number of additional molecules also participate in the activation process. The TCR and other cell surface molecules contribute to the initiation of T cell activation by inducing signal transduction events and by contributing to the overall avidity of the T cell–APC interaction (Lanzavecchia *et al.*, 1999).

Following the current two-signal hypothesis (Figure 1.4), the activation of T cells requires at least two distinct signals (Weiss, 1999). T cells receive a first, antigen-induced signal through specific binding of their TCR to the peptide–MHC complex on specialized APC (Cantrell, 1996; Clements *et al.*, 1999; Lanzavecchia *et al.*, 1999). Mononuclear phagocytes, macrophages, dendritic cells, and B cells function as professional antigen-presenting cells. Molecules on the APC that are called costimulatory molecules, such as CD80 (B7.1), CD86 (B7.2), and CD40, provide the second signal for T cell activation. This signal is transmitted into the T cell via costimulatory ligand molecules such as CD28 and CD40L (Lenschow *et al.*, 1996). Together, these two signals (TCR engagement plus costimulation) initiate a cascade of signaling events within the T cell, which leads to proliferation, differentiation into effector T cells and secretion of cytokines. Moreover, new studies suggest that even a third signal might be mandatory for optimal

activation of naïve CD8 T cells. This additional signal could be provided by either certain cytokines such as interleukin-12 (IL-12), which directly acts on the naïve T cells (Schmidt and Mescher, 2002), or by exogenous factors like lipopolysaccharide (LPS), which enhances maturation of antigen presenting dendritic cells (Schuurhuis *et al.*, 2000). IL-12 and LPS both indicate, that components of the innate immune system might be crucial to optimal priming conditions for naïve CD8 T cells. T cells that encounter antigen in the absence of costimulation and/or other required signals either fail to becoming activated and undergo apoptosis, or enter a state of unresponsiveness called anergy (Harding *et al.*, 1992; Schwartz, 1997), or could become tolerant to the presented antigen (Schmidt and Mescher, 2002).

Following optimal activation, the primary virus-specific T cell response shows distinct kinetics and is composed of three separate phases (Ahmed and Gray, 1996) (Figure 1.5):

(a) Expansion phase

(b) Death phase

(c) Memory phase

The kinetics of CD8 and CD4 responses have been documented in a variety of experimental and clinical systems.(Ahmed and Gray, 1996; Blattman, 2001; Callan *et al.*, 1998; Ewing *et al.*, 1995; Murali-Krishna *et al.*, 1998; Topham *et al.*, 1996; Whitmire and Ahmed, 2000). Emerging evidence indicates that CD8 and CD4 T cell immunity is differentially regulated (Homann *et al.*, 2001). However, the magnitude of virus-specific CD8 T cell expansion is generally larger than that seen for virus-specific CD4 T cells (Figures 1.5 and 1.10). In the following, we will focus on CD8 T cells.

(a) Expansion Phase

The first phase begins when peripheral naïve CD8 T cells encounter antigen, become activated and differentiate into effector cytotoxic T lymphocytes (CTL) (Ahmed and Biron, 1999; Ahmed and Gray, 1996). Simultaneously, antigen recognition also initiates T cell proliferation that can be tightly coupled with changes in gene expression (Agarwal and Rao, 1998; Bird *et al.*, 1998). Several studies have shown that the strength and duration of TCR signaling and costimulatory receptor signaling are important parameters regulating T cell activation (Iezzi *et al.*, 1998; Iezzi *et al.*, 1999; Lenschow *et al.*, 1996). There is new evidence that initial antigen encounter triggers developmental programs and therefore is crucial to a T cell's faith. Once the parental naïve CD8 T cell had been activated, it becomes committed to divide and to differentiate (Kaech and Ahmed, 2001; Wong and Pamer, 2001). This is a process, which cannot be interrupted

and occurs in the absence of further antigenic stimulation. But not all specific precursor T cells are automatically subjected to this program. The amount of presented antigen and the duration of antigen presence determine, how many precursors are recruited into the response (Kaech and Ahmed, 2001). This phenomenon is reflected in different burst sizes: The higher the antigenic load, the larger is the initial burst and programmed expansion, and *vice versa*. This allows the immune response to generate sufficient/appropriate numbers of antigen-specific effector cells that are essentially required to resolve the infection (Butz and Bevan, 1998; Cardin *et al.*, 1996; Doherty, 1996; Doherty *et al.*, 1997; Kägi and Hengartner, 1996). Furthermore, the program itself cannot be changed but modulated by microenvironmental factors like hormones and cytokines. For example, it has been shown that the growth-promoting interleukin-2 (IL-2) propels antigen-independent CD8 T cell proliferation and differentiation (Kaech and Ahmed, 2001), which further can be augment by IL-7 and IL-15 (Wong and Pamer, 2001).

Generated virus-specific CTL now exert their effector functions by direct cytolysis of infected cells as well as by the production of antiviral cytokines (Figure 1.6). Effector CD8 T lymphocytes (CTL), are able to directly kill virally infected targets by at least two distinct mechanisms: a secretory and membranolytic pathway involving perforin-dependent vectoral exocytosis of granzymes-containing granules and/or a non-secretory receptor-mediated pathway involving the interaction of CD95L (FasL) on the surface of effector T cells with CD95 (Fas) expressed on infected target cells (Berke, 1994; Kägi *et al.*, 1994b).

Perforin-mediated cytolytic pathways facilitate the entry of granzyme B into target cells and the subsequent activation of intracellular caspases. CD95 engagement leads to activation of intracellular adapter molecules including FADD (Fas-associated death domain)-containing proteins that directly activate pro-caspase 8. In either case, the activation of the caspase cascade results in the Bcl-2-mediated release of mitochondrial cytochrome C, fragmentation of cellular DNA, and ultimately in cellular apoptosis (Thompson, 1999).

In addition to cytotoxic effector functions, effector CD8 T cells are also capable of producing cytokines that directly inhibit viral replication (Biron, 1994). The most extensively characterized cytokines are interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). These cytokines target virus by increasing antigen presentation (IFN-γ) and induction of cell death (TNF-α). All viruses require the host protein synthesis machinery for the production of progeny virions. Cytokines produced by CD4 and CD8 T cells act in concert with type I interferons (αβ-interferons), produced by cells of the innate immune system, to shut down protein synthesis in order to prevent further viral assembly.

Beside CD8, specific CD4 T cells play a central role in antiviral immunity and con-

tribute to viral control in many different ways: they are necessary for optimal antibody and cytotoxic CD8 T cell responses and can also act as effectors themselves by producing antiviral cytokines such as IFN-γ and TNF-α (Biron, 1994; Doherty *et al.*, 1997) (Figure 1.6). An important role of CD4 T cells in virus infections is to provide help for clonal expansion and differentiation of virus-specific B cells. The CD4 response in viral infections is often dominated by a Th1-type profile, characterized by IFN-γ and IL-2 production and specific IgG isotypes. Th2-type responses are characterized mainly by IL-4 and IL-5 secretion and different IgG isotypes, and are less frequent during virus infections. Although CD4 help is dispensable for induction of CTL responses during some acute viral infections, CD4 T cells are essential for sustaining CD8 T cell responses during chronic viral infections (Cardin *et al.*, 1996; Matloubian *et al.*, 1994; Rosenberg *et al.*, 1997; Saha and Wong, 1992). CD4 T cells make cytokines, such as IL-2, that are necessary for survival of CD8 T cells. Additionally, CD4 T cells increase expression of costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) on APC through CD40L–CD40 interaction (Abbas *et al.*, 2000; Bluestone *et al.*, 1999; Janeway *et al.*, 2001). Upregulation of these costimulatory molecules by CD4 T cells is essential for activation of CD8 T cells. Moreover, the presence of CD4 T cells can help to reduce functional inactivity of CTL under conditions of chronic stimulation (Matloubian *et al.*, 1994; Moskophidis *et al.*, 1993; Wodarz *et al.*, 1998).

(b) Death Phase

Following clearance of the virus, the majority (up to 95-99%) of virus-specific effector T cells undergo apoptosis. The contraction of the T cell response is as dramatic as the rapid clonal expansion seen in the early expansion phase. This phenomenon, termed activation-induced cell death (AICD), serves as a mechanism for regulating cell numbers and maintaining homeostasis. However, some virus-specific T cells undergo further differentiation to become memory cells. The exact mechanisms that operate to downregulate the effector response remain unknown and are an area of intense research. One mechanism could be that the selection of memory cells from the effector pool is a stochastic process in which the repertoire of memory T cells directly reflects that of the virus-specific T cells present during the expansion phase (Busch *et al.*, 1998; Maryanski *et al.*, 1996; Sourdive *et al.*, 1998; Vijh and Pamer, 1997).

(c) Memory Phase

The third phase of the T cell response is characterized by a stable pool of memory cells that can persist for many years (Ahmed and Gray, 1996; Lau *et al.*, 1994). The generation and maintenance of CD8 T cell memory is highly complex and thus is discussed in detail in the following section.

Figure 1.4

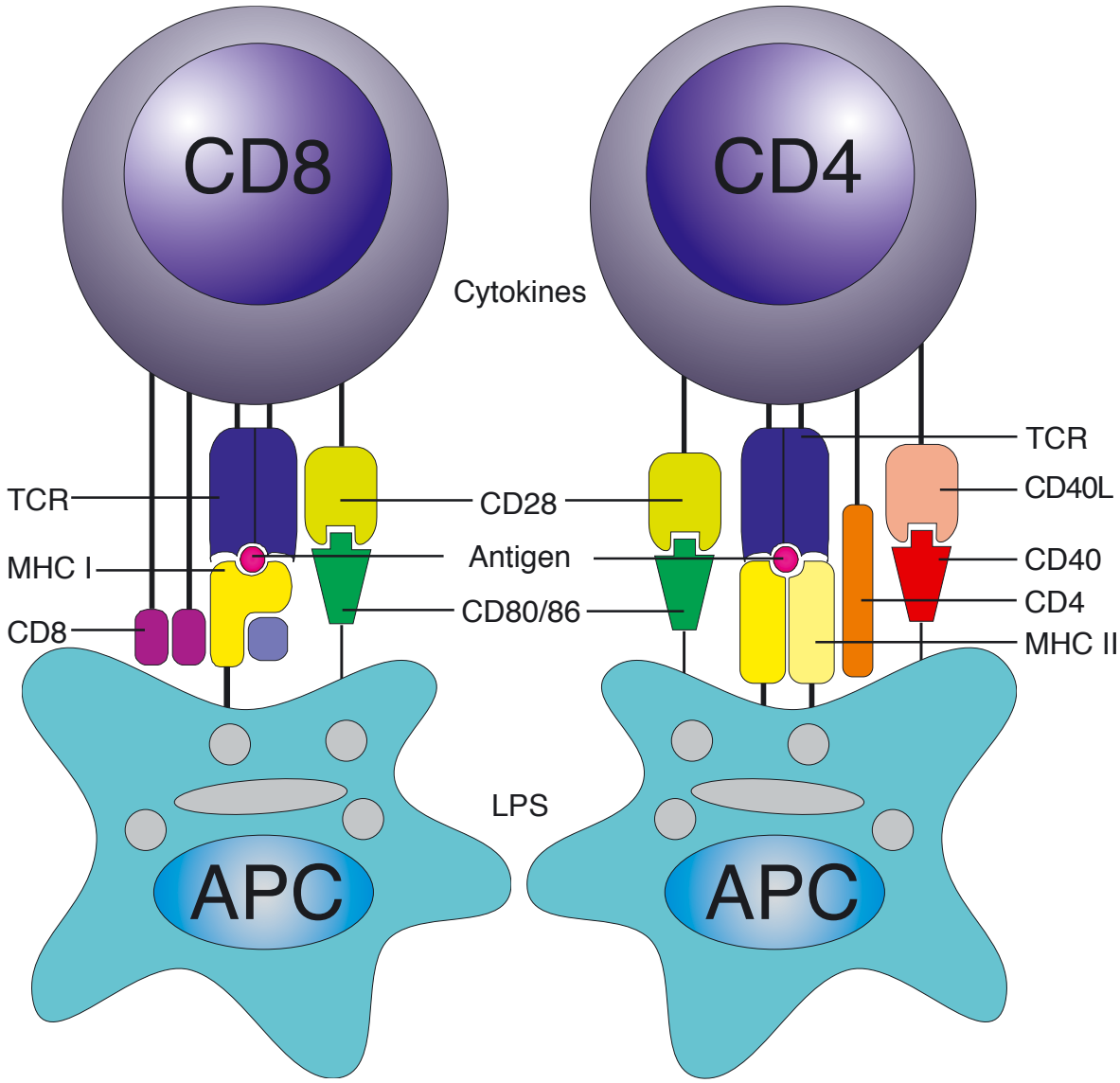


Figure 1.4: Activation of T Cells Requires Three Signals.
Signal 1 is the antigen-induced signal delivered to the T cell via the TCR–peptide–MHC interaction. CD8 T cells (left panel) bind to MHC class I molecules and CD4 T cells (right panel) recognize antigen in association with MHC class II molecules. Signal 2 for CD4 and CD8 T cells is conducted through the costimulatory molecule CD28 on the T cell, which binds to CD80 (B7.1) and/or CD86 (B7.2) on the APC. An additional second signal for CD4 T cells represents the CD40L–CD40 interaction. Cytokines and exogenous factors (LPS) provide the third signal for naïve T cells and APC, respectively. APC, antigen-presenting cell; TCR, T cell receptor; LPS, lipopolysaccharide.

Figure 1.5

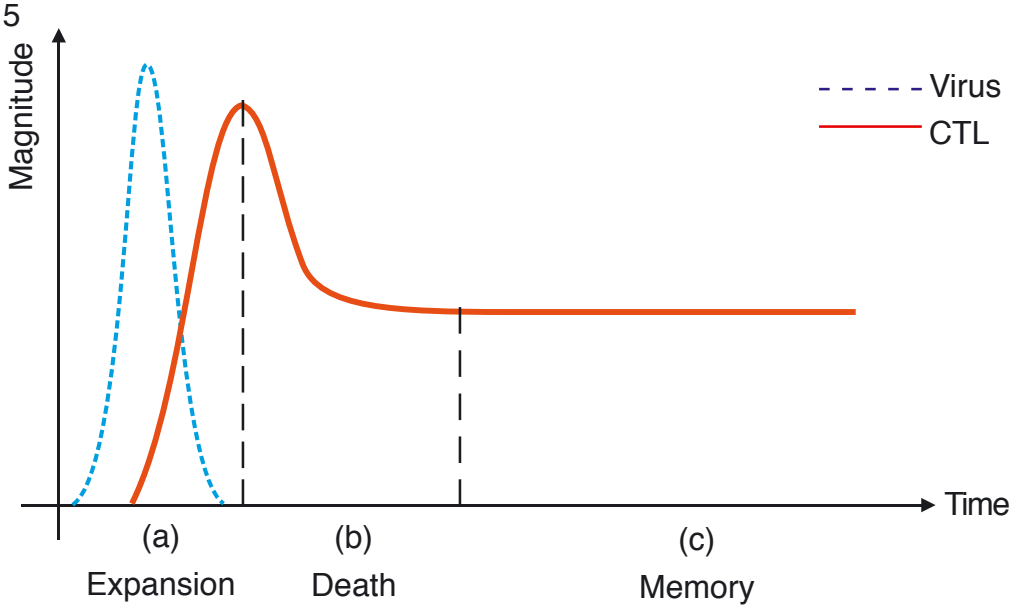


Figure 1.5: T Cell Response to Virus Infection.

Upon activation by viral antigens (virus, dotted line), T cells progress trough three different phases: (a) effector phase, (b) death phase and (c) memory phase. The total number of antigen-specific T cells (CTL, solid line) is shown schematically.

Figure 1.6

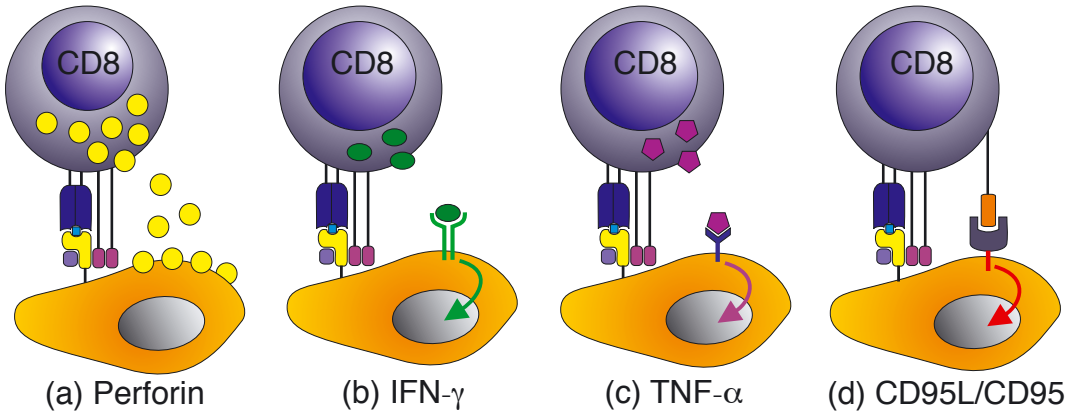


Figure 1.6: Effector Mechanisms Used by T Cells to Control Viral Infections.

The different effector functions are illustrated for the example of CD8 T cells. (a) Fusion of secreted perforin/granzyme B vesicles with the target membrane induces perforation and subsequent lysis. The cytokines IFN-γ (b) and TNF-α (c) bind to their receptors on the target cell and lead to intracellular signaling causing variant structural and metabolic changes. (d) Binding of CD95L (FasL) to its receptor CD95 (Fas) induces apoptosis in the target cell by triggering intracellular signaling pathways and cascades. CD8 T cells can employ all shown mechanisms to exert antiviral function whereas CD4 T cells are not capable to produce and secrete perforins (a). T cells can maintain and exert more than one effector function simultaneously or successively.

Immunity to Viruses II – The Immunological Memory

The hallmark of immunological memory is the ability of the host's immune system to remember a previous antigen encounter (primary infection) and to rapidly respond to the identical (homotypic) antigen when it is presented again during reinfection (Bevan and Goldrath, 2000; Doherty, 1996; Farber, 2000). The secondary (anamnestic) response induced during reinfection is usually quantitatively and qualitatively improved, that means greater in magnitude and faster in resolution of antigen than the primary response. Following most acute viral infections, immunological memory is generated that will confer protective immunity to the host from future homotypic infections. Acute viral infections induce both, T and B cell memory (Ahmed and Gray, 1996; Doherty *et al.*, 1992; Zinkernagel *et al.*, 1996).

B Cell Memory

Antiviral B cell memory is comprised of both, persisting memory B cells and long-lived plasma cells (LLPC) (Figure 1.7). LLPC are responsible for the continuous antibody production to sustain protective levels of immunoglobulins in the serum after the resolution of primary infection (Kelsoe, 2001; Slifka *et al.*, 1998; Slifka *et al.*, 1995). They can persist for long periods – even for a lifetime – in special niches like the bone marrow (Manz and Radbruch, 2002; Manz *et al.*, 1997). In spite of this longevity of LLPC, the maintenance of long-term antibody production additionally might be in part due to continuous memory B cell differentiation into plasma cells. Recent data indicate that memory B cells seem to have two response modes (Bernasconi *et al.*, 2002). On the one hand an antigen-dependent mode, which allows them after reencounter of antigen to undergo massive expansion and differentiation toward mostly short-lived plasma cells (SLPC) and a few LLPC. On the other hand a polyclonal, antigen-independent mode where memory B cells respond to environmental stimuli like LPS and cytokines by undergoing continuous turnover and differentiation. For the latter mode, the plasma cell population could theoretically be maintained for extended periods without reexposure of memory B cells to specific antigen.

T Cell Memory

After the expansion phase has subsided, a stable pool of resting memory T cell is found. Virus-specific memory T cells (CD4 and CD8) are capable of responding to subsequent homotypic viral infection with enhanced kinetics due to both, quantitative as well as qualitative changes. Quantitative enhancement is due to an increase in the specific precursor frequency upon secondary infection. Accelerated CTL kinetics and

cytokine secretion reflect some qualitative changes in the recall response. In particular, memory T cells express a distinct pattern of adhesion and accessory molecules compared to naïve T cells (Dutton *et al.*, 1998). This may influence the migration or surveillance of these cells resulting in an increased capacity to respond to viral antigen. In this context, particularly changes in the expression of the lymph node homing receptors CD62L and CCR7 play an important role. Alternatively, increased expression of adhesion molecules may allow memory T cells to respond to lower amounts of antigen. However, recent evidence also shows that memory cells are able to exert effector functions prior to proliferation (Kaech *et al.*, 2002b; Lanzavecchia and Sallusto, 2002) while naïve virus-specific cells require proliferation before exerting effector functions (Murali-Krishna *et al.*, unpublished data)(Brenchley *et al.*, 2002; Lanzavecchia and Sallusto, 2000).

Generation of Immunological Memory

The source of the memory cells is still unclear (Ahmed and Gray, 1996; Dutton *et al.*, 1998; Dutton *et al.*, 1999; Sprent and Surh, 2001; Sprent and Tough, 2001). Currently, there are basically two models to explain the generation of T cell memory (Figure 1.8): (a) Following the linear differentiation model, they may derive from a subset of effector cells, which is not prone to apoptosis after the antigen is cleared. (b) The divergent differentiation model proposes that they may derive from some cohort of the activated population that is either precommitted to become memory cells or that is driven by directive processes into the memory cell pool, analogous to what is known about the generation of B cell memory (Figure 1.7). The factors involved in the transition of activated T cells into resting memory cells are largely unknown. In addition, the developmental lineage that is followed during different types of immune responses is also not certain. Several studies suggest that the lineage of memory CD8 T cell development is linear and memory cells directly descend from effector cells (naïve → effector → memory)(Kaech and Ahmed, 2001; Kaech *et al.*, 2002a; Kaech *et al.*, 2002b). Conversely, other studies have also suggested that activated CD8 T cells can bypass the effector stage and develop directly into memory cells (Jacob and Baltimore, 1999; Lauvau *et al.*, 2001; Manjunath *et al.*, 2001; Oehen and Brduscha-Riem, 1998; Opferman *et al.*, 1999). Whether short-lived effector cells and long-lived memory cells are generated by different developmental programs or whether the same program is utilized but a fraction of the cells selectively survive and become memory cells is not clear. The duration and strength of exposure to antigen could be a crucial factor that triggers different developmental programs (Busch and Pamer, 1999; Iezzi *et al.*, 1998; Kaech and Ahmed, 2001; Lanzavecchia *et al.*, 1999; Savage *et al.*, 1999). Lastly, it has not been carefully determined when memory cells arise following antigenic stimulation. Recently, molecular

and functional profiling of memory CD8 T cell differentiation revealed that memory T cell precursors are generated during the expansion phase, but initially do not display functional memory cell traits (Kaech *et al.*, 2002a). Memory cell properties are acquired gradually several weeks after antigen clearance. Together, these data strongly support a model of linear and progressive T cell memory differentiation.

Maintenance of Immunological Memory

It is not only important to study the generation of memory, but it is also indispensable to understand how immunological memory is maintained at a constant and functional size throughout the greater part of the host's life. By knowing the mechanisms that are involved in this phenomenon, it might be possible to influence and extend the longevity of memory B and T cells.

Currently there is much debate concerning the need of specific antigen in the maintenance of memory (Ahmed and Gray, 1996). One model proposes that persistent antigen is needed to maintain immune memory by periodic restimulation of antigen-specific cells (Zinkernagel *et al.*, 1996). Further, it is known that periodic reexposure to antigen enhances the level of T cell memory (Ahmed and Biron, 1999; Ahmed and Gray, 1996), but considerable amount of data has been published recently describing the persistence of memory B and T cells in the absence of antigen (Lau *et al.*, 1994; Maruyama *et al.*, 2000). In studies involving memory T cells, it has been demonstrated by adoptive transfer experiments that memory T cells can persist indefinitely without any detectable antigen (Lau *et al.*, 1994; Murali-Krishna *et al.*, 1999; Swain *et al.*, 1999; Tanchot *et al.*, 1997). In addition, it has been recently shown that memory T cells can survive without interactions with the appropriate MHC molecules: memory CD4 T cells can persist in the absence of MHC class II (Swain *et al.*, 1999) and memory CD8 T cells can persist without MHC class I molecules (Murali-Krishna *et al.*, 1999). Together these data provide strong evidence that antigen and MHC is not required for the maintenance of memory T cells.

It has been hypothesized that memory T cells are maintained by periodic "tickling" of their T cell receptor (TCR) or by specific cytokines (Dai *et al.*, 2000; Ku *et al.*, 2000; Marks-Konczalik *et al.*, 2000; Zhang *et al.*, 1998). Recent studies now provide strong evidence that particularly IL-7 and IL-15 are essential for homeostatic proliferation in order to maintain CD8 T cell memory (Becker *et al.*, 2002; Goldrath *et al.*, 2002; Jameson, 2002; Judge *et al.*, 2002; Schluns *et al.*, 2000; Schluns *et al.*, 2002; Tan *et al.*, 2002; Weng *et al.*, 2002).

Memory T Cell Subsets

A phenotypic heterogeneity among memory T cells has long been known to exist (Doherty *et al.*, 1996). Recently, a memory model comprised of two distinct subpopulations – "central memory" and "effector memory" T cells – has been proposed (Sallusto *et al.*, 1999). This discrimination is based on the expression of the lymphocytic adhesion molecule L-selectin (CD62L) and the CC-chemokine receptor 7 (CCR7) which both determine the homing properties of T cells. Additionally, functional distinctions between $CD62L^{hi}CCR7^{+}$ central memory and $CD62L^{lo}CCR7^{-}$ effector memory T cell subsets have been described.

CD62L interacts with peripheral-node addressins (PNAd) (for example, GlyCAM-1, CD34, MAdCAM-1) on high endothelial venules, which mediate attachment and rolling (Arbones *et al.*, 1994; Warnock, 1998). CCR7 binds the chemokines CCL19 and CCL21, which are expressed on the luminal surface of endothelial cells in the lymph nodes, and establishes firm arrest and the initiation of extravasation (Campbell *et al.*, 1998). Consequently, $CD62L^{hi}CCR7^{+}$ and $CD62L^{lo}CCR7^{-}$ T cells would be expected to have distinct recirculatory properties *in vivo*.

Indeed, several studies have shown that $CD62L^{hi}CCR7^{+}$ T cells migrate efficiently to peripheral lymph nodes and other secondary lymphoid tissues, whereas T cells lacking these two molecules do not (Iezzi *et al.*, 2001; Weninger *et al.*, 2001). Rather, $CD62L^{lo}CCR7^{-}$ T cells can be found in other sites, such as the liver and lungs (tertiary lymphoid tissues) (Weninger *et al.*, 2001).

When the functional properties of $CD62L^{hi}CCR7^{+}$ and $CD62L^{lo}CCR7^{-}$ subsets of memory T cells were examined, an interesting dichotomy was observed (Sallusto *et al.*, 1999). Stimulation of human $CD62L^{hi}CCR7^{+}$ memory CD4 T cells *in vitro* resulted in the production of IL-2, but little interferon-γ, IL-4 or IL-5. In contrast, $CD62L^{lo}CCR7^{-}$ T cells rapidly produced these effector cytokines, but produced less IL-2. Further, only the $CD62L^{lo}CCR7^{-}$ subpopulation of CD8 T cells was found to contain intracellular perforins. Therefore, a model was proposed which appoints different protective functions to the subsets (Sallusto *et al.*, 1999): on the one hand, the tissue-homing effector memory T cells – endowed with immediate effector functions – for rapid control of invading pathogens at the site of entry. On the other hand, the central memory T cells – capable of homing efficiently to lymph nodes and secondary lymphoid organs – with the task to stimulate dendritic cells, provide B cell help and/or generate a second wave of T cell effectors. Several recent reports have confirmed the presence of antigen-specific memory T cells in non-lymphoid compartments long after priming, which supports the notion of an effector memory subset of T cells (Marshall *et al.*, 2001; Masopust *et al.*, 2001; Reinhardt *et al.*, 2001). However, these studies did not address the phenotype of tissue-

derived memory T cells with respect to CD62L and CCR7. Although some interesting functional differences were observed (Masopust *et al.*, 2001; Reinhardt *et al.*, 2001), many aspects of the central memory–effector memory model await confirmation or direct examination. For example, it is unclear whether the dichotomy in rapid effector functions observed between $CD62L^{hi}CCR7^{+}$ and $CD62L^{lo}CCR7^{-}$ memory phenotype T cells in human blood will also hold true for T cells of similar phenotype in other tissues. In addition, the role of these individual subpopulations during secondary immune responses *in vivo* remains untested. When restimulated *in vitro*, $CD62L^{hi}CCR7^{+}$ memory CD4 T cells became $CD62L^{lo}CCR7^{-}$, which suggests that central memory cells can give rise to effector T cells or potentially to effector memory cells (Sallusto *et al.*, 1999). However, the precise developmental relationship between central memory and effector memory subsets is not understood (Figure 1.9). Furthermore, the mechanisms and conditions that maintain each subset and the signals that determine their generation during a primary immune response are areas that remain to be explored.

Figure 1.7

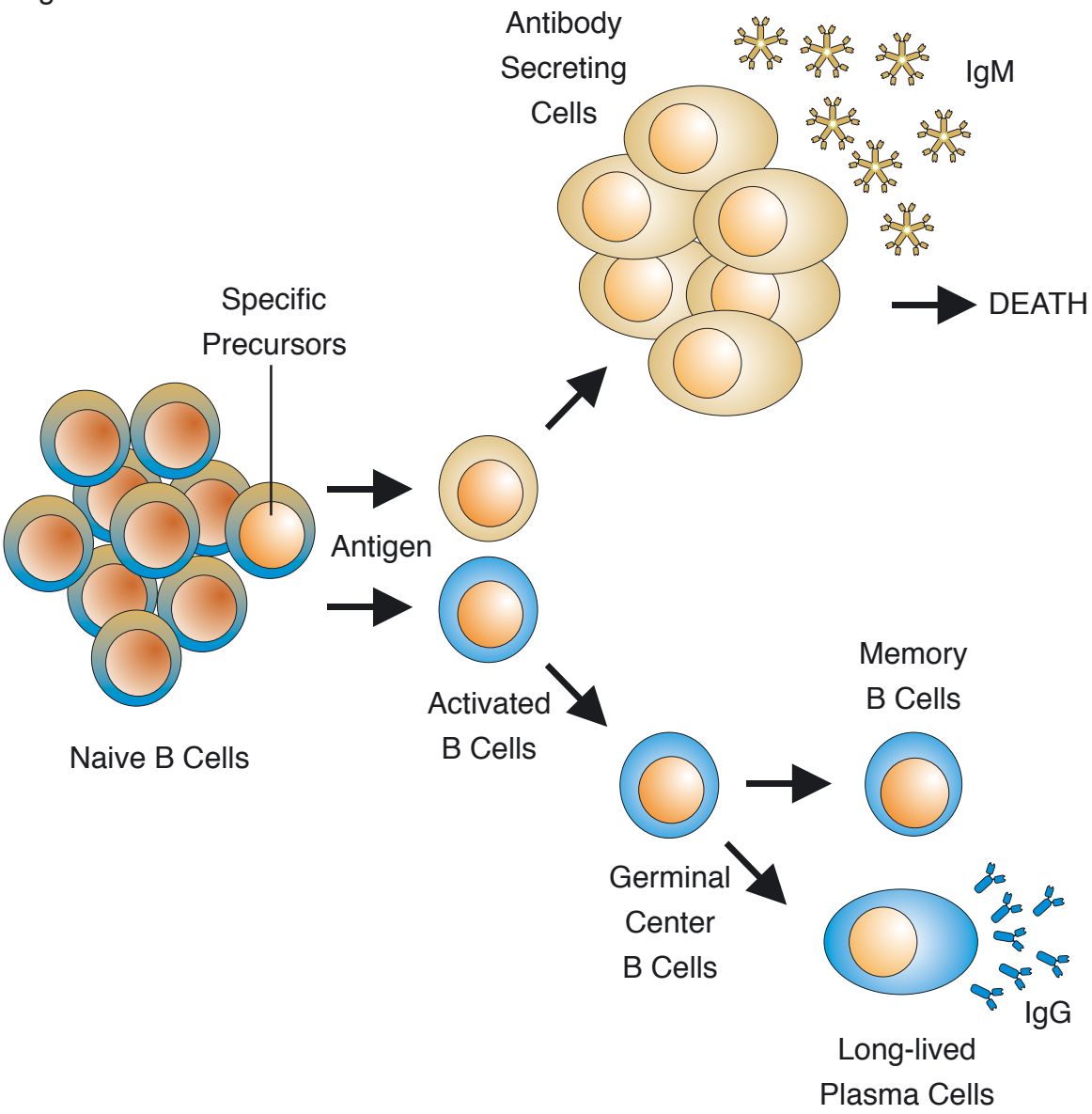


Figure 1.7: Differentiation of B Cell Memory.

The current model explaining the generation of B cell memory is almost unanimously accepted. In this model, the effector and memory B cells differentiate along separate lineages. Upon activation, the antigen-specific B cells form follicles (follicular B cells). Within the follicles, the low affinity B cells become short-lived antibody secreting cells (effector B cells). Some B cells are migratory and form germinal centers where they continue to differentiate and undergo isotype switching and affinity maturation (germinal center B cells). The germinal center B cells then give rise to the memory B cells and the long-lived plasma cells.

Figure 1.8

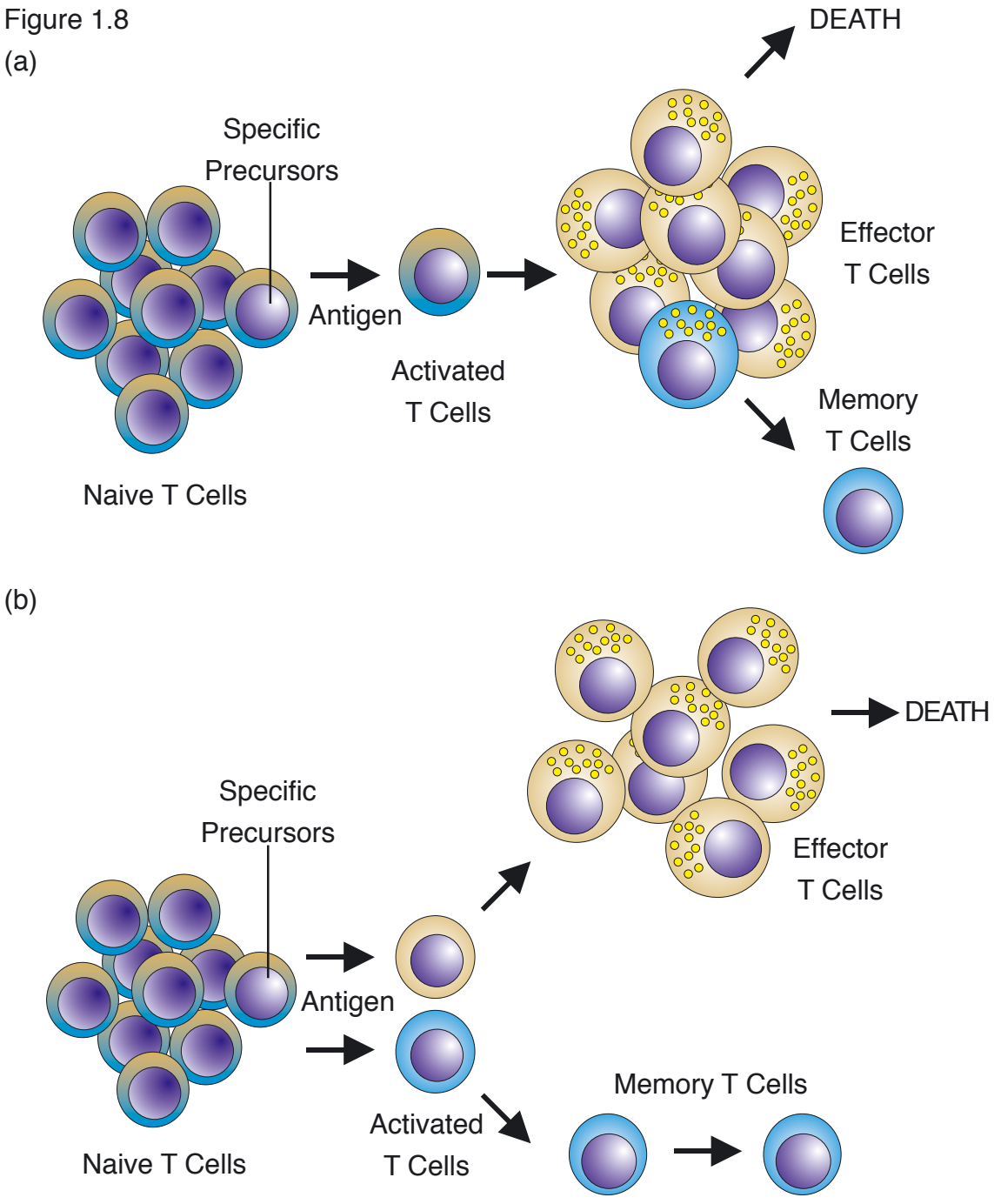
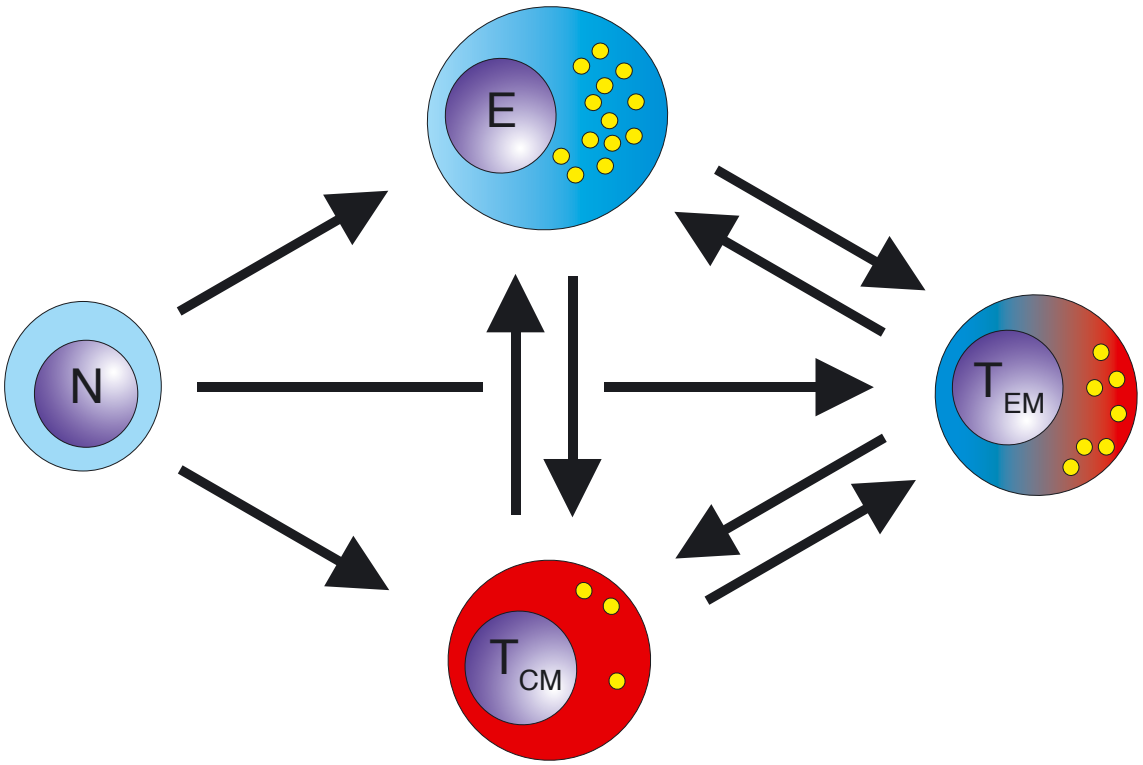


Figure 1.8: T Cell Memory Differentiation.

(a) The linear model of memory generation states that upon activation with antigen, naïve T cells differentiate into effector cells. From this effector population, the majority of the expanded specific cells will undergo apoptosis, but a subset of these cells will survive and become memory T cells. (b) The divergent differentiation model proposes that after naïve T cells encounter antigen and become activated, two distinct populations arise: the effector T cell population and the memory T cell population. After the antigen is cleared, the effector population will die off while the memory T cell population persists.

Figure 1.9



Naive	Effector	Central Memory	Effector Memory
CD62L ^{hi}	CD62L ^{lo}	CD62L ^{hi}	CD62L ^{lo}
CCR7 $^{+}$	CCR7 $^{-}$	CCR7 $^{+}$	CCR7 $^{-}$
CD44 ^{lo}	CD44 ^{hi}	CD44 ^{hi}	CD44 ^{hi}
CD27 ^{lo}	CD27 ^{lo}	CD27 ^{hi}	CD27 $^{lo/int}$

Figure 1.9: Memory T Cell Subsets.

The precise relationship between effector memory (T_{EM}) and central memory T cells (T_{CM}) remains unknown. Several different models have been proposed. Naïve T cells (N) could bypass an effector cell stage and develop directly into memory subsets. More likely, T cells of the memory subsets are direct descendants of effector cells (E). Herein, it would be possible that T_{EM} develop from T_{CM}. Conversely, T_{CM} cells could give rise to T_{EM}. Upon antigen exposure both, T_{CM} and T_{EM} could generate effector cells. Whether this occurs on direct routes ($T_{CM} \rightarrow E$ or $T_{EM} \rightarrow E$) or is detoured through the subset stages ($T_{CM} \rightarrow T_{EM} \rightarrow E$ or $T_{EM} \rightarrow T_{CM} \rightarrow E$) is not known.

Lymphocytic Choriomeningitis Virus

The lymphocytic choriomeningitis virus (LCMV) model provides a rich source of insight into the biology of host–virus interactions (Buchmeier and Zajac, 1999). For the last 60 years, this model system has been particularly useful in laboratory mice to study various aspects of the immune system, of protective immunity, and of immunopathology. The LCMV mouse model allows immunologists to achieve great insights into mechanisms of MHC-restriction, immunotolerance and autoimmunity, antiviral immune responses, and especially about T and B cell memory.

LCMV – The Virus

LCMV is the prototypic member of the Old World arenavirus family. It is an enveloped ambisense bi-segmented RNA genome virus. The LCMV genome encodes four gene products, two from each strand. The nucleoprotein (NP) and glycoprotein (GP) are produced from the smaller (3.4 kb) S strand while the polymerase or L protein (L) and the zinc-binding Z protein (Z) are produced from the larger (7.2 kb) L strand (Buchmeier and Zajac, 1999; Southern, 1996). In the viral life cycle, the NP and L proteins are produced first, with the GP and Z gene products first requiring complementary template synthesis and transcription from the complementary strand. However, there is also a discrepancy in the relative amounts of the L and S strands in infected cells. This may in part explain the dominant expression of NP and GP gene products produced. LCMV infects a variety of cell types utilizing the α-dystroglycan receptor for cell entry (Cao *et al.*, 1998). A-dystroglycan is expressed on all tissues, which explains the wide tropism of LCMV. However, various strains of LCMV exhibit differences in tissue and cell type preference, which might be due to sequence differences in the viral GP, influencing their affinity for α-dystroglycan (Sevilla *et al.*, 2000; Smelt *et al.*, 2001).

Following viral entry into permissive cells, the entire non-cytopathic life cycle of LCMV occurs in the cytoplasm. Although surprisingly little is known about the assembly of progeny virions, budding of LCMV viral particles has been observed at the plasma membrane (Figure 1.10).

LCMV Infection Models

LCMV infection of laboratory mice provides an excellent system for the study of antiviral T cell responses and the generation and maintenance of T cell memory. Because many of the MHC class I and class II restricted T cell epitopes are known (Buchmeier and Zajac, 1999; van der Most *et al.*, 1997; van der Most *et al.*, 1996) (Table 1.2) and due to innovative techniques (Altman *et al.*, 1996; Murali-Krishna *et al.*, 1998) it has been

made possible to characterize and visualize LCMV-specific T cell responses very precisely.

Furthermore, different strains of LCMV facilitate the investigation and comparison of immune responses to different types of viral infection. Principally two types of infection are observed: acute and chronic; and different LCMV strains can cause either an acute or a chronic infection:

(a) Acute LCMV infection, caused by the Armstrong strain and

(b) Chronic LCMV infection, induced by the Clone-13 strain.

(a) Acute LCMV Infection

Infection of adult immunocompetent mice with the Armstrong strain of LCMV results in an acute infection (Figure 1.11). The virus replicates rapidly in various tissues and virus titers peak approximately two to three days after infection. The infection induces a vigorous T and B cell response and virus is completely cleared from the host after seven to eight days. LCMV-specific CD8 T cells play a pivotal role in the resolution of the infection. An intact CD8 response is essential for elimination of the virus because $\beta 2m^{-/-}$ (Quinn *et al.*, 1995) and $perforin^{-/-}$ (Kägi *et al.*, 1994a; Kägi *et al.*, 1994b; Walsh *et al.*, 1994) deficient mice with intact CD4 T cell and B cell responses fail to clear the infection and the virus becomes widely disseminated throughout the infected animal. Conversely, acute viral clearance is not dependent on CD4 T cells and/or B cells because mice deficient in both cell types eliminate virus very efficiently (Cerny *et al.*, 1988). In addition, LCMV-specific CD8 T cells are generated to equivalent frequencies in the absence of either CD28 (CD8) or CD40L (CD4 help), suggesting a less stringent role for costimulation in the activation and generation of LCMV-specific CD8 responses (Whitmire and Ahmed, 2000).

Since almost exclusively LCMV-specific CD8 T cells are responsible for the viral clearance, their expansion following activation is enormous (Butz and Bevan, 1998; Murali-Krishna *et al.*, 1998). At the peak of the antiviral immune response eight days after infection, greater than 50% of the CD8 T cells are specific for the LCM-virus. These CD8 T cells produce antiviral cytokines such as IFN-γ and TNF-α, secrete autocrine growth factors like IL-2, and exhibit high levels of direct perforin-mediated *ex vivo* cytolytic activity. Following rapid expansion during the effector phase, a period of cell death ensues and 90 to 95% of the LCMV-specific CD8 T cells undergo apoptosis. The surviving specific CD8 T cells persist and form the LCMV-specific CD8 memory pool. These virus-specific memory CD8 T cells henceforth provide protection for the lifetime of the mouse from reinfection/rechallenge with LCMV.

Along with the generation of virus-specific CD8 T cells, there is also a strong induction of LCMV-specific CD4 T and B cell responses (Slifka *et al.*, 1998; Whitmire *et al.*, 1998). At late time points after infection, both memory CD4 T cells and B cells can be detected as well. Furthermore, LCMV-immune mice are equipped with persistent high serum titers of LCMV-specific antibodies that provide additional protection upon reexposure.

(b) Chronic LCMV Infection

Strains derived from lymphoid tissues of congenital carrier mice are capable of generating persistent infections in adult immunocompetent mice, despite containing only a few point mutations (Ahmed *et al.*, 1984; Matloubian *et al.*, 1990). In particular, the Clone-13 strain of LCMV only contains two amino acid substitutions from the parental Armstrong strain. These mutations result in a higher affinity interaction with the cellular receptor (α-dystroglycan) and thus in a wider tropism as well as more rapid replication, allowing enhanced spread and dissemination during infection *in vivo* (Cao *et al.*, 1998). No mutations are found in the T cell epitopes. Therefore, infections induced with chronic isolates of LCMV at least initially also generate an acute T cell response. Following infection of intact mice with these chronic strains of LCMV, virus can persist for up to three month in the serum and in most tissues. Clone-13 is even never cleared from some immunoprivileged tissues, such as the kidneys and the testes. Immunocompromised $CD4^{-/-}$ and/or B cell deficient mice that are infected with these virulent strains fail to resolve viremia and high levels of serum virus persist for life. In contrast, chronic strains of LCMV are rapidly cleared from the serum and from all tissues after infection of Armstrong-immune mice. Thus, virulent LCMV-strains provide an excellent system to study the capability of memory CD8 T cells to mediate protection from chronic viral infection.

Figure 1.10

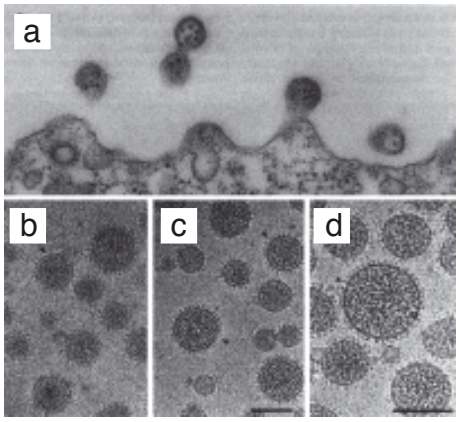


Figure 1.10: Electronmicroscopic Images of LCMV.
(a) Thin section showing several virions budding from the surface of an infected BHK-21 cell. Numerous electron-dense granules characteristic of arenaviruses are evident within the virions. Original magnification 30000-fold. (b)–(d) Cryo-electronmicroscopic images of purified unstained virions frozen in vitreous ice. Original magnification 35000-fold, bars indicate 100 nm. Adapted from (Buchmeier and Zajac, 1999).

Figure 1.11

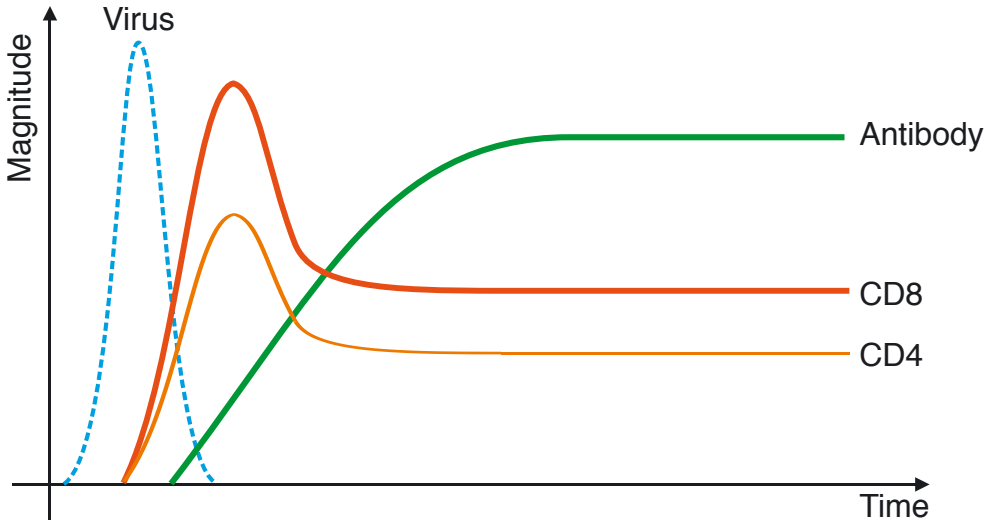


Figure 1.11: Acute LCMV Infection of Mice.
Infection of immunocompetent mice with LCMV-Armstrong induces virus titers in the spleen that peak approximately two to three days after infection and is quickly cleared from the mouse (blue dotted line). There is a potent LCMV-specific CD8 T cell response that peaks on day eight post infection. A significant LCMV-specific CD4 T cell response shows similar kinetics but a much smaller magnitude. Both of these cell populations undergo apoptosis and 90-95% of the LCMV-specific T cells die. The remaining T cells comprise the LCMV-specific memory compartment. In addition, LCMV infection induces a strong B cell response that results in LCMV-specific memory B cells and plasma cells and the production of LCMV-specific antibodies.

Table 1.2: Defined LCMV-derived T Cell Epitopes

MHC Class I	Epitope	Sequence	Frequency*
H-2D^b	NP396-404	FQPQNGAFI	30 (B6)
H-2D^b	GP33-41/43	KAVYNFATC/GI	20 (B6)**
H-2K^b	GP34-43	AVYNFATCGI	
H-2D^b	GP276-286	SGVENPGGYCL	10 (B6)
H-2K^b	NP205-212	YTVKYPNL	8 (B6)
H-2D^b	GP92-101	CSANNSHHYI	1 (B6)
H-2L^d	NP118-126	RPQASGVYM	55 (BALB)
H-2K^d	GP283-291	GYCLTKWMIL	1 (BALB)
H-2K^d	GP99-108	HYISMGTSGL	0.1 (BALB)

MHC Class II	Epitope	Sequence
I-A^b	NP309-328	SGEGWPYIACRTSIVGRAWE
I-A^b	GP61-80	GLKGPDIYKGVYQFKSVEFD

Abbreviations: GP, glycoprotein; NP, nucleoprotein; B6, C56BL/6; BALB, BALB/c. Frequency: Numbers shown represent the percent of epitope-specific CD8 T cells among all CD8 T cells at the peak of the immune response (day eight after infection) in indicated hosts.

*adapted from (Murali-Krishna *et al.*, 1998)

**combined immune response to H-2D^b-GP33 and H-2K^b-GP34

Goals

Natural and experimental exposure to viruses provides long-lived or even life-long immunity to subsequent homotypic viral infections. This phenomenon represents the basis for many of the currently employed strategies in the prevention of viral diseases. In addition, the concept of immunological memory remains central to the understanding and development of vaccination strategies for many current major public health concerns including many acute (Yellow Fever and Ebola) and chronic viral infections, caused by agents such as human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV, HCV), as well as members of the herpes virus family. These viruses have developed a multitude of strategies for evading or overwhelming the immune system. Therefore, understanding the mechanisms that generate and maintain immune responses and induce immunological memory during acute and chronic viral infections remains crucial to the prevention and cure of these diseases.

The goal of this dissertation is to add new perspectives to the complex phenomenon of immunological memory. It addresses questions of memory generation and maintenance, particularly in respect to the phenotypic and functional characterization, the proliferative capacity, in vivo persistence, lineage relationship, programmed differentiation, and the mediation of protective immunity of memory T cell subsets. Furthermore, properties of memory T cell subsets of multiple specificities are characterized. In addition, genotypic comparison is used to illustrate and confirm emerged differences between memory T cell subsets on a molecular basis. Altogether, these studies shall provide new assistance to decipher the complexity and heterogeneity of the immunological memory in order to support the design of more potent vaccines and to optimize their administration protocols, and to provide new targets for therapeutic manipulations of the immune system to enhance protection from infectious and tumor diseases.

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2

Materials and Methods

Materials

All materials, including chemicals, solutions, buffers, detergents, proteins, peptides, and other diverse agents of biochemical nature, expendable items, basic commodities, laboratory machinery, and scientific devices were used as described below and according to the manufacturers' instructions.

Experimental Procedures

Mice, Virus and Infections

C57BL/6 (henceforth referred to as B6) and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Thy1.1^{+} P14 mice bearing the D^{b}-gp33 specific TCR were fully backcrossed to C57BL/6 and maintained in our animal colony (Kaech and Ahmed, 2001). LCMV Armstrong and clone-13 strains, the recombinant *Listeria monocytogenes* (LMgp3) strain and the recombinant vaccinia virus (VVgp33), the latter two of which both express the gp33 epitope, were propagated, titered and used as previously described (Harrington *et al.*, 2002; Kaech and Ahmed, 2001). B6 and BALB/c mice were directly infected with LCMV Armstrong (2 x 10^5 plaque-forming units (p.f.u.) intraperitoneally (i.p.)). P14 chimeric immune mice were generated by adoptively transferring ~5 x 10^4 to 7.5 x 10^4 naïve TCR transgenic T cells into naïve B6 mice followed by LCMV Armstrong infection (these mice are henceforth referred to as P14 chimeras) (Kaech and Ahmed, 2001). Infection of P14 transgenic chimeras with LCMV Armstrong and LMgp33 has been previously described (Kaech and Ahmed, 2001). All LCMV or LMgp33 immune mice were used at least 30 days post infection (d.p.i.). Mice were challenged with 2 x 10^6 p.f.u. LCMV clone-13 intravenously (i.v.), 1 x 10^3 p.f.u. LCMV clone-13 in the footpad subcutaneously (s.c.), or 5 x 10^6 p.f.u. VVgp33 i.p. or intranasally (i.n.). LCMV stocks were grown and plaque assays performed as previously described (Murali-Krishna *et al.*, 1998). Vaccinia virus expressing the LCMV gp33 epitope has been previously described (Harrington *et al.*, 2002). Vaccinia plaque assays were performed essentially as described for LCMV (Murali-Krishna *et al.*, 1998), except after 2.5 days of incubation monolayers were overlayed with crystal violet (0.1% w/v in 20% methanol) and plaques counted. For footpad challenge, footpad thickness was measured using a Mituoyo Micrometer (Mitutoyo Corporation, Japan). All mice were used in accordance with NIH and the Emory University Institutional Animal Care and Use Committee guidelines.

Isolation of T Cell Subsets

T_{CM} and T_{EM} subsets were purified by fluorescence-activated cell sorting (FACS) of

$CD8^+D^b$-$gp33^+CD62L^{hi}$ or $CD8^+D^b$-$gp33^+CD62L^{lo}$ cells. Alternatively, T_{CM} and T_{EM} or total CD8 cells were purified using anti-CD62L or anti-CD8 magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of FACS-sorted samples (Figure 3.3, (d)) was 93% for T_{CM} and 95% for T_{EM}, and ranged from 80 to 99% pure for T_{CM} and T_{EM} purified by magnetic beads. Lymphocytes were isolated from non-lymphoid tissues as previously described (Becker *et al.*, 2002; Masopust *et al.*, 2001). Briefly, mice were euthanized, the hepatic vein cut and 5 ml ice cold phosphate-bufffered saline (PBS) injected directly into the hepatic artery to perfuse the liver or the left ventricle cut, and PBS injected in the right ventricle to perfuse the lungs. Liver or lung tissue was homogenized using a wire screen. Homogenized lung was first incubated in 1.5 mM ethyldiamine tetraacetate (EDTA) at 37 ºC for 30 min and both, liver and lung, then incubated in 0.25 mg/ml collagenase B (Boehringer Mannheim) and 1 U/ml DNase (Sigma) at 37 ºC for 45 min. Digested tissue was applied to a 44%/56% PBS/Percoll-gradient and centrifuged at 850*g* for 20 min. at 20 ºC. The intrahepatic lymphocyte population was harvested from the interface layer and red blood cells (RBC) were lysed using 0.83% ammonium chloride and washed, and the resulting lymphocytes counted. This procedure was found to have little impact on the expression of most cell surface molecules including CD62L (data not shown). Splenocytes isolated in the same manner as liver lymphocytes exhibited similar functional properties to splenocytes isolated by standard procedures (data not shown).

Flow Cytometry

After single-cell suspensions from the different tissues were prepared, ~10^6 cells were stained in phosphate-buffered saline containing 1% bovine serum albumin and 0.02% sodium azide (FACS buffer) for 30 min at 4 ºC, followed by three washes in FACS buffer. Samples were acquired on a FACSCalibur instrument (BectonDickinson, San Jose, CA). The data were analyzed using CELLQuest software (Becton Dickinson Immunocytometry Systems).

Surface Staining

MHC class I peptide tetramers were made and used as previously described (Murali-Krishna *et al.*, 1998). All antibodies were purchased from Pharmingen (San Diego, CA) and were used according to the manufacturer's instructions. Antibodies were conjugated with one of the following fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) or allophycocyanin (APC). Staining for granzyme B expression was performed using PE-labeled anti-human granzyme B (Caltag, Burlingame, CA). Though a mouse antibody, isotype control staining was low (Figure 3.2, (i)). The specificity of this reagent was further confirmed by the

absence of staining in granzyme $B^{-/-}$ mice (T. Ley, personal communication). CCR7 staining was performed using CCL19-Ig as described (Manjunath *et al*., 2001).

Intracellular Cytokine Staining

For intracellular cytokine staining (ICS), lymphocytes (10^6/well) were cultured for the indicated periods of time in 96-well flat-bottomed plates (Costar, Cambridge, MA) in a volume of 0.2 ml complete medium supplemented with 10 units human recombinant IL-2 and 1 μl/ml Brefeldin A (Golgistop, Pharmingen) per well with gp33-41 peptide (0.2 μg/ml) in the presence of bovine fetal albumin (BFA). After the indicated times of culture, the cells were harvested, washed once in FACS buffer, and surface stained in FACS buffer with a PE- or PerCP-conjugated monofindings clonal rat anti mouse CD8a antibody. After washing, cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (Pharmingen). For intracellular IFN-γ, TNF-α, or IL-2 staining we used FITC-, PE-, and APC-conjugated monoclonal rat anti-mouse antibodies and their isotype control antibodies (rat IgG1, Pharmingen), respectively.

Cytotoxicity Assays

Chromium (^{51}Cr) release assays were performed as previously described (Murali-Krishna *et al*., 1998), except in all cases the starting effector:target (E:T) ratio was adjusted to obtain identical ratios of D^b-gp33 specific CD8 T cells to target cells for all T cell populations. In addition, the total number of cells/well was kept constant by the addition of naïve C57BL/6 splenocytes. Background cytotoxicity against non-peptide-pulsed targets was similar for different effector populations and in some cases (Figure 3.2, (h)) has been subtracted. Briefly, the murine fibrosarcoma cell line MC57 provided the target cells (Butz and Bevan, 1998). The culture was grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine and antibiotics (RP10). Target cells were prepared by incubation for 1 to 2 h with or without peptide in the presence of sodium ^{51}Cr chromate, washed three times in PBS, and resuspended in RP10. For the assay, 10^4 target cells were added to 96-well U-bottom plates along with adjusted numbers of effector cells in a total volume of 200 μl. After indicated periods of time, 100 μl of supernatant was removed and assayed for ^{51}Cr content in a gamma counter. Specific lysis was calculated as: (experimental release - spontaneous release)/(maximum release - spontaneous release). Spontaneous release was determined for target cells in medium alone and maximum release was determined by incubating target cells in 1% Triton X-100.

Chemotaxis Assay

Transwell migration assays were performed essentially as previously described (Laouar and Crispe, 2000). Briefly, LCMV-immune splenocytes (3 x 10^6) from chimeric mice were incubated in the top of a 5 μm transwell plate. We added 100 nM, 10 nM or no chemokine to the lower well and cells were incubated at 37 ºC for 4 h. Following incubation, lymphocytes were counted in the upper and lower wells and stained for CD8, CD62L, and D^b-gp33-tetramer$^+$ memory cells. The percent of the gp33-specific CD62L^{hi} and CD62L memory cells added to the upper well that migrated into the lower well was calculated. The chemokines CCL19 (MIP-3β) and CCL21 (SLC; 6Ckine) were purchased from R & D Systems (Minneapolis, MN).

Proliferation Assays

Cells were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) as previously described (Murali-Krishna *et al*., 1999). For *in vitro* proliferation, 1 x 10^4 of either CD62L^{hi} or CD62L^{lo} D^b-gp33$^+$ cells were cultured in a total of 1 x 10^6 splenocytes in the presence of 0.2 μg/ml gp33 peptide for 60 h. For *in vivo* proliferation, ~1.5 x 10^5 (irradiated recipients) or 5 x 10^5 (non-irradiated recipients) CFSE-labeled P14 memory CD8 T cells were adoptively transferred. For bromodeoxyuridine (BrdU) labeling, LCMV-infected mice were fed continuously with drinking water containing BrdU (0.8 mg/ml) for 8 days. On day 8 post infection, freshly prepared lymphocytes were surface stained with PE-conjugated anti-mouse CD8, Cychrome-conjugated anti-mouse CD44 (Pharmingen), and APC-conjugated MHC tetramer followed by intracellular BrdU staining. For BrdU staining, a FITC-conjugated mouse anti-BrdU antibody (Clone B44, Becton Dickinson) and/or its isotype control (mouse IgG1 antibody) were used.

Statistics

For all data, P-values were calculated using the paired student's t-test if not otherwise specified. Erorr bars represent the standard error or the mean. Mean divison numbers (D_M) and Recruitment (R) used to evaluate proliferation were calculated as follows:

$$D_M = \sum_{i=0}^{k} N(D_i) \cdot \left(\sum_{i=0}^{k} x(D_i) \right)^{-1} \qquad R = \sum_{i=1}^{k} \frac{N(D_i)}{2^i} \cdot \left(\sum_{i=1}^{k} \frac{N(D_i)}{2^i} + N(D_0) \right)^{-1}$$

with being

	D_i	division(i)
	k	highest division number
	N	absolute number of cells in division(i)
	x	relative number of cells in division(i)

RNA Isolation and cRNA Synthesis

Cells recovered from FACS were counted using Trypan blue exclusion on a hemacytometer. Subsequently, cells were washed two times in PBS and then lysed in Trizol (GIBCO/BRL Life Technologies, Rockville, MD). Total RNA was isolated according to the manufacturer's protocol and resuspended in 5ml DEPC H_2O per 1 x 10^6 cells. cDNA was synthesized from 5ml total RNA (~1 x 10^6 cells) using the SuperScript Choice cDNA synthesis kit (GIBCO/BRL Life Technologies, Rockville, MD) and an oligo(dT) primer containing a T7 promoter (5'-GGCCAGTGAATTGTAATACGACTCAC-TATAGGGAGGCGG-$(dT)_{24}$-3').

Four hour *in vitro* transcription reactions with T7 RNA polymerase were used to amplify poly(A) +RNA (referred to as cRNA) from the cDNA using the MEGAscript T7 kit (Ambion, Austin, TX). The generated cRNA was treated with DNase I for 10 min, extracted with phenol-chloroform and precipitated. A fraction of the cRNA was analyzed on a 1% agarose gel stained with ethidium bromide. The cRNA samples were compared to purified poly(A) +mRNA standards to quantify cRNA synthesis. A total of 600 to 1000 ng cRNA was sent to Incyte Genomics, St. Louis, MO, for DNA microarray hybridization.

DNA Microarray Hybridization and Analysis

DNA microarray hybridizations were performed using Incyte mouse gene expression microarrays (mouse GEM 1; Incyte Genomics, St. Louis, MO.). The cRNA was converted to Cy-chrome 3 (Cy3) or Cy-Chrome 5 (Cy5) fluorescently labeled cDNA probes using random primers and reverse transcriptase (the T_{CM} subset was consistently labeled with Cy5, whereas the T_{EM} subset was labeled with Cy3). The fluorescent cDNA was competitively hybridized onto the DNA microarrays. After hybridization, the microarrays were laser-scanned to quantify the fluorescent intensity of bound probes per DNA spot. Defective cDNA spots (that is a signal/noise ratio <2.5, irregular geometry or a spot area smaller than 40% compared to average spot size) were eliminated from the data set of 8799 sequenced DNA elements. The data sets were subjected to normalization. First, the Cy5-channel of each microarray was multiplied with a balance coefficient to equal median gene signal values to those of the Cy3-channel. Second, a balanced differential expression ratio (Cy3/Cy5) was calculated for each gene (Yue *et al.*, 2001). The ratios were averaged across multiple experiments (*n=3*) to generate a data set comprised of genes, which are differentially regulated. Genes with an average differential expression ratio equal to or larger than 1.5 were selected for further study.

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3

Lineage Relationship and Protective Immunity of Memory CD8 T Cell Subsets

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Lineage Relationship and Protective Immunity

Memory CD8 T cells can be divided into two subsets, central memory (T_{CM}) and effector memory (T_{EM}), but their lineage relationships, ability to persist and confer protective immunity are not well understood. Our results show that T_{CM} have a greater capacity than T_{EM} to persist *in vivo* and are more efficient in mediating protective immunity because of their increased proliferative potential. We also demonstrate that following antigen clearance T_{EM} convert to T_{CM} and that the duration of this differentiation is programmed within the first week after immunization. We propose that T_{CM} and T_{EM} do not necessarily represent distinct subsets, but are part of a continuum in the Naïve → Effector → T_{EM} → T_{CM} linear differentiation pathway.

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Introduction

Memory T cells are well suited to combat pathogens because they are present at higher numbers than naïve cells, they persist for extended periods due to antigen-independent homeostatic turnover, and they respond rapidly upon reencounter with pathogen (Kaech *et al*., 2002b). Recently, two subsets of memory T cells were described based on their anatomical location, expression of cell surface markers and effector functions (Sallusto *et al*., 1999). Memory T cells that express molecules such as CD62L and CCR7 which allow efficient homing to lymph nodes (LN) are termed central memory cells (T_{CM}), whereas memory T cells that lack expression of these LN homing receptors and are located in non-lymphoid tissues are termed effector memory cells (T_{EM}). However, both T cell subsets are present in the blood and spleen. Some studies have also shown that T_{EM} acquire effector functions such as cytokine production and killing more rapidly than T_{CM} (Masopust *et al*., 2001; Reinhardt *et al*., 2001; Sallusto *et al*., 1999).

The existence of T_{CM} and T_{EM} subsets raises several important questions about memory T cell differentiation as well as protective immunity. Recently, considerable interest has focused on memory T cell differentiation, but it is unclear how T_{CM} and T_{EM} subsets are generated and whether they represent separate or related lineages. One study examining T cell differentiation in vitro showed that T_{CM} and T_{EM} generation can be influenced by cytokines such as interleukin-2 (IL-2) and IL-15 (Manjunath *et al*., 2001). Based on this study a model was proposed in which T_{CM} and T_{EM} can arise as separate branches during T cell differentiation (Sallusto and Lanzavecchia, 2001). However the lineage relationship between these two subsets following infection *in vivo* has not been examined. A hallmark feature of memory T cells is the ability to undergo antigen-independent homeostatic turnover and thus, maintain a stable pool of antigen-specific memory T cells (Jameson, 2002; Kaech *et al*., 2002b). It remains to be determined which memory T cell subset has the greater capacity to persist long-term *in vivo* and undergo homeostatic proliferation. A second defining characteristic of memory T cells is rapid responsiveness to antigen upon secondary infection (Kaech *et al*., 2002b). T_{EM} could provide a first line of defense in non-lymphoid tissues and therefore represent a more effective population for protection from reinfection (Sallusto *et al*., 1999), but a direct *in vivo* comparison of the protective capacity of T_{CM} and T_{EM} is lacking.

To examine these questions we have used two well-studied models of T cell immunity, acute infection of mice with lymphocytic choriomeningitis virus (LCMV) or the intracellular bacterium *Listeria monocytogenes* (LM). LCMV and LM represent the prototypical viral and intracellular bacterial pathogens used to study CD8 T cell immunity to intracellular pathogens. Infection with either LCMV or LM results in long-term protec-

tive immunity and the generation of a memory CD8 T cell population that is maintained in the absence of antigen (Busch *et al*., 1998; Kaech and Ahmed, 2001; Lau *et al*., 1994; Murali-Krishna *et al*., 1998). In the present study we have taken advantage of the P14 transgenic mouse bearing a T cell receptor (TCR) specific for the D^b-restricted LCMV gp33 epitope as well as a recombinant LM expressing the LCMV gp33 epitope (LMgp33). Our results demonstrate that following pathogen clearance there is a linear differentiation from T_{EM} into T_{CM}, indicating that these memory T cell subsets are part of a continuum of T cell differentiation rather than separate lineages that arise early during infection. In the absence of antigen T_{EM} convert directly into T_{CM} and only then gain the ability to undergo efficient homeostatic turnover. The rate at which the T_{EM} to T_{CM} conversion occurs is determined during the first week of stimulation in vivo and may depend upon the magnitude of the infection. Finally, protective immunity is more efficiently conferred by T_{CM} than T_{EM} due to the greater proliferative capacity of T_{CM}. Thus, CD8 T cell differentiation following acute infection follows a linear Naïve → Effector → T_{EM} → T_{CM} pathway that culminates in the generation of a cell type, T_{CM}, that has acquired the two hallmark characteristics of memory T cells: rapid responsiveness to antigen and the stem cell-like quality of self-renewal.

Results

Effector and Memory T Cell Characterization

Normal B6 mice as well as P14 transgenic mice expressing a TCR specific for the LCMV D^b-gp33 epitope were used in this study. B6 mice were infected directly with LCMV Armstrong, whereas P14 transgenic chimeras were generated by adoptively transferring naïve P14 cells (~7.5 x 10^4) into naïve B6 mice and then infecting these chimeric mice with the Armstrong strain of LCMV (Kaech and Ahmed, 2001). Viral titers in the spleen peaked at ~3 days post infection and virus was eliminated from all tissues by ~day 8 (Harrington *et al.*, 2000) (Figure 3.1, (a) and data not shown). The LCMV-specific CD8 T cell response peaked on days 7 to 8, underwent a contraction phase between days 8 to 30 and resulted in a memory pool whose numbers remain stable over time (Lau *et al.*, 1994; Murali-Krishna *et al.*, 1998) (Figure 3.1, (a)). Effector CD8 T cells at the peak of the response were highly cytolytic directly *ex vivo*, but this lytic capacity per cell decreased gradually over time (Harrington *et al.*, 2000) (Figure 3.1, (b)). The amount of *ex vivo* cytotoxicity correlated well with the intensity of staining for granzyme B protein in D^b-gp33 specific CD8 T cells, which decreased as cells transitioned from effectors, through the contraction phase and into memory (Figure 3.1, (c)). Tissue homing properties also changed during the differentiation of naïve CD8 T cells into effectors and finally into memory cells (Figure 3.1, (d)). Naïve CD8 T cells efficiently homed to lymph nodes (LN), but not to non-lymphoid sites such as the lung and liver. In contrast effector CD8 T cells had a reduced ability to localize to LN, but gained the ability to enter non-lymphoid organs. Resting memory T cells retained an enhanced capacity to home to the lung and liver compared to naïve cells, but in contrast to effectors, regained the ability to enter LN. All three populations homed to the spleen equally well. A similar pattern of activation and memory CD8 T cell generation was observed following infection of P14 chimeras with LMgp33 (Kaech and Ahmed, 2001) (see Chapter 4).

The memory T cell compartment can be divided into T_{CM} and T_{EM} subsets based on the expression of several cell surface molecules such as the LN homing receptors (Hislop *et al.*, 2001; Sallusto *et al.*, 1999; Tussey *et al.*, 2000). Consistent with these reports, we found that LCMV D^b-gp33 specific memory CD8 T cells present in the spleen and peripheral blood mononuclear cells (PBMC) could be readily distinguished based on CD62L and CCR7 (Figure 3.2, (a), (b) and data not shown). One subset, representative of T_{CM}, expressed high amounts of CD62L and CCR7 and the other subset, representative of T_{EM}, expressed low amounts of these two homing molecules. CD27 expression was also useful in distinguishing between these two subsets because T_{CM} cells were mostly $CD27^{hi}$ whereas T_{EM} cells were $CD27^{lo/int}$ (Figure 3.2, (b)). We also examined ex-

pression of several canonical cell surface markers of memory CD8 T cells on these two subsets and found that CD44, CD11a, Ly6C, CD122 and CD132 were highly expressed by both subsets (Figure 3.2, (c)). Neither subset showed evidence of recent TCR activation and both expressed low amounts of CD25 and CD69 and were not blasting based on cell size (Forward Scatter, FSC; Figure 3.2, (c)). Thus, both subsets represented "resting" memory CD8 T cells and the $CD62L^{hi}CCR7^{+}CD27^{hi}$ subset corresponded to what is termed T_{CM}, whereas the $CD62L^{lo}CCR7^{-}CD27^{lo/int}$ subset to what is called T_{EM}.

T_{CM} and T_{EM} have also been defined by anatomical location. Specifically, T_{CM} localize to LN while T_{EM} are found in non-lymphoid organs (Masopust *et al.*, 2001; Reinhardt *et al.*, 2001; Weninger *et al.*, 2001). As expected D^{b}-gp33 specific memory CD8 T cells found in LN were $CD62L^{hi}$ T_{CM}, whereas the majority of those in non-lymphoid tissues were of a $CD62L^{lo}$ T_{EM} phenotype (Figure 3.2, (d)). Both T_{CM} and T_{EM} subsets were present in the spleen and PBMC (Figure 3.2, (a) and data not shown). Also, the $CD62L^{hi}$ D^{b}-gp33-specific T_{CM}, but not the $CD62L^{lo}$ T_{EM} subset had the capacity to respond to the lymphoid chemokines CCL19 and CCL21 in transwell migrations assays (Figure 3.2, (e); similar results were observed at multiple chemokine concentrations) consistent with its capacity to localize efficiently to LN *in vivo*.

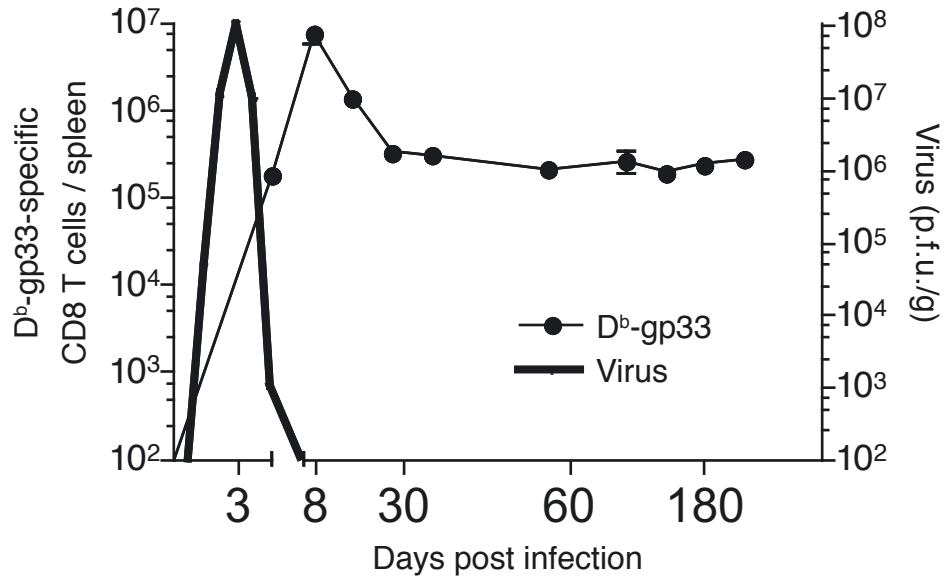
We next analyzed the functional properties of gp33-specific T_{CM} and T_{EM} cells following peptide stimulation *in vitro*. For these experiments we used two approaches to obtain these memory T cell subsets. T_{CM} and T_{EM} cells were either purified from the spleen based on CD62L expression (T_{CM} 92% pure and T_{EM} 97% pure) or T_{CM} from the LN were compared to T_{EM} isolated from the liver (similar results were observed for T_{EM} derived from the lung; data not shown). The ability to produce the antiviral cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) was very similar for T_{CM} and T_{EM} whether they were derived from the LN, spleen or liver; all populations produced these cytokines with remarkable rapidity (Figure 3.2, (f) and (g)). In other words, both T_{CM} and T_{EM} were extremely efficient in producing the effector cytokines IFN-γ and TNF-α upon restimulation. However, only T_{CM} (LN or spleen) were capable of producing IL-2 (Figure 3.2, (f) and (g)). Little virus-specific cytotoxicity was detected in 5 hours directly *ex vivo* for either T_{CM} or T_{EM} and neither subset expressed high levels of granzyme B (Figure 3.2, (h) and (i)). Similar low levels of granzyme B staining were observed from memory cells isolated from the liver (data not shown). However, both memory T cell subsets were equally capable of rapidly acquiring cytotoxic function upon restimulation with peptide since equivalent levels of target cell lysis were observed at 12 and 18 hours (Figure 3.2, (h) and data not shown).

Thus, the LCMV-specific memory CD8 compartment contained two subsets that resembled T_{CM} and T_{EM}. Gp33-specific T_{CM} were $CD62L^{hi}CCR7^{+}CD27^{hi}$, capable of re-

sponding to CCL19 and CCL21, present in LN, spleen and PBMC and were able to produce IL-2 upon restimulation. T_{EM}, on the other hand, were $CD62L^{lo}CCR7^{-}CD27^{lo/int}$, less responsive to CCL19 and CCL21, absent from LN but present in spleen, PBMC and non-lymphoid tissues and did not produce IL-2. However, the canonical memory cell markers CD44, CD11a, Ly6C and CD122 were highly expressed by both subsets. In contrast to some reports (Hislop *et al.*, 2001; Masopust *et al.*, 2001; Reinhardt *et al.*, 2001; Sallusto *et al.*, 1999; Tussey *et al.*, 2000), but consistent with others (Champagne *et al.*, 2001; Masopust *et al.*, 2001; Unsoeld *et al.*, 2002) we found that both memory T cell subsets were equally efficient in acquiring effector functions (IFN-γ and TNF-α production and cytotoxicity) upon restimulation with peptide *in vitro*.

Figure 3.1

(a)



(b)

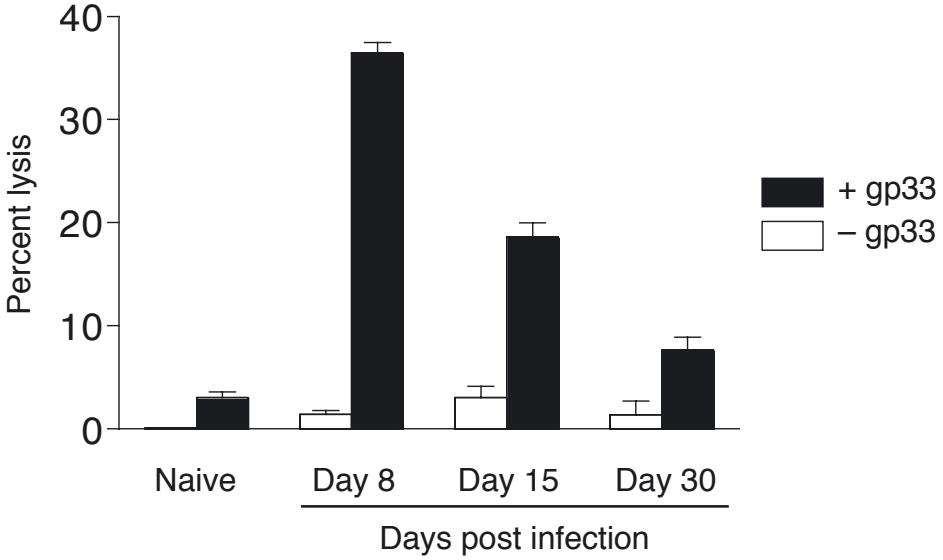


Figure 3.1: Characterization of Effector and Memory T Cells.

(a) Viral load (Virus, measured in plaque-forming units per gramm tissue (p.f.u./g), bold solid line) and D^b-gp33-specific CD8 T cell numbers (D^b-gp33, connected filled circles) in the spleen following LCMV Armstrong infection of B6 mice. (b) Cytotoxicity of D^b-gp33-specific CD8 T cells (effector cells) at days 8, 15, and 30 post LCMV Armstrong infection and in naive animals. Target cells were either labeled with gp33 peptide (+gp33, filled bars) or unlabled (-gp33, open bars). Effector:target ratio (E:T ratio) was 2:1 in all cases.

Figure 3.1

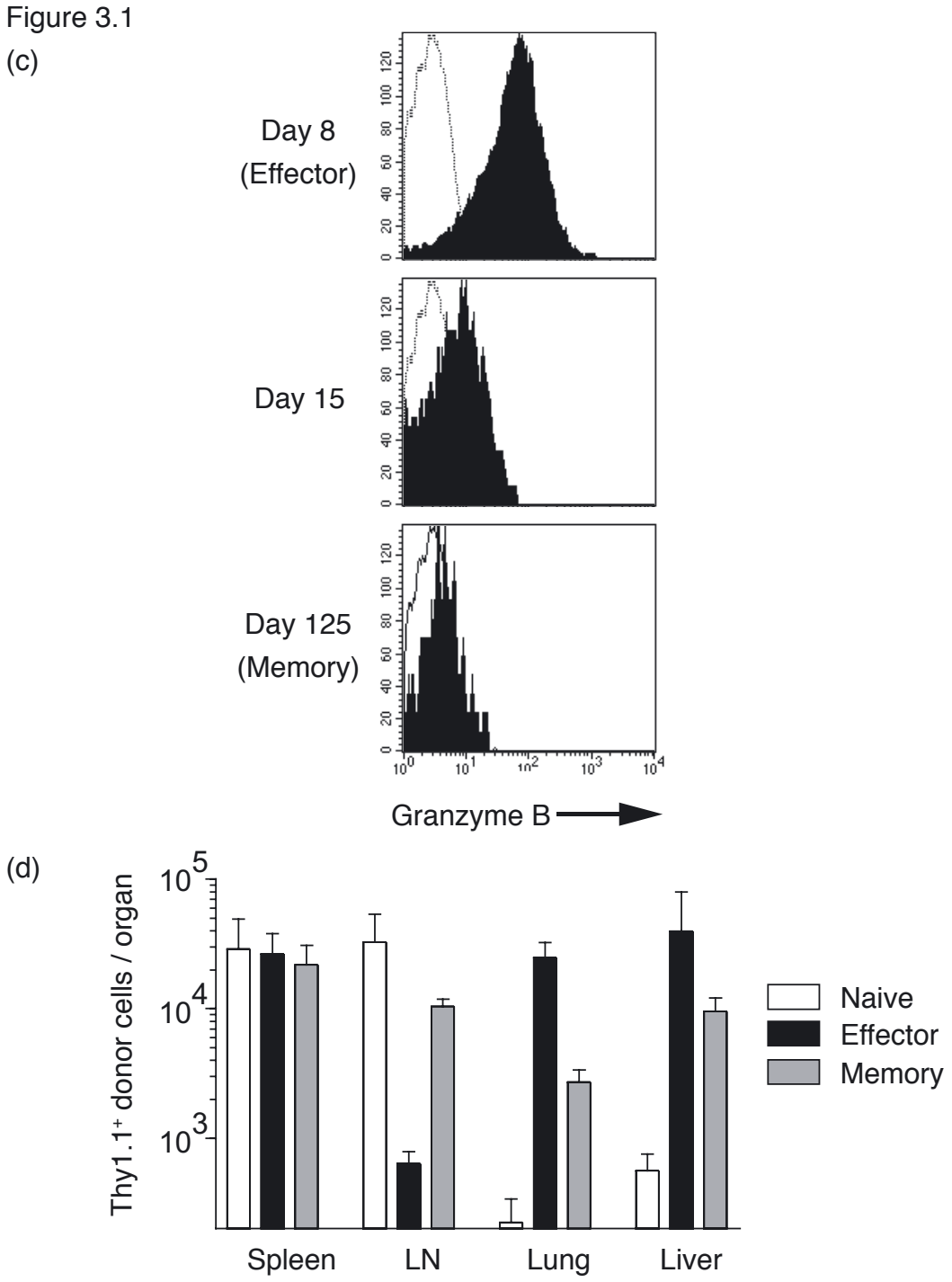


Figure 3.1: Characterization of Effector and Memory T cells.

(c) Intracellular granzyme B staining of D^b-gp33-specific CD8 T cells (filled histogram) at 8, 15, and 125 (memory) days post infection. Open histogram indicates naïve cells. (d) *In vivo* homing of naïve, effector and memory T cells. Naïve (open bars), effector (day 8 p.i., black bars) and memory (~day 60 p.i., gray bars) P14 cells ($Thy1.1^+$) were adoptively transferred into naïve B6 ($Thy1.2^+$) recipeints. 12 hours later, the number of donor ($Thy1.1^+$) gp33-specific CD8 T cells was determined in the indicated organs by flow cytometry.

Figure 3.2

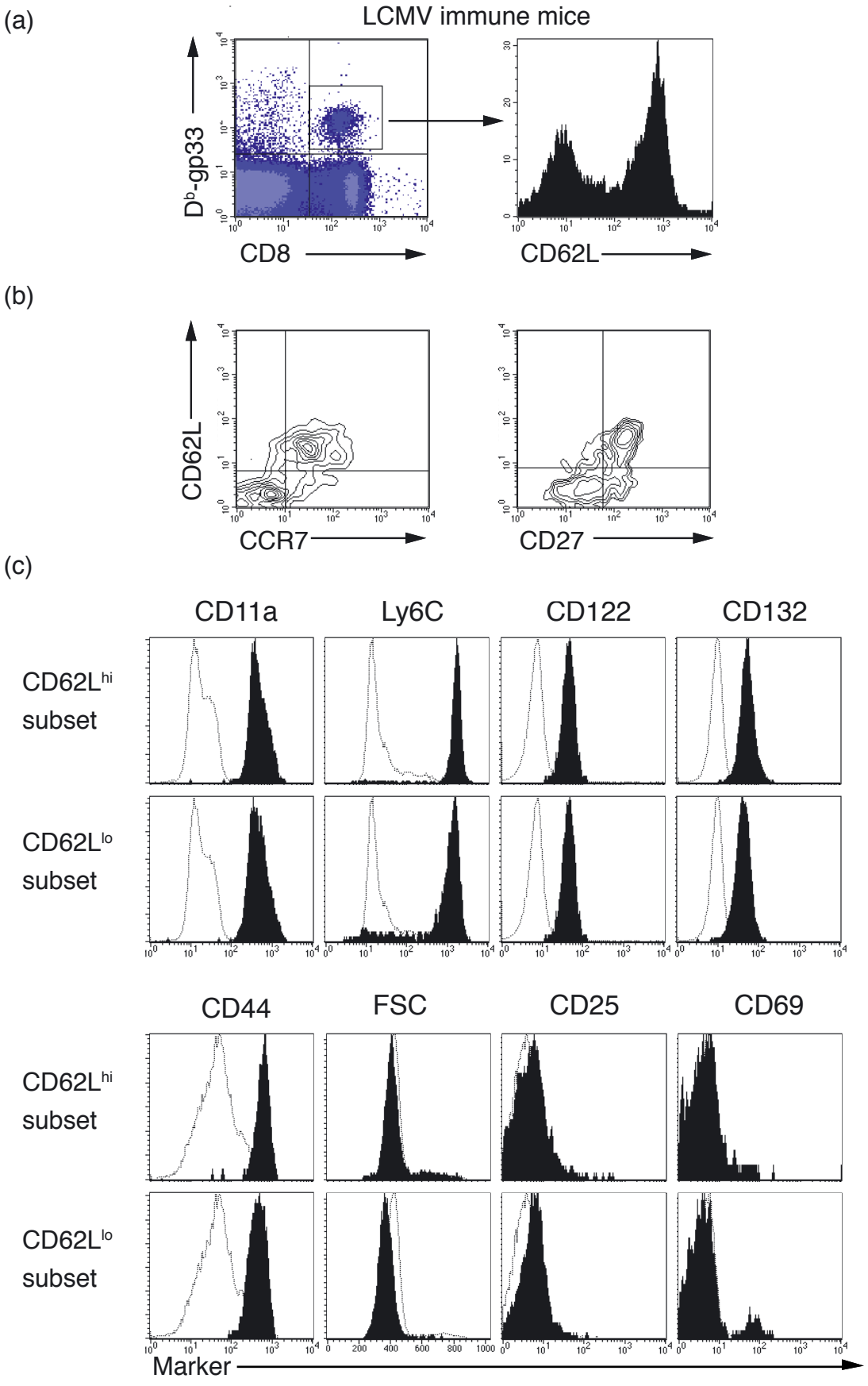

(a)
LCMV immune mice
Db-gp33
CD8
CD62L
(b)
CD62L
CCR7
CD27
(c)
CD11a
Ly6C
CD122
CD132
CD62Lhi subset
CD62Llo subset
CD44
FSC
CD25
CD69
CD62Lhi subset
CD62Llo subset
Marker

Figure 3.2

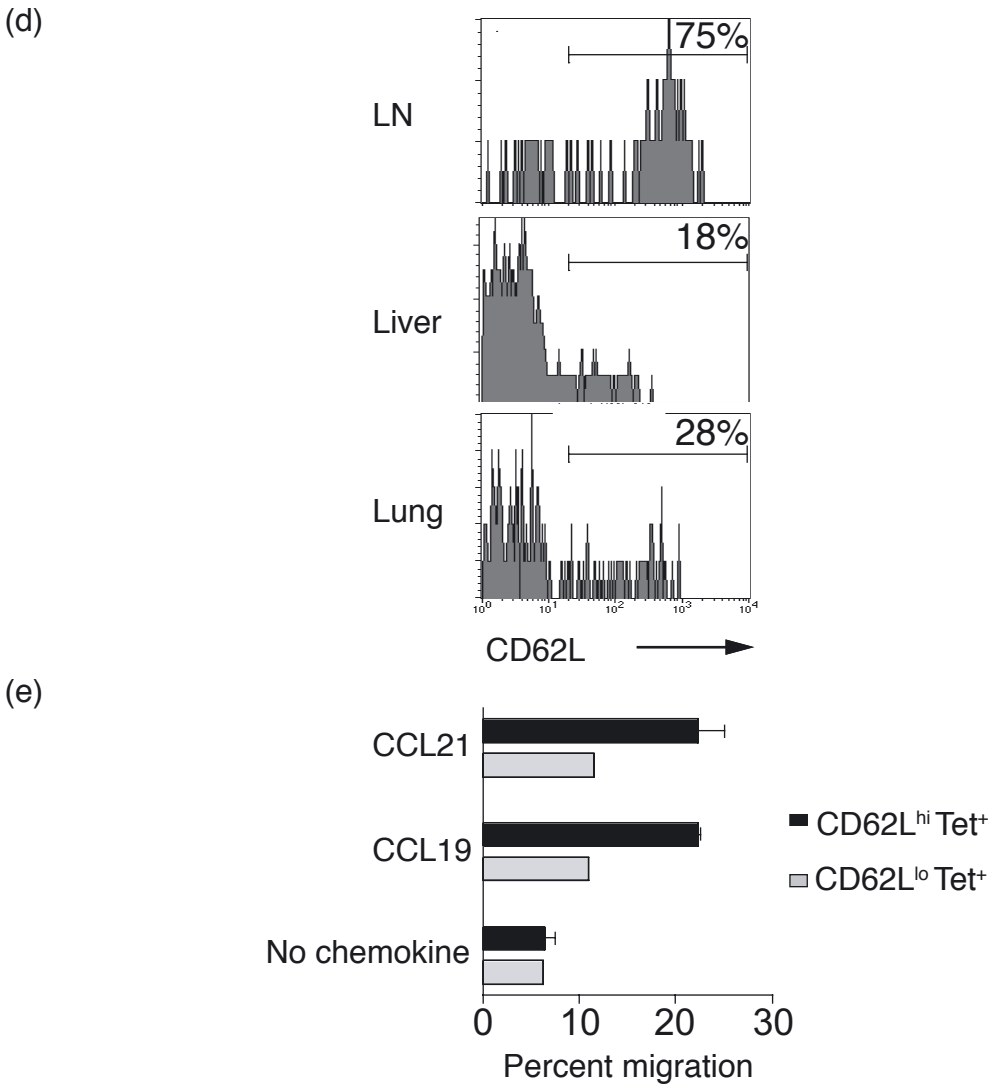


Figure 3.2: Characterization of Memory T Cell Subsets.

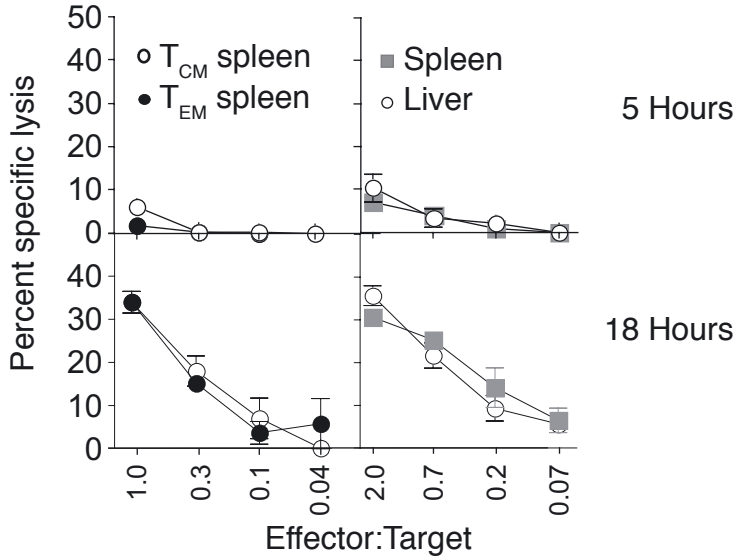
(a) LCMV D^b-gp33-specific memory CD8 T cells (~2-3 months p.i.) were costained for CD62L expression (histogram is gated on $CD8^+D^b$-$gp33^+$ cells). (b) D^b-gp33-specific memory T cells (2-4 months p.i.) were costained for CD62L and CCR7 expression (left panel) or CD62L and CD27 expression (right panel). Plots are gated on $CD8^+D^b$-$gp33^+$ cells. (c) Phenotypic analysis of $CD62L^{hi}$ and $CD62L^{lo}$ subsets of D^b-gp33-specific memory CD8 T cells. Histograms are gated on either $CD62L^{hi}$ (top rows) or $CD62L^{lo}$ (bottom rows) $CD8^+D^b$-$gp33^+$ memory cells (~1-2 months p.i.). Open histograms indicate naïve cells. (d) LCMV gp33-specific memory (~60 d.p.i.) cells from LN, liver and lung of P14 chimeras were stained for CD62L expression. Histograms are gated on $CD8^+D^b$-$gp33^+$ memory cells. Similar results were observed for normal B6 mice. (e) Splenocytes from P14 LCMV-immune chimeras (> 30 d.p.i.) were added to a transwell plate and migration in the presence or absence of added chemokine (100 nM) was assessed.

Figure 3.2

(f)
(g)
Percent Db-gp33+
IFN-γ
TNF-α
IL-2
Hours
TCM spleen
TEM spleen
TCM LN
TEM liver

Figure 3.2

(h)



(i)

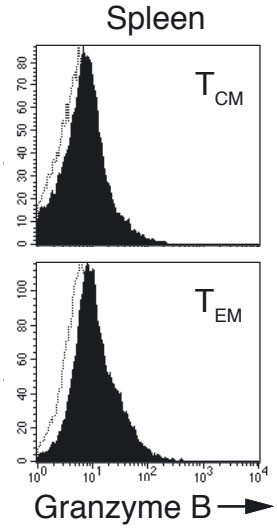


Figure 3.2: Characterization of Memory T Cell Subsets.

(f) (previous page) IFN-γ, TNF-α, and IL-2 production by gp33-specific T_{CM} and T_{EM} P14 splenocytes separated using magnetic beads (92% and 97% pure, respectively) was assessed by intracellular cytokine staining (ICS) following gp33 peptide stimulation. (g) (previous page) ICS of T_{CM} from LN and T_{EM} from the liver. (h) 5- and 18-h gp33-specific ^{51}Cr release assay using splenic T_{CM} and T_{EM} purified as in (f) (left panel) or using memory T cells from spleen *versus* liver (right panel). All immune mice used in functional experiments were > 30 d.p.i.; background lysis in the absence of gp33 peptide was similar for T_{CM} and T_{EM} and has been subtracted. (i) Granzyme B staining of memory T cell subsets from the spleen. T_{CM} are gated on $CD62L^{hi}$ and T_{EM} on $CD62L^{lo}$ D^b-$gp33^+CD8$ T cells at ~60 d.p.i.; open histograms indicate staining with an isotype control antibody.

Protective Immunity by T_{CM} and T_{EM}

To address the question how T_{CM} and T_{EM} compare in their ability to mediate protective immunity, the following experimental design was used (Figure 3.3, (a)). First, to provide a source of T_{CM} and T_{EM} cells, B6-P14 chimeric mice were infected with LCMV Armstrong or LMgp33 (Kaech and Ahmed, 2001). Between 30 and 60 days after resolution of the acute infection, memory T cells subsets were isolated from various tissues of these mice. Then, equal numbers of these memory T cell subsets were adoptively transferred to separate naïve recipients and these mice tested for their ability to control viral infection. Splenic T_{CM} and T_{EM} were purified on the basis of CD62L expression by either flow cytometry or positive magnetic bead separation (Figure 3.3, (b); purity ranged from 80% to 99%). To compare protective capacity of these memory T cell subsets on a per cell basis, it was critical to demonstrate that the total number of T_{CM} and T_{EM} was the same after adoptive transfer to naïve recipients. After transfer, the total number of $CD62L^{hi}$ and $CD62L^{lo}$ memory gp33-specific CD8 T cells recovered from several organs (LN, lung, liver, spleen and bone marrow (BM)) was composite equivalent (Figure 3.3, (c)). As expected, T_{CM} homed more efficiently to the LN, whereas T_{EM} had a modest advantage in homing to the lung and liver, confirming the known recirculation properties of these memory T cell subsets (Kunkel and Butcher, 2002; Weninger *et al.*, 2001). Both subsets were equally efficient in homing to the spleen and BM.

To evaluate the protective capacity of T_{CM} and T_{EM}, we used four different challenge models that assess viral control by LCMV- or LM-induced memory CD8 T cells. First, after intravenous infection with a virulent strain of LCMV (clone-13), T_{CM} cells mediated considerably more rapid control of the viral infection than did the T_{EM} subset (Figure 3.3, (d)). Though less effective than T_{CM}, T_{EM} administered to mice caused a more rapid reduction in viremia compared with control mice. Reduced viremia in this model represents viral control in multiple tissues. To examine control of viral replication in a more localized tissue, naïve recipients of purified T_{CM} and T_{EM} were challenged intraperitoneally with vaccinia virus (VV) expressing the gp33 epitope (VVgp33) and viral control was examined in the ovary on day 5. T_{CM} again provided better control of viral replication than did the T_{EM} cells (Figure 3.3, (e)). Thus, whether viral titers were determined in the blood after LCMV clone-13 infection or in a peripheral tissue (ovary) after VV infection, the T_{CM} subset controlled virus more effectively than did T_{EM}. To determine whether memory T cell subsets induced by a different pathogen also displayed similar properties, T_{CM} and T_{EM} were generated by immunization with LMgp33, and purified populations of T_{CM} and T_{EM} were adoptively transferred to naïve recipients. These mice were then challenged with LCMV clone-13. Serum viral titers 8 days after systemic LCMV challenge were substantially lower in mice that received T_{CM} than in those that received T_{EM} cells

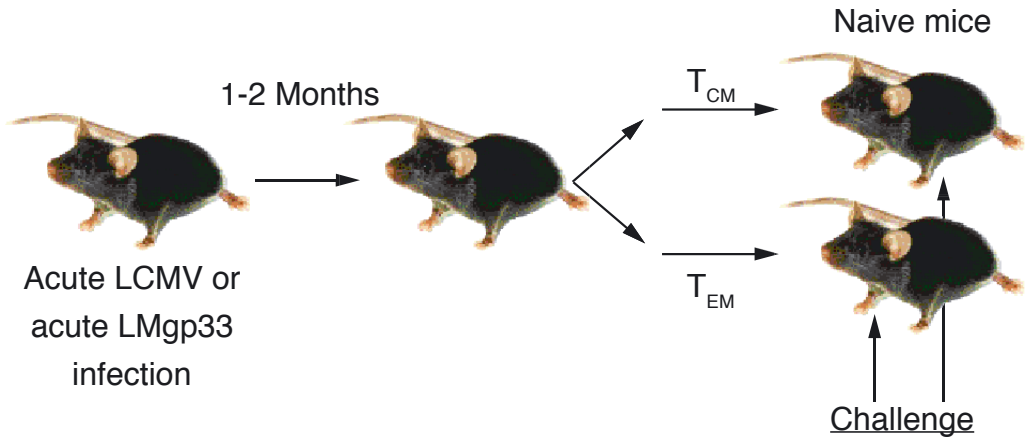
(Figure 3.3, (f)). T_{CM} also elicited a more rapid virus-specific delayed-type hypersensitivity (DTH) response (Figure 3.3, (g)) after subcutaneous infection of the footpad with LCMV, demonstrating that T_{CM} could more rapidly initiate a response and accumulate at a site of peripheral challenge than T_{EM}. Thus, whether memory CD8 T cell subpopulations were generated following an acute viral or bacterial infection, T_{CM} consistently demonstrated more effective and rapid pathogen control.

Our next experiment was designed to address protection using an intranasal (i.n.) challenge and to test the capacity of memory T cells derived from a non-lymphoid tissue, the lung, to mediate protection. Spleen-derived T_{CM} or T_{EM} and T_{EM} isolated from the lung (70% $CD62L^{lo}$) were adoptively transferred and recipients infected i.n. with VVgp33. After 5 days, recipients of T_{CM}, but not T_{EM} (either lung- or spleen-derived), showed significant control of viral replication in the lungs (Figure 3.3, (h)). In an additional experiment, liver-derived T_{EM} were compared to T_{CM} in their ability to control virus after a systemic LCMV infection and, once again, the T_{CM} cells were superior (data not shown).

These data from the four challenge models showed that T_{CM} more effectively control viral replication (either LCMV or vaccinia virus) on a per cell basis than T_{EM}, irrespective of the route of infection (intravenous, intraperitoneal, intranasal or subcutaneous) or the site of infection (lung, ovary or viremia). This was true whether T_{CM} and T_{EM} were defined phenotypically and purified from the same tissue (splenic $CD62L^{hi}$ $CCR7^{hi}$ *versus* $CD62L^{lo}$ $CCR7^{lo}$) or defined anatomically and isolated from non-lymphoid tissue (lung *versus* liver). It should also be noted that these experiments were designed to test the contribution of only the adoptively transferred, gp33-specific T_{CM} or T_{EM} cells to protective immunity, as either highly purified gp33-specific CD8 T cells were transferred or the only shared determinant between the immunizing and challenge pathogen was the gp33 epitope itself (for example, LCMV primed → VVgp33 challenge).

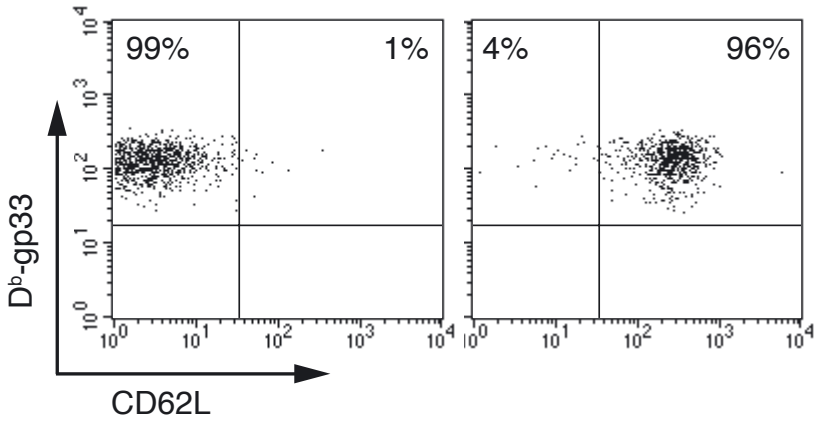
Figure 3.3

(a)



1. Systemic LCMV (Clone-13 i.v.)
2. VV in ovaries (VVgp33 i.p.)
3. DTH (LCMV Clone-13 s.c. footpad)
4. Respiratory infection (VVgp33 i.n.)

(b)



(c)

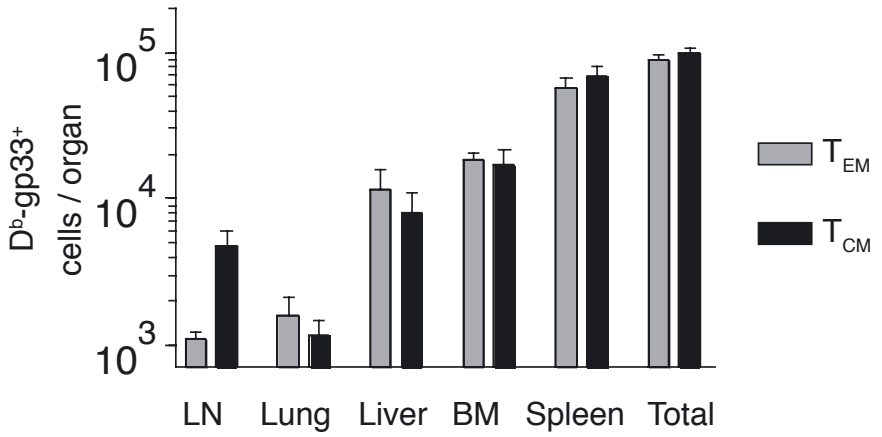
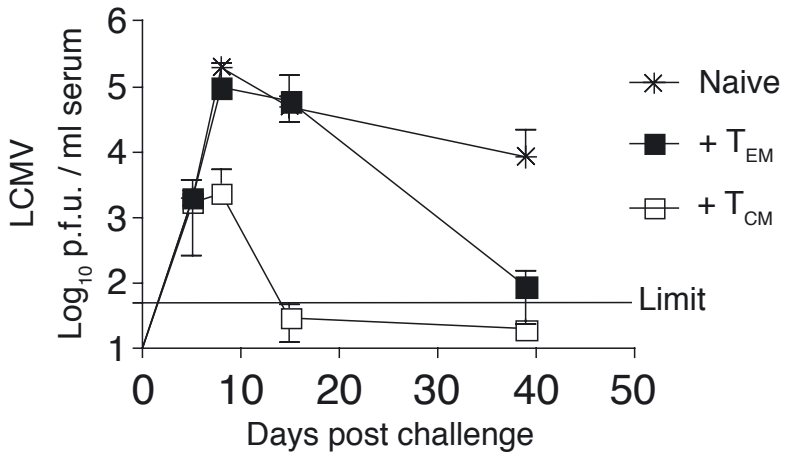


Figure 3.3

(d)



(e)

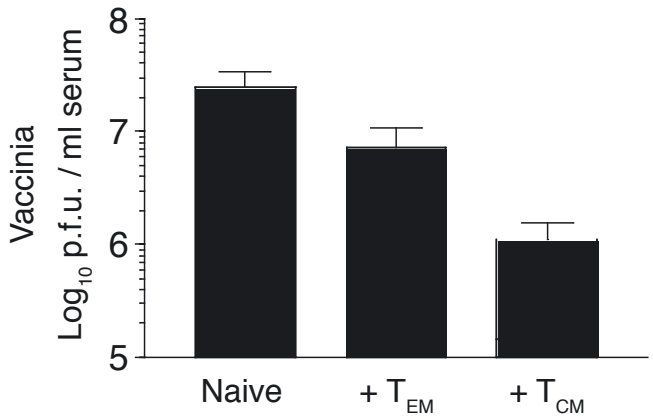


Figure 3.3: Protective Immunity by Memory T Cell Subsets.

(a) Thy1.1^{+} gp33 specific P14 transgenic memory cells were generated by infecting B6 chimeric mice containing P14 cells with LCMV (Armstrong) or recombinant LM expressing the gp33 epitope (LMgp33). We purified 1- to 2-months p.i. CD62L^{hi} and CD62L^{lo} splenocytes by flow cytometry or magnetic bead separation. Equal numbers of CD62L^{hi} or CD62L^{lo} gp33-specific P14 cells were adoptively transferred to separate naïve mice. Two days later, recipients were challenged as indicated. *n* = 3-6 mice in all groups for all experiments. (b) Purity of CD62L^{hi} and CD62L^{lo} populations before transfer. (c) To determine the number of T_{CM} and T_{EM} present in recipient mice following adoptive transfer, the number of D^{b}-gp33^{+}Thy1.1^{+}CD8^{+} cells in the indicated organs was measured by flow cytometry (2 days after transfer into naïve Thy1.2^{+} mice). (d) Control of LCMV clone-13 infection by T_{CM} or T_{EM} (7.5 x 10^{4} of each) following intravenous (i.v.) challenge. (e) Control of VVgp33 infection by T_{CM} or T_{EM} (2.5 x 10^{5} of each) following intraperitoneal (i.p.) challenge. VV ovary titers were determined on day 5 (T_{EM} *versus* T_{CM}, P = 0.08).

Figure 3.3

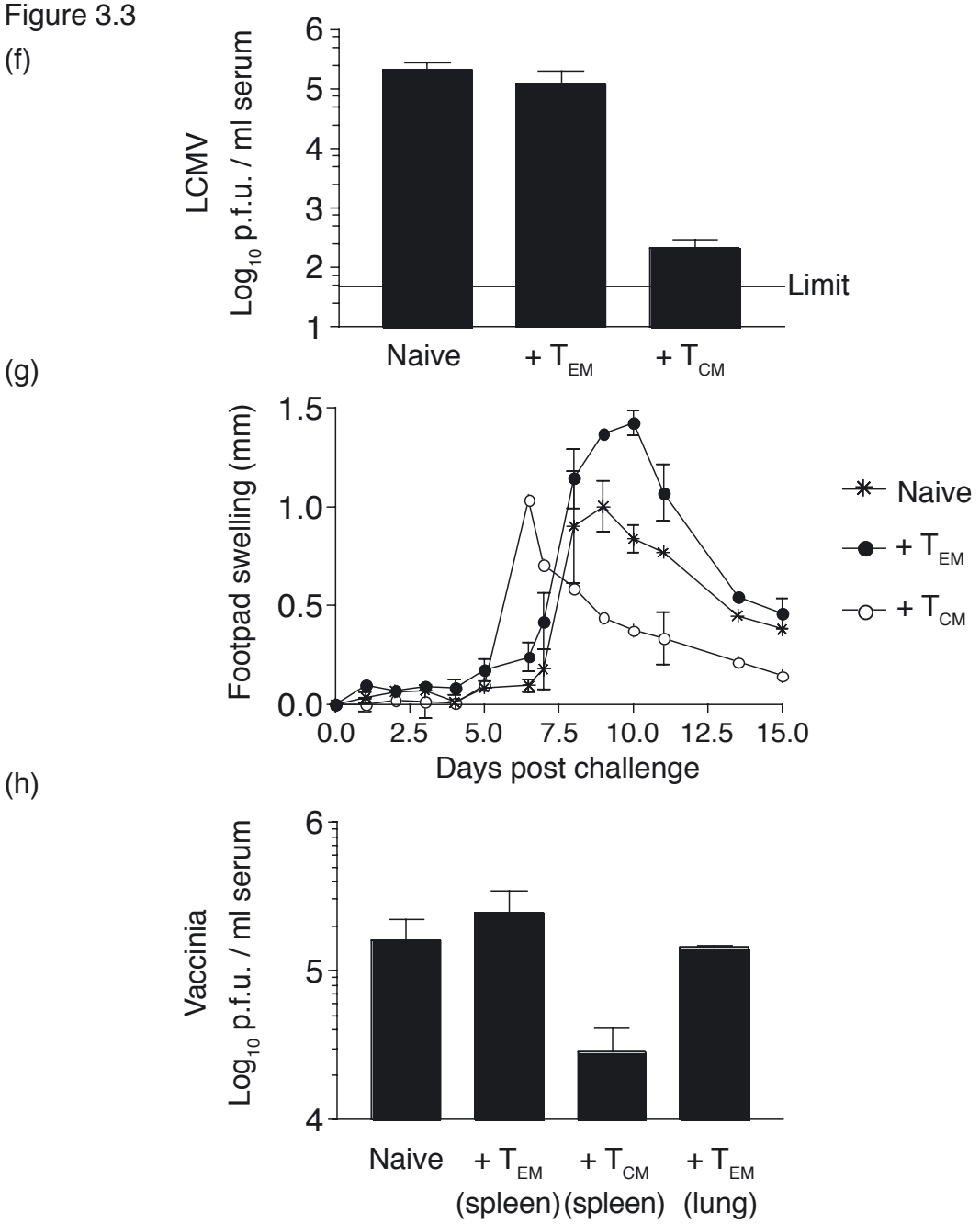


Figure 3.3: Protective Immunity by Memory T Cell Subsets.

(f) Control of LCMV clone-13 infection by LMgp33-induced T_{CM} or T_{EM} (1 x 10^5 of each) following i.v. challenge. Day 8 serum viral titers shown (T_{EM} *versus* T_{CM}, P = 0.02). (g) Induction of DTH response by LMgp33-induced T_{CM} or T_{EM} (2 x 10^5 of each) following footpad injection of LCMV clone-13. Footpad thickness was measured daily. (h) Control of VVgp33 infection by LCMV-induced T_{CM}, T_{EM} from the spleen or lung-derived T_{EM} (3 x 10^5 of each) following i.n. challenge (T_{EM} spleen *versus* T_{CM} spleen, P = 0.04; T_{EM} lung *versus* T_{CM} spleen, P = 0.003). VV lung titers were determined on day 5. For all protection experiments at least two doses of cells were transferred.

Antigen-driven Proliferation of T_{CM} and T_{EM}

What properties endow T_{CM} with greater protective capacity than T_{EM}? Because effector functions were very similar between T_{CM} and T_{EM} (Figure 3.2, (f)-(i) and data not shown), we examined the *in vivo* expansion of these subsets after viral challenge. Donor (Thy1.1$^+$) gp33-specific CD8 T cells were enumerated after either systemic or respiratory viral challenge of recipients of T_{CM} or T_{EM}. Five days after systemic challenge, T_{CM} expanded substantially more (2- to 5-fold higher frequencies; 2.7- to 4.4-fold greater total numbers/organ) in all tissues examined (Figure 3.4, (a)). Following respiratory challenge the difference in *in vivo* expansion of T_{CM} and T_{EM} was even more dramatic. T_{CM} recipients contained 10- to 13-fold more virus-specific CD8 T cells in the lung than recipients of T_{EM} cells (Figure 3.4, (b)). Several recent studies have demonstrated that initial T cell activation *in vivo* occurs in draining LN despite, in some cases, the presence of T cells at the site of inoculation (Mueller *et al.*, 2002; Norbury *et al.*, 2002; Ostler *et al.*, 2001). Therefore, it is likely that the greater expansion of T_{CM} cells after infection is a reflection of their ability to localize to the LN. However, it is also possible that the T_{CM} cells have a stronger intrinsic capacity to proliferate following antigenic stimulation than T_{EM} cells. To directly test this hypothesis, we stimulated D^b-gp33 specific T_{CM} or T_{EM} *in vitro* with gp33 peptide and analyzed cell division by 5,6-carboxyfluorescein diacetate succinimidyl diester (CFSE) dilution (Figure 3.4, (c)). T_{CM} proliferated considerably better than T_{EM}, indicating that T_{CM} have an inherent proliferative advantage over T_{EM} following antigenic stimulation. Thus, it is likely that the substantially greater expansion of T_{CM} *in vivo* after infection is due to both, their inherent capacity to proliferate and their ability to localize to the LN.

We next examined the phenotype of transferred T_{CM} and T_{EM} in several tissues after infection (Figure 3.4, (d)). Each transferred population was greater than 95% CD62L^{hi} or CD62L^{lo} at the time of transfer (Figure2.4, (d), pre-challenge). As expected, when CD62L^{lo} T_{EM} cells were transferred they remained CD62L^{lo} after infection (left column). However, when CD62L^{hi} T_{CM} cells were transferred, nearly 90% of all gp33-specific CD8 T cells in the spleen, PBMC and liver had become CD62L^{lo} by 5 days post infection. Even in the LN, approximately 65% of the gp33-specific memory cells were CD62L^{lo}. Similar results were observed following respiratory challenge (data not shown). This conversion from CD62L^{hi} to CD62L^{lo} required reexposure to antigen because cells recovered from all organs after adoptive transfer in uninfected mice maintained their pre-transfer phenotype. In the absence of infection, transferred T_{CM} in the spleen, LN, liver and lung remained 94%, 91%, 82%, and 84% CD62L^{hi}, respectively (data not shown). Collectively, these results show that, following antigen challenge, T_{CM} can rapidly convert to CD62L^{lo} effector cells and that T_{CM}-derived secondary effectors

can efficiently localize to non-lymphoid tissues including the site of infection. Thus, the major difference in T_{CM} and T_{EM} seems to be not a difference in immediate effector functions (both subsets were equally good), but rather the ability of T_{CM} to rapidly proliferate and expand after reencountering antigen.

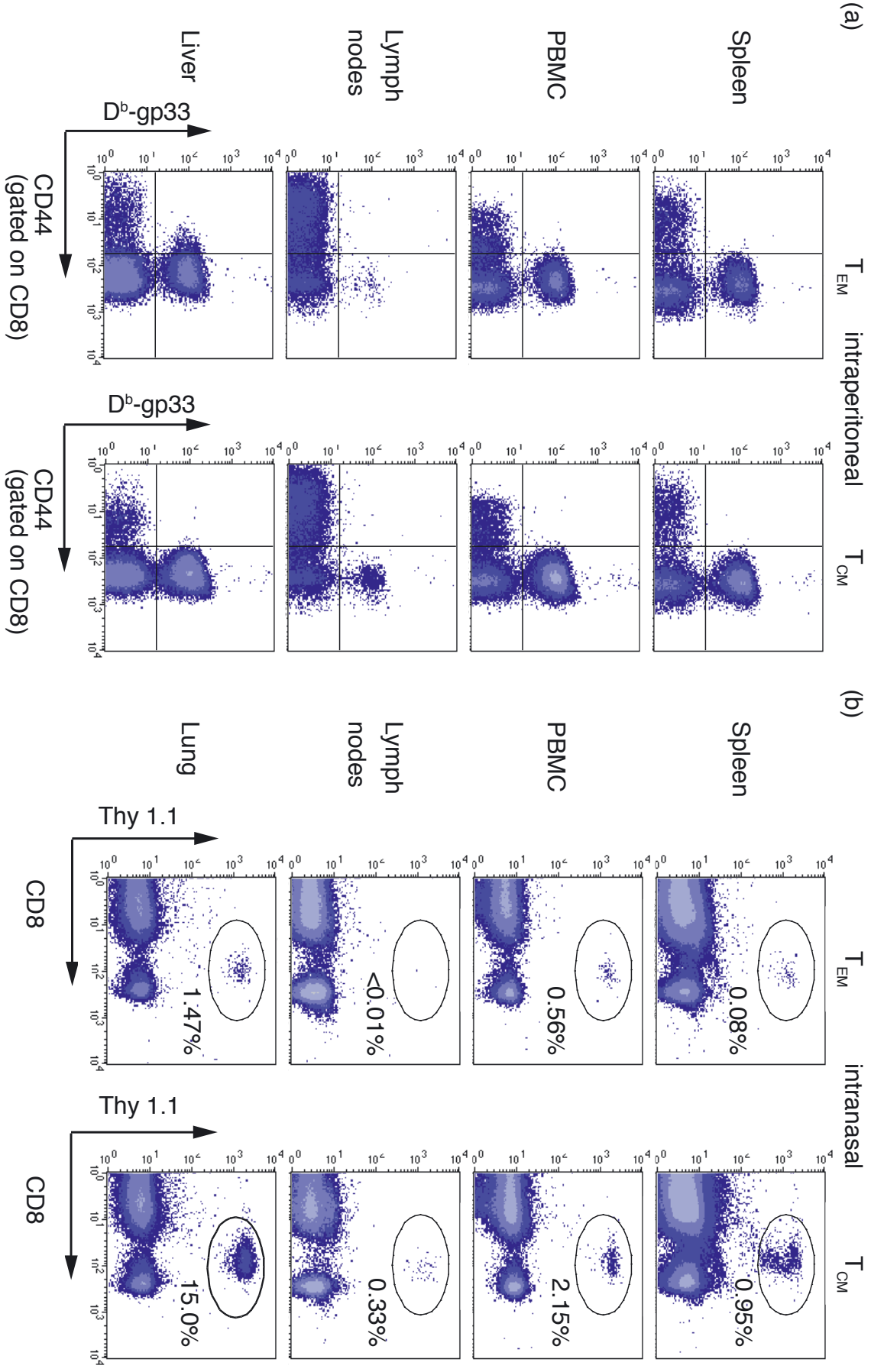

(a)
Spleen
PBMC
Lymph nodes
Liver
T_{EM}
T_{CM}
intraperitoneal
D^b-gp33
CD44 (gated on CD8)
(b)
Spleen
PBMC
Lymph nodes
Lung
intranasal
Thy 1.1
CD8
0.08%
0.56%
<0.01%
1.47%
0.95%
2.15%
0.33%
15.0%

Figure 3.4

Figure 3.4

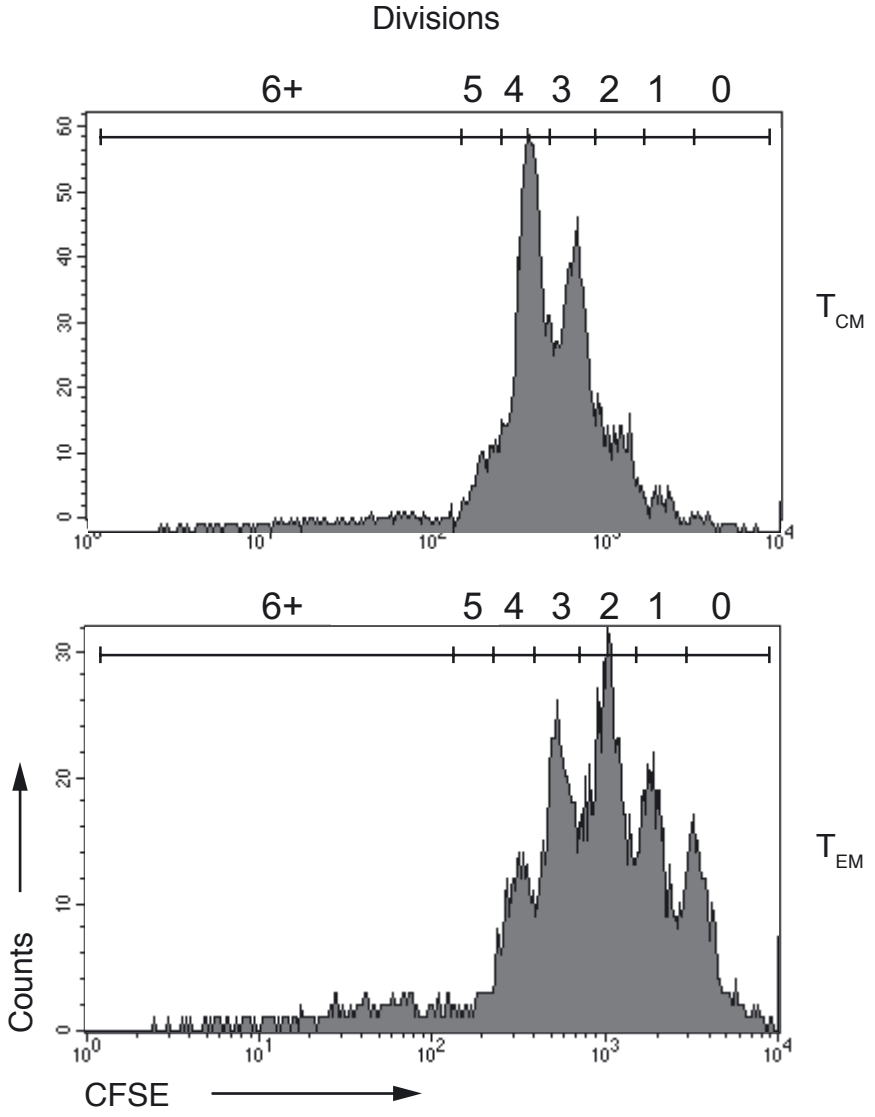


Figure 3.4: Antigen-driven Proliferation of Memory T Cell Subsets.

(a) (previsous page) *In vivo* T cell expansion following systemic challenge. D^b-$gp33^+CD8^+$ T cells were enumerated in spleen, PBMC, LN and liver of T_{CM}- and T_{EM}-recipients 5 days after i.p. VVgp33 challenge (Figure 3.3, (e)). All D^b-$gp33^+CD8^+$ T cells were donor derived ($Thy1.1^+$; data not shown). T_{CM} recipients had significantly more total $Thy1.1^+D^b$-$gp33^+CD8^+$ T cells in all locations examined ($P < 0.05$). (b) *In vivo* T cell expansion following respiratory challenge. $Thy1.1^+$ (donor) D^b-$gp33^+CD8^+$ T cells were enumerated in spleen, PBMC, LN, and lung of T_{CM} and T_{EM} recipients 5 days after i.n. VVgp33 challenge (Figure 3.3, (h)). T_{CM} recipients had significantly more total $Thy1.1^+$Db-$gp33^+CD8^+$ T cells in lung, spleen and PBMC ($P < 0.05$). (c) *In vitro* proliferation of T_{CM} and T_{EM} P14 cells in response to gp33 peptide. The mean division number for T_{CM} and T_{EM} was 3.4 and 2.0, respectively. No division was observed in the absence of gp33 peptide (data not shown).

Figure 3.4

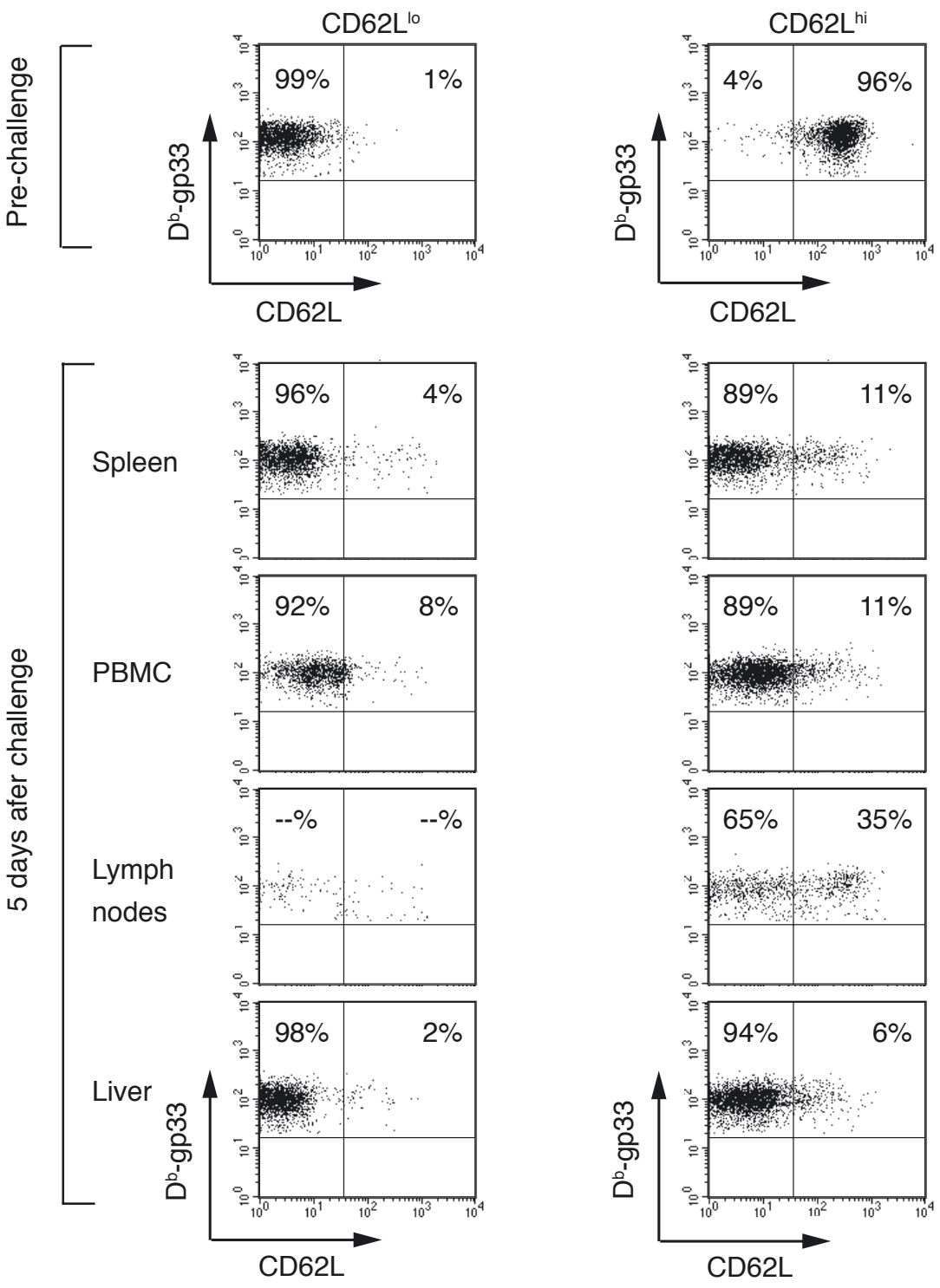


Figure 3.4: Antigen-driven Proliferation of Memory T Cell Subsets.

(d) Five days after i.p. VVgp33 challenge, recipients of T_{CM} or T_{EM} were sacrificed and the expression of CD62L on secondary effectors in the spleen, PBMC, LN and liver was assessed by flow cytometry. Initially, transferred populations of gp33-specific memory cells are shown at the top. Data is representative of 3 to 4 mice per group.

In vivo Persistence and Lineage Relationship

One of the cardinal properties of memory T cells is their long-term antigen-independent persistence (Ahmed and Gray, 1996; Fearon *et al.*, 2001; Murali-Krishna *et al.*, 1999; Swain *et al.*, 1999). Given the different properties of T_{CM} and T_{EM}, it is important to determine which population persists for extended periods and to understand the lineage relationship between these subsets. The total number of gp33-specific memory T cells in the spleen remained constant between 1 and 3 months post infection (p.i.) with LCMV (Figure 3.5, (a)) and were stably maintained even at 400 d.p.i. (data not shown). However, during this time the absolute number of T_{EM} cells declined, whereas the number of T_{CM} increased proportionally (Figure 3.5, (a), top). At very late time points (for example, day 400) >95% of the LCMV-specific memory CD8 T cells were $CD62L^{hi}$ (see Chapter 3). A similar trend was also observed for CCR7 expression (Figure 3.5, (a), bottom) and CD27 expression (data not shown). This pattern of the number of T_{EM} cells decreasing and the number of T_{CM} cells increasing was seen in both, the spleen and PBMC. This suggested that either death of the T_{EM} subset was compensated by a reciprocal increase in the T_{CM} population or that there was conversion of T_{EM} to T_{CM}. To investigate this issue, gp33-specific memory CD8 T cells were again separated into T_{CM} and T_{EM} on the basis of CD62L and adoptively transferred into naïve recipients (Figure 3.5, (b)). After 25 days, the transferred $CD62L^{hi}$ T_{CM} population remained uniformly $CD62L^{hi}$, but approximately half of the transferred $CD62L^{lo}$ T_{EM} cells had converted to $CD62L^{hi}$ cells, demonstrating that the T_{CM} subset can arise directly from T_{EM} (Figure 3.5, (b)).

A principal attribute of memory T cells is their ability to undergo homeostatic proliferation to maintain their numbers (Becker *et al.*, 2002; Fearon *et al.*, 2001; Goldrath *et al.*, 2002; Kieper *et al.*, 2002; Ku *et al.*, 2000; Murali-Krishna *et al.*, 1999; Schluns *et al.*, 2000; Schluns *et al.*, 2002; Sprent and Surh, 2001; Swain *et al.*, 1999; Tan *et al.*, 2002; Tanchot *et al.*, 1997; Tough *et al.*, 1996). To examine homeostatic proliferation of memory T cell subsets, purified T_{CM} and T_{EM} were labeled with CFSE and transferred to naïve mice (non-irradiated). Thirty days later the division profile of the transferred cells revealed that T_{CM} cells had undergone more divisions (69% divided) compared to T_{EM} cells (36% divided; Figure 3.5, (c)). As our data (Figure 3.5, (b)) suggested that the T_{EM} population could give rise to T_{CM}, we next examined the phenotype of CFSE-labeled transferred T_{CM} and T_{EM} cells during homeostatic division. One day following adoptive transfer of purified T_{CM} and T_{EM}, each population maintained its phenotype, and at this early time point no cell division had occurred (Figure 3.5, (d)). By day 30 post transfer the T_{CM} population had undergone efficient homeostatic proliferation and also retained its phenotype ($CD62L^{hi}$; Figure 3.5, (d)). These cells also remained $CCR7^{hi}$ and $CD27^{hi}$ (data not shown). In contrast, T_{EM} cells again showed a phenotypic change and by day 30

a substantial proportion (~42%) of T_{EM} cells had converted to $CD62L^{hi}$. It was predominantly this population that had divided (Figure 3.5, (d)). It should be noted that one day after transfer there were very few, if any, $CD62L^{hi}$ cells in the T_{EM} population, but on day 30 there were a substantial number of memory T cells that had not yet divided but had already converted to $CD62L^{hi}$ (Figure 3.5, (d), box). This shows that the emergence of $CD62L^{hi}$ T_{CM} cells from the $CD62L^{lo}$ T_{EM} population truly represents a conversion of the two subsets and is not simply due to proliferation of a few contaminating T_{CM} cells in the purified T_{EM} population. This $T_{EM} \rightarrow T_{CM}$ conversion was also accompanied by increased CCR7 and CD27 expression (Figure 3.5, (e)). Similar results were observed in multiple tissues (data not shown). These results demonstrate that long-term persistence of memory T cells is primarily in the form of T_{CM}. Further, the T_{EM} subset does not appear to be a permanent memory population, but rather converts to T_{CM} and in so doing acquires the ability to undergo efficient, antigen-independent homeostatic proliferation. This advantage of T_{CM} in proliferative renewal was confirmed using three additional approaches: Purified T_{CM} and T_{EM} were CFSE labeled and adoptively transferred to naïve irradiated recipients in which, after 8 days, substantially more division was observed by the transferred T_{CM} than T_{EM} cells (Figure 3.5, (f)). Additionally, bromodeoxyuridine (BrdU) labeling was used to analyze memory cell turnover in an unmanipulated mouse (that is, no adoptive transfer). Gated $CD62L^{hi}$ gp33-specific T_{CM} contained ~24% $BrdU^{+}$ cells after a week of BrdU pulse compared to only 6.5% for T_{EM} cells (Figure 3.5, (g)). Finally, high forward scatter can be used to identify T cells that are currently, or have recently been, in cycle (Razvi *et al.*, 1995). The D^{b}-gp33-specific T_{CM} subset contained approximately four-fold higher frequency of cells with high forward scatter than the T_{EM} population (Figure 3.5, (h)).

Thus, during this memory T cell differentiation not only does the expression of LN homing receptors convert from $CD62L^{lo}CCR7^{-}$ to $CD62L^{hi}CCR7^{+}$, but the memory pool also acquires both, homeostatic and antigen-driven proliferative potential. A third functional quality of T_{CM} is the ability to produce IL-2 after antigenic stimulation. To test whether this functional property also changed during this memory T cell differentiation, the ability of the memory T cell population to produce IL-2 was examined over time. The proportion of the memory pool capable of IL-2 production gradually increased consistent with an accumulation of T_{CM} cells in the memory pool (Figure 3.5, (i)). These results demonstrate that, over time, the memory T cell pool converts both, phenotypically and functionally from a T_{EM} population that is $CD62L^{lo}CCR7^{-}$, has reduced antigen-driven and little homeostatic proliferative potential and does not produce IL-2 to a T_{CM} subset that is $CD62L^{hi}CCR7^{+}$, proliferates vigorously to antigen, is capable of efficient homeostatic proliferation and has gained the ability to make IL-2 following antigen stimulation.

Figure 3.5

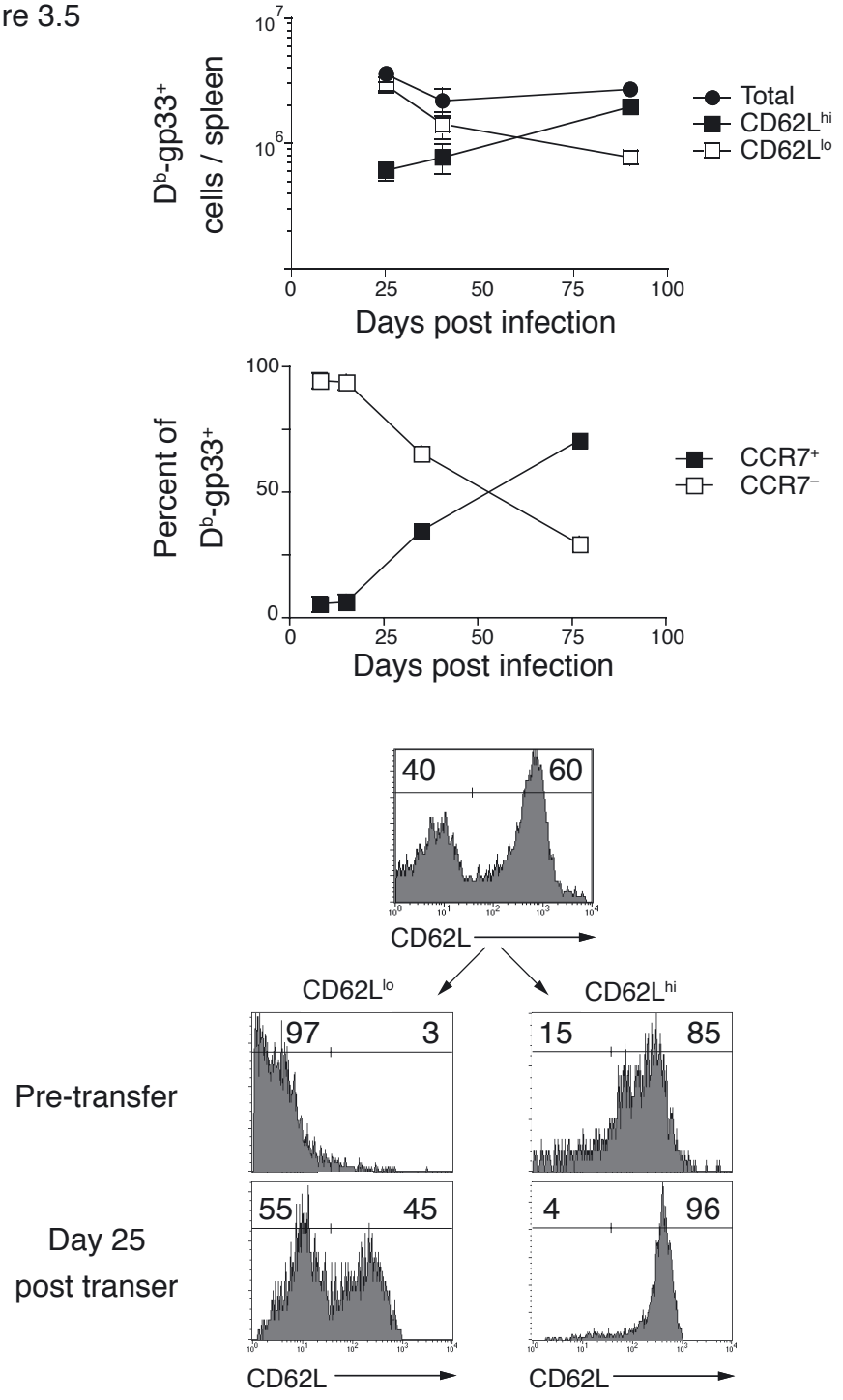


Figure 3.5: Lineage Relationship between Memory T Cell Subsets.

(a) The number of total and $CD62L^{hi}$ and $CD62L^{lo}$ memory D^b-$gp33^+CD8^+$ T cells and the percentage of $CCR7^+$ and $CCR7^-$ D^b-$gp33^+$ CD8 T cells in the spleens of LCMV immune P14 chimeric mice is plotted over time. n = 2 to 4 mice / timepoint. (b) Column purified $CD62L^{hi}$ or $CD62L^{lo}$ D^b-$gp33^+$ memory T cells were adoptively transferred into separate naïve mice. After 25 days, CD62L expression on splenic D^b-$gp33^+CD8^+$ T cells of recipients was determined.

Figure 3.5

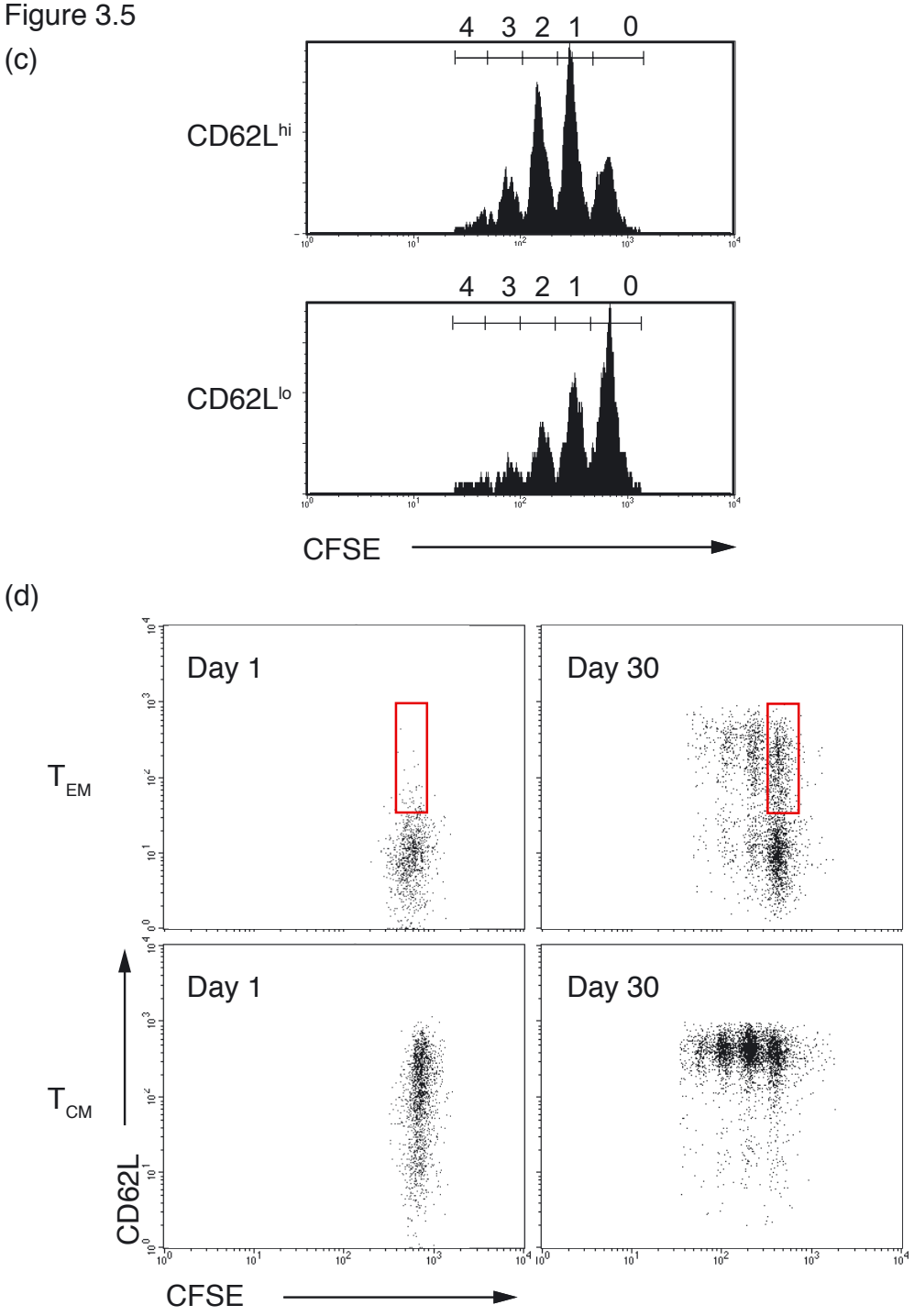
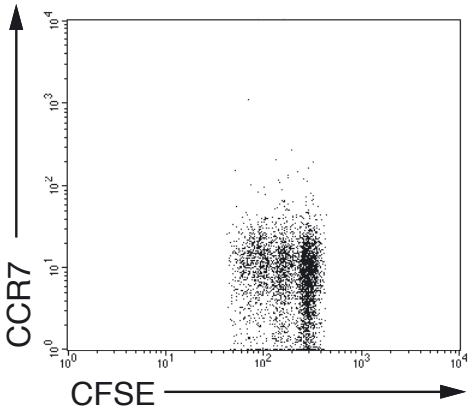


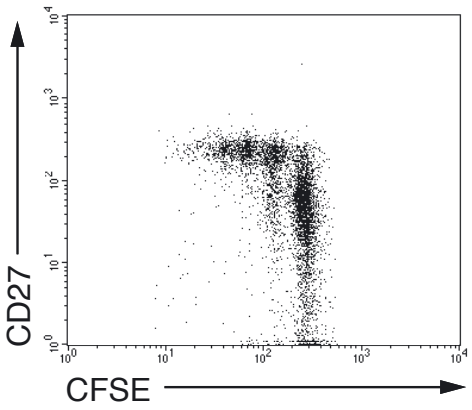
Figure 3.5: Lineage Relationship between Memory T Cell Subsets.

(c) Purified T_{CM} and T_{EM} cells were CFSE-labeled and transferred into separate naïve recipients (non-irradiated). Division of the transferred Thy1.1$^+$ P14 memory cells was assessed after 30 days. (d) Purified T_{CM} or T_{EM} D^b-gp33$^+$ cells were CFSE labeled and transferred to naïve mice (non-irradiated). After 1 and 30 days, CD62L expression was examined as a function of division. Dot plots are gated on Thy1.1$^+$ P14 memory CD8 T cells from the spleen.

Figure 3.5

(e)





(f)

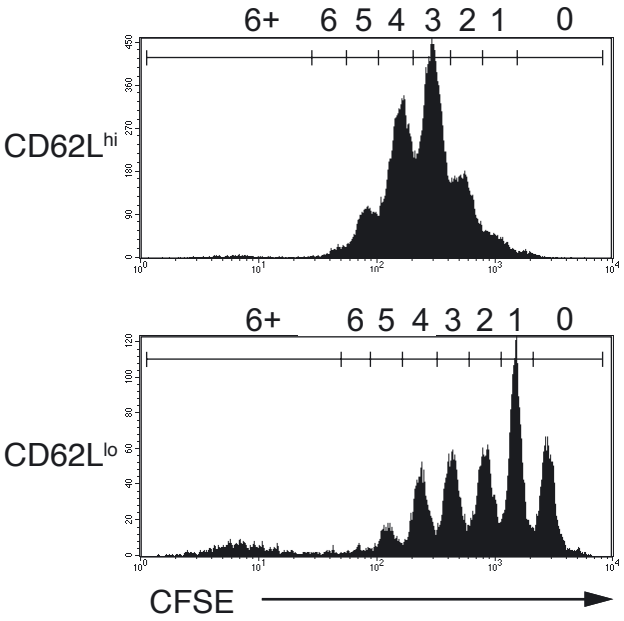


Figure 3.5: Lineage Relationship between Memory T Cell Subsets.

(e) CCR7 and CD27 expression was examined as a function of cell division on transferred T_{EM} cells 30 days post transfer. (f) Purified T_{CM} and T_{EM} D^b-gp33$^+$ CFSE-labeled memory CD8 T cells were transferred separately into naïve irradiated recipients. Division of the transferred Thy1.1$^+$ P14 memory cells was analyzed after 8 days.

Figure 3.5

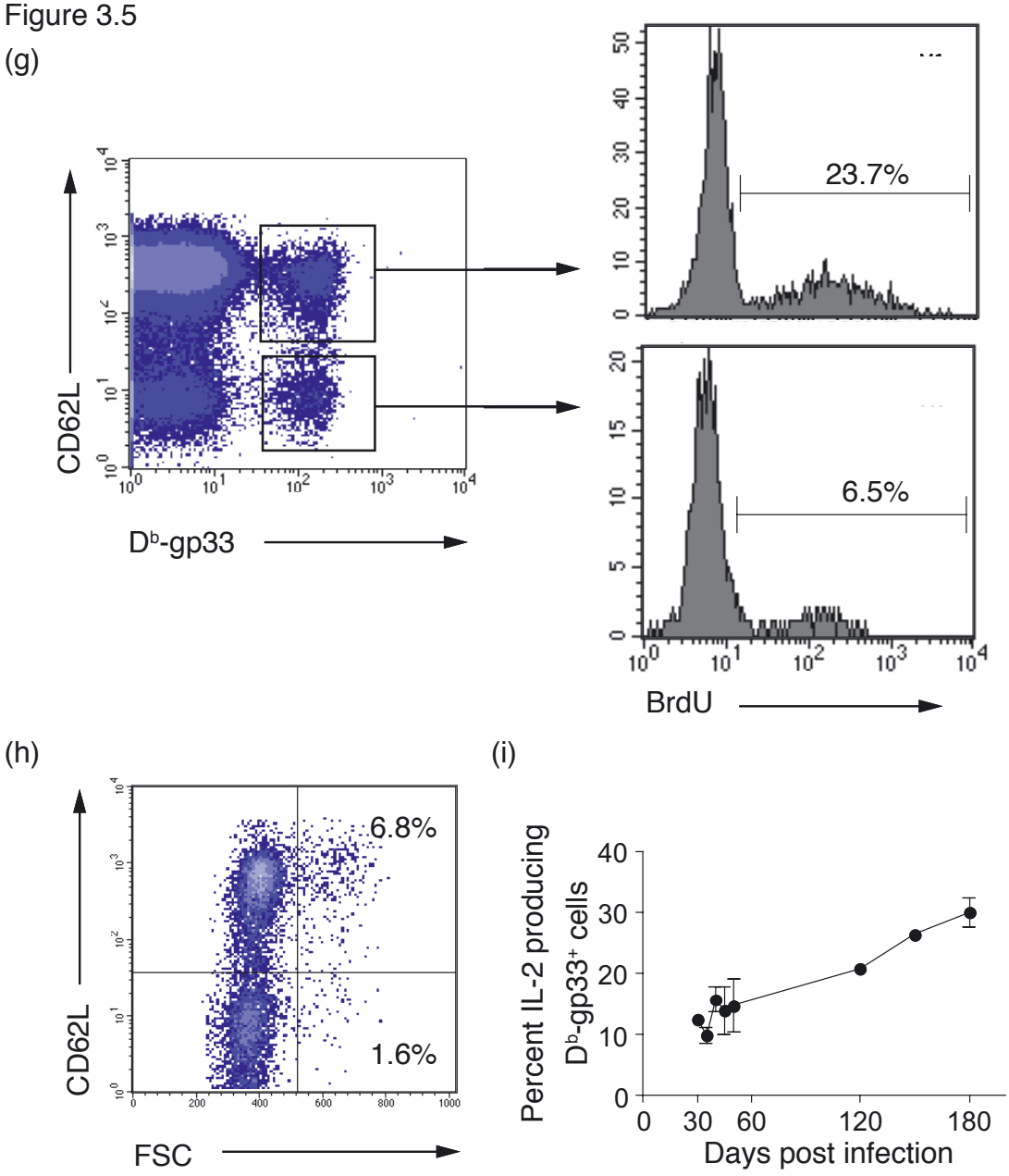


Figure 3.5: Lineage Relationship between Memory T Cell Subsets.

(g) LCMV immune (~85 d.p.i.) mice were fed BrdU in their drinking water for 8 days and splenocytes stained for BrdU incorporation. D^b-gp33 tetramer staining *versus* CD62L is shown for gated CD8 cells. Histograms are gated on D^b-gp33$^+$CD8$^+$CD62L^{hi} (top) or CD62L^{lo} (bottom) memory T cells. (h) D^b-gp33$^+$CD8$^+$ memory T cells (~30 d.p.i.) from a P14 chimera were stained for CD62L expression and the percentage of blasting cells indicated by high forward scatter was assessed. Plots are gated on D^b-gp33$^+$CD8$^+$ cells. (i) IL-2 production by D^b-gp33-specific memory CD8 T cells was assessed at the indicated times post infection by intracellular cytokine staining following gp33 peptide stimulation.

Programmed $T_{EM} \rightarrow T_{CM}$ Conversion Rate

We next determined if the differentiation from T_{EM} to T_{CM} was affected by the magnitude of the infection and the duration of antigenic stimulation *in vivo*. We used conditions of low dose (LD) and high dose (HD) immunization that resulted in relatively short (2 to 3 days) or more prolonged (at least 5 to 7 days) exposure to antigen. After infection with a LD (500 colony-forming units (c.f.u.)) of LMgp33, antigen can be detected for only 48 to 72 hours (Kaech and Ahmed, 2001). In contrast, following HD (3×10^4 c.f.u.) LMgp33 or LCMV infection, antigen can be detected for at least 5 to 7 days (Kaech and Ahmed, 2001) (unpublished observations). P14 chimeric mice were immunized with either a LD or HD of LMgp33, or with an alternative HD immunization, with 2×10^5 p.f.u. LCMV (Armstrong). The rate of reversion of gp33-specific T cells from $CD62L^{lo}$ to $CD62L^{hi}$ was monitored in the PBMC of individual mice over time (Figure 3.6, (a)). The reversion from T_{EM} to T_{CM} occurred much more rapidly in LD immunized mice compared with the HD immunized group. To investigate whether this property of reversion was programmed during the phase of initial T cell priming or was a result of persisting antigen or the environment, mice containing $Thy1.2^+$ P14 cells were immunized with LCMV (HD) and a separate group of mice containing $Thy1.1^+$ P14 cells was immunized with LD LMgp33 (Figure 3.6, (b)). On day 8 post infection, effector CD8 T cells were purified from each group, mixed and adoptively transferred to naïve recipients. If a low amount of persisting antigen in HD infected mice or the environment was responsible for the slower conversion after HD infection, then after mixing the LD and HD primed effector CD8 T cells and transferring them to a new naïve recipient, the two cell populations should both revert at the same rate. If, however, the $T_{EM} \rightarrow T_{CM}$ conversion was programmed during the period of initial T cell priming, then the rate of reversion of HD- and LD-primed T cells parked in the same recipient should remain as observed in the original mice. The results of our experiment were consistent with this latter model. The conversion rate of the LD- and HD-primed cells in the mixed recipients was nearly identical to that observed in the original mice; that is, the HD effectors still reverted slowly and the LD effectors reverted faster (Figure 3.6, (b)).

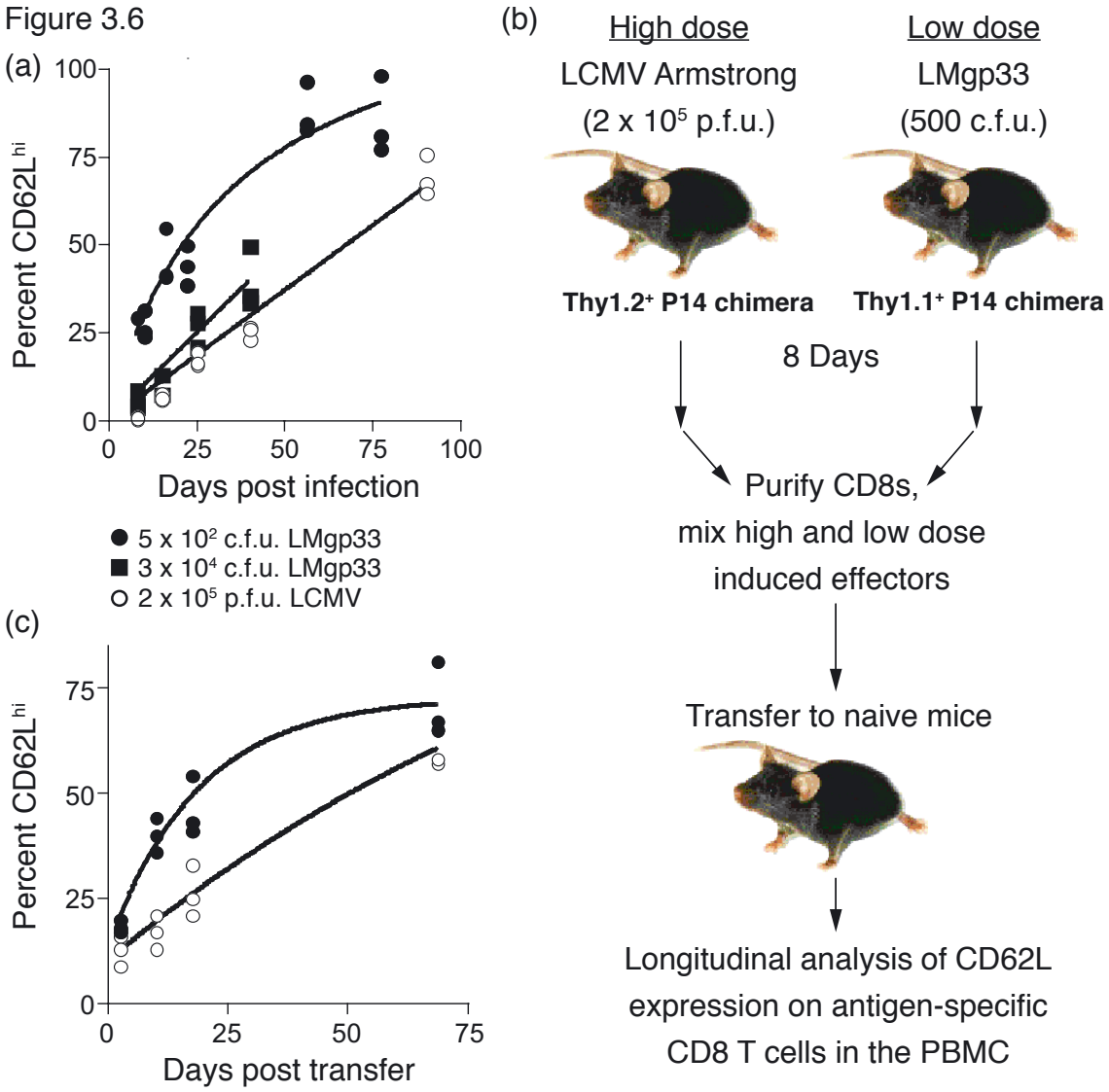


Figure 3.6: The Effect of High Dose *versus* Low Dose Infection on the Duration of T_{EM} to T_{CM} Conversion.

(a) P14 chimeras were infected with a low dose (500 c.f.u.) or high dose (3 x 10^4 c.f.u.) of LMgp33 or with LCMV and the percentage of gp33-specific CD8 T cells (P14 cells) that were $CD62L^{hi}$ or $CD62L^{lo}$ in the blood was determined longitudinally in individual mice. (b) Naïve Thy1.1$^+$ P14 chimeras were infected with low dose LMgp33 and separate naïve Thy1.2$^+$ P14 chimeras were infected with LCMV. After 8 days post infection spleens were harvested, CD8 T cells column purified (both > 96% pure), and CD8 T cell populations from LMgp33 and LCMV infected mice were mixed and transferred into the same recipients. (c) Reexpression of CD62L was monitored on LD LMgp33-induced (Thy1.1$^+$) and HD LCMV-induced (Thy1.2$^+$) P14 cells parked in the same mice. P14 cells in the PBMC were analyzed over time. Data are representative of 4 independent experiments.

Figure 3.7

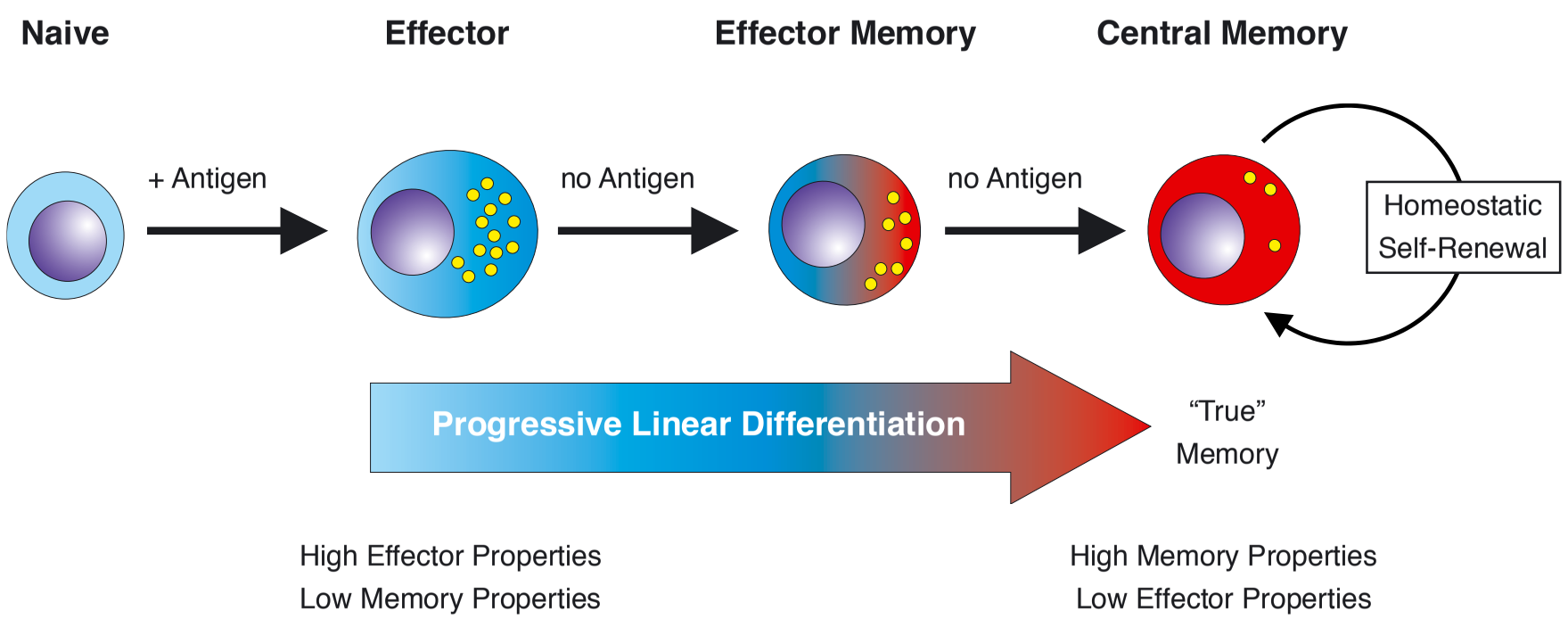


Figure 3.7: Progressive Linear Differentiation of T Cell Memory.

T cell memory formation is the result of a programmed, progressive linear differentiation (N → E → T_{EM} → T_{CM}). Naive T cells (N) encounter antigen, which triggers developmental programs. They acquire CTL functions, become effector T cells (E) and resolve the infection. In the absence of antigen, effector T cells progress further through differentiation toward effector memory (T_{EM}). Effector memory represents an intermediate developmental stage. Successful differentiation of T_{EM} results in the formation of Central Memory (T_{CM}).

Discussion

There has been considerable interest in understanding the developmental pathways of memory T cells (Hamann *et al.*, 1997; Jacob and Baltimore, 1999; Kaech and Ahmed, 2001; Kaech *et al.*, 2002b; Lauvau *et al.*, 2001; Manjunath *et al.*, 2001; Opferman *et al.*, 1999; Sallusto and Lanzavecchia, 2001; Tomiyama *et al.*, 2002; van Stipdonk *et al.*, 2001; Wong and Pamer, 2001). The results of our study now allow us to propose a model of T cell differentiation that incorporates the recently defined memory T cell subsets (Sallusto *et al.*, 1999). The essence of this model is that T_{EM} are a transitory population representing an "intermediate" cell type in the effector to memory transition. Thus, according to this model T_{CM} and T_{EM} cells are not distinct subsets but part of a continuum that ends with the development of T_{CM} cells. T_{CM} are the "true" memory cells because it is only this population that exhibits both of the two hallmark characteristics of memory T cells: long-term persistence *in vivo* by self-renewal and the ability to rapidly expand upon reencounter with pathogen. This model also predicts that memory development is a gradual process and that memory cells only develop several weeks after clearance of the acute infection. Our recent data analyzing global gene expression patterns during memory T cell development are consistent with this model of progressive differentiation (Kaech *et al.*, 2002a and see Chapter 4). Our study also shows that the rate at which a T cell population converts from $T_{EM} \rightarrow T_{CM}$ can vary depending upon the nature of the immunization (high antigen dose *versus* low antigen dose), and that this conversion rate is programmed during the initial period of encounter with antigen *in vivo*. A strong initial antigenic stimulus imprinted a $T_{EM} \rightarrow T_{CM}$ differentiation program that occurred over several months, whereas a lower amount of priming antigen resulted in more rapid differentiation of T_{EM} to T_{CM}. Thus, the duration of $T_{EM} \rightarrow T_{CM}$ conversion is not constant, but is imprinted during effector generation and varies depending on the magnitude of the initial stimulation.

Our results demonstrate that both, T_{CM} and T_{EM} can rapidly elicit effector functions *in vitro* and can both become effectors *in vivo* following reinfection. However, our results also demonstrate that T_{CM} convert to effectors and subsequently to T_{EM} only in the presence of antigen. We found no evidence for T_{CM} converting to T_{EM} in the absence of antigen – even in non-lymphoid tissues after adoptive transfer. While this does not formally exclude that in some locations (such as the intestinal mucosa) or in response to some cytokines an antigen-independent $T_{CM} \rightarrow T_{EM}$ reversion may occur, our results suggest that this reversion back to effectors or T_{EM} is primarily an antigen-driven process.

Many characteristics of T cells change as they differentiate from naïve cells to effectors and subsequently to memory cells (Kaech *et al.*, 2002a). Our results demonstrate

that this differentiation process continues long after infection has been resolved as T_{EM} convert to T_{CM}. During this $T_{EM} \rightarrow T_{CM}$ conversion memory T cells gradually acquire the ability to undergo efficient homeostatic turnover and to rapidly respond to antigen, and gain the ability to produce IL-2. In contrast, effector-like qualities such as granzyme B expression and the ability to rapidly kill *ex vivo* are lost during the $E \rightarrow T_{EM} \rightarrow T_{CM}$ transition. During this differentiation process hallmark phenotypic changes also occur, some of which (such as CD62L and CCR7 reexpression) affect homing and recirculation properties (Arbones *et al.*, 1994; Bradley *et al.*, 1994; Campbell *et al.*, 2001; Kunkel and Butcher, 2002; Weninger *et al.*, 2001). Effector cells have an increased capacity to migrate to non-lymphoid tissues, but do not home to LN following adoptive transfer (Iezzi *et al.*, 2001; Weninger *et al.*, 2001). During the transition from effector to memory cells, the ability to migrate to non-lymphoid sites is reduced, though it is still much greater than for naïve T cells, but these cells regain the ability to enter LN, a property mainly of T_{CM}. It is likely that the various functional and phenotypic characteristics that change during the $E \rightarrow T_{EM} \rightarrow T_{CM}$ transition do so at different rates. For example, *ex vivo* lytic activity and granzyme B expression are lost before conversion from $CD62L^{lo}$ to $CD62L^{hi}$, whereas the acquisition of the ability to produce IL-2 is tightly coupled with the $CD62L^{hi}$ phenotype. A key component of this $N \rightarrow E \rightarrow T_{EM} \rightarrow T_{CM}$ model of progressive differentiation (Figure 3.7) is that these qualitative changes occur gradually as the memory population converts to T_{CM}. The ultimate outcome is the formation of a memory population with all of the characteristic properties of a self-renewing, antigen-responsive T_{CM}.

Several recent studies have proposed lineage relationships between memory T cell subsets based on the phenotypic analysis of T cells from human PBMC and analyses of T cells restimulated *in vitro* (Appay *et al.*, 2002; Campbell *et al.*, 2001; Sallusto and Lanzavecchia, 2001; Sallusto *et al.*, 1999; Tomiyama *et al.*, 2002; Wills *et al.*, 2002). Although these reports provide valuable information characterizing human T cell populations and on the antigen-driven conversion of memory T cells into effectors, it is difficult to draw conclusions about the differentiation of memory T cells *in vivo* from such experiments. Our study has two advantages over these approaches. First, the timing and duration of antigen exposure is known. After acute LCMV or LM infection antigen, is eliminated in approximately one week (Harrington *et al.*, 2000; Kaech and Ahmed, 2001) and the memory T cells examined several months later have been differentiating in the absence of antigen for a defined period. In the studies analyzing human T cell responses to persisting viruses such as EBV, CMV or HIV, the frequency and amount of stimulation with antigen can vary considerably. Not only do these viruses vary substantially in their level of viral load, but there can also be considerable variation among different individu-

als. Without precise information about antigen levels, it is difficult to determine whether the memory T cells being analyzed are going through an antigen-independent process of $E \rightarrow T_{EM} \rightarrow T_{CM}$ differentiation that is likely to occur after acute infections, or through an antigen-driven $T_{CM} \rightarrow E$, or $T_{EM} \rightarrow E$ activation of memory T cells. Thus, T cell populations in the PBMC specific for persisting viruses may contain one population of T cells that has not encountered antigen for several days or weeks and another that has been recently exposed to antigen, resulting in a mixture of recently generated effector cells and T_{EM} and T_{CM} cells. The second advantage of our study is that the differentiation of a labeled (Thy1.1$^+$ and/or CFSE-labeled) memory CD8 T cell population was tracked *in vivo*. Such longitudinal studies are essential for defining lineage relationships between different cell populations. Using this approach, the lineage relationship between T_{EM} and T_{CM} *in vivo* was directly demonstrated in our studies. In contrast to previous proposals based on *in vitro* studies (Sallusto and Lanzavecchia, 2001; Sallusto *et al*., 1999; Wills *et al*., 2002), our results demonstrate that the T_{EM} subset is not continually replenished from T_{CM} in the absence of antigen, but rather that T_{CM} cells undergo this differentiation primarily as a result of reencounter with antigen.

Thus, the findings of our study and the proposed model of linear differentiation ($N \rightarrow E \rightarrow T_{EM} \rightarrow T_{CM}$, Figure 3.7) are likely to provide the paradigm for acute infections. We propose that this will be the natural course of memory T cell differentiation in the absence of antigen. It is possible, however, that under certain conditions (Lauvau *et al*., 2001; Manjunath *et al*., 2001), especially chronic infections where antigen persists at high amounts (Appay *et al*., 2002; Champagne *et al*., 2001), one may see a different pattern of memory T cell differentiation. Perhaps in these circumstances T cells are caught in a cycle of transition between effector cells and T_{EM}. This could ultimately lead to terminal differentiation as has been proposed (Champagne *et al*., 2001) or exhaustion and/or deletion (Moskophidis *et al*., 1993; Zajac *et al*., 1998).

One of the findings of this study was that, on a per cell basis, T_{CM} controlled systemic and even peripheral or mucosal challenge infections substantially better then T_{EM}. The enhanced protection by T_{CM} cells did not correlate with a difference in effector functions, as both T_{CM} and T_{EM} (defined both, phenotypically and anatomically) produced the effector cytokines IFN-γ and TNF-α rapidly and also quickly acquired CTL function upon reexposure to antigen. Rather, more effective protective immunity by T_{CM} was likely a result of greater expansion of this subset after infection. This greater expansion is at least in part due to an inherent difference in proliferative capacity of T_{CM} and T_{EM}. This may be related to IL-2 production by T_{CM} or could represent additional developmental changes that favor rapid proliferation. Localization of T_{CM} to LN *in vivo* may provide an additional advantage to this subset because dendritic cells efficiently drain from infected

sites to LN (Banchereau and Steinman, 1998) and *in vivo* T cell responses seem to be initiated in draining LN rather than at the site of primary infection – even in the presence of tissue resident memory T cells (Mueller *et al.*, 2002; Norbury *et al.*, 2002; Ostler *et al.*, 2001). The proliferative advantage of T_{CM} is therefore likely the result of a combination of intrinsic differences in proliferative potential and more efficient antigen presentation *in vivo*.

It should be noted that in all of the challenge models tested in this study, proliferation of antigen-specific CD8 T cell was critical to efficient pathogen control. In some circumstances, for example after a low dose infection in which pathogen control can be achieved without substantial expansion of antigen-specific memory T cells, T_{EM} cells present at the site of initial infection may be more efficient than T_{CM} cells in protective immunity. However, in many instances of protective recall responses, such as vaccine-induced memory T cells responding to a virulent infection, substantial clonal expansion of memory T cells is often necessary for efficient pathogen control. Thus, the increased proliferative potential is likely to be one of the most important properties acquired as T_{EM} differentiate into T_{CM}.

The findings of this study have implications for vaccination especially in terms of determining the optimal time for boosting. Because T_{CM} cells proliferate substantially better than T_{EM} cells upon reexposure to antigen, our results suggest that one should wait until a sufficient number of memory cells have acquired the T_{CM} phenotype before giving the booster immunization. Also, the optimal time interval between the first and second immunization is likely to vary depending upon the strength of the primary vaccination. Based on our results, we would predict that stronger vaccines will require a longer interval between the "prime" and "boost" than weaker vaccines. A kinetic analysis of the rate of $T_{EM} \rightarrow T_{CM}$ conversion in the blood after vaccination may allow one to design optimal boosting regimens tailored for individual T cell vaccines. Such an approach would be particularly useful for designing therapeutic vaccination of HIV-infected individuals on antiretroviral therapy. Consistent with this idea, the proliferative capacity of HIV-specific CD8 T cells is significantly greater in long-term non-progressors who maintain HIV control than in other HIV^+ individuals (Migueles *et al.*, 2002), suggesting that CD8 T cells with strong proliferative potential should be the goal of vaccination approaches.

Acknowledgements

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4

Conversion Rates of CD8 Memory T Cells

Conversion Rates of CD8 Memory T Cells

Recent evidence indicates that the two subsets of memory CD8 T cells, effector memory cells (T_{EM}) and central memory cells (T_{CM}), are related along a linear differentiation pathway. Upon antigen encounter, naïve T cells become activated and differentiate into effector T cells. Following antigen clearance most of these effectors die, but those that survive give rise to T_{EM} and in the continued absence of antigen, T_{EM} differentiate into T_{CM}. It is upon differentiation into T_{CM} that memory T cells acquire rapid responsiveness to antigen, the ability to produce IL-2, and the ability to undergo homeostatic turnover for long-term maintenance and persistence of memory. We have previously demonstrated that the rate of conversion from T_{EM} to T_{CM} within the memory pool is programmed during the first week after immunization. In the present study we examined the rate of reversion from T_{EM} to T_{CM} for several different epitope-specific T cell populations induced during the same infection. In addition, we compared the reversion rates of these different virus-specific CD8 T cell populations in multiple tissues. Our findings demonstrate that memory conversion is a distinct function of time and that the rate at which this differentiation occurs after resolution of infection, is characteristic for each single epitope-specific T cell population. We further show that memory conversion occurs in all tissues and that the conversion rates are different for each location. Moreover, our study demonstrates that conversion occurs at similar rates after different types of infection.

Introduction

The successful clearance of infectious pathogens by the host's immune system results in the formation of immunological memory (Ahmed and Gray, 1996). Memory formation is also the goal of vaccination strategies because memory cells are the mediators of protective immunity against infection and disease (Ahmed and Gray, 1996). Depending on the nature of the infectious or immunizing agent, the immune response is either a balanced interplay of B cells, CD4 and CD8 T cells, or is dominated by one these populations (Abbas *et al.*, 2000; Ahmed and Biron, 1999). For example, some viruses characteristically induce a strong CD8 T cell response upon infection and thus result primarily in the formation of a long-lived CD8 T cell memory (Ahmed and Biron, 1999; Doherty *et al.*, 1994). During such a primary infection, virus-specific naïve CD8 T cells become activated, differentiate into effector cells and undergo a phase of massive clonal expansion. Even though the vast majority of these activated effector CD8 T cells dies from apoptosis following the peak of the response, this contraction results in the formation of a stable memory population and virus-specific memory CD8 T cells can be found at increased numbers in the immune host (Murali-Krishna *et al.*, 1998). For example, after infection of mice with lymphocytic choriomeningitis virus (LCMV) there is up to a 1000-fold increase in the precursor frequency of virus-specific CD8 T cells (Blattman *et al.*, 2002; Murali-Krishna *et al.*, 1998). During a recall response, when they reencounter specific antigen, memory CD8 T cells proliferate vigorously, secrete antiviral cytokines, and kill virus-infected cells more rapidly than naïve CD8 T cells, and thus mediate efficiently protection from reinfection and disease (Bachmann *et al.*, 1999; Harrington *et al.*, 2000; Kaech and Ahmed, 2001; Veiga-Fernandes *et al.*, 2000; Zimmermann *et al.*, 1999). Thus, it is this combination of increased numbers and faster responsiveness that forms the cellular basis of long-term T cell immunity.

It has been shown that memory T cell populations are heterogeneous, both in humans (Sallusto *et al.*, 1999) and in mice (Chapter 3). Memory CD8 T cells can be divided into two subsets, effector memory (T_{EM}) and central memory (T_{CM}), based on the expression of the lymph node homing receptors CCR7 and CD62L (Sallusto *et al.*, 1999). T_{CM} express high levels of CCR7 and CD62L ($CCR7^{+}CD62L^{hi}$) which allow efficient homing to lymph nodes, whereas T_{EM} lack expression of these lymph node homing receptors ($CCR7^{-}CD62L^{lo}$) and are located in blood, spleen and non-lymphoid tissues (Chapter 3), (Sallusto *et al.*, 1999). Besides the different phenotype and migration pattern, these subsets are further distinguished by their ability to persist and to confer protective immunity. T_{CM} have a greater capacity than T_{EM} to persist *in vivo* and are more efficient in mediating protective immunity because of their increased proliferative potential (Chapter 3). More-

over, the proportion of each of these subsets within a memory population is not constant. We have demonstrated that despite functional and phenotypical differences, T_{EM} and T_{CM} are developmentally linked (Chapter 3). T_{EM} resemble an intermediate stage and directly convert to T_{CM} over time after the antigen has been cleared. We showed previously that this differentiation is programmed within the first week after immunization and is dependent on the magnitude of infection (high dose *versus* low dose immunization) (Chapter 3). These findings suggest antigen load as a major trigger of the developmental program but the strength of antigenic signal, provided costimulation and cytokines, as well as the type of infection may influence the rate at which conversion from T_{EM} to T_{CM} occurs.

To assess the involvement of these latter factors in programming memory differentiation, we used the model of acute infection of mice with lymphocytic choriomeningitis virus (LCMV) or the intracellular bacterium *Listeria monocytogenes* (LM) (Chapter 3). We examined in the present study the rate of reversion from T_{EM} to T_{CM} for several different (D^b-np396, D^b-gp33, D^b-gp276) epitope-specific T cell populations induced during the same infection. Our findings demonstrate that conversion is a distinct function of time and that the rate at which this differentiation occurs after resolution of infection is characteristic for each single epitope-specific T cell population.

T_{EM} traffic preferentially to non-lymphoid tissues and it is not clear whether they become resident and persist or recirculate to those tissues (Masopust *et al.*, 2001; Sallusto *et al.*, 1999; Weninger *et al.*, 2001). Our preceeding findings showed that after the clearance of infection both T_{EM} and T_{CM} are present in peripheral as well as in lymphoid tissues. Therefore, we further investigated whether conversion of virus-specific CD8 T cells occurs in multiple lymphoid and non-lymphoid tissues and if so, how conversion rates compared between these tissues. Indeed, our results indicate that conversion occurs in all tissues and that conversion rates are different for each location. T_{EM} convert much faster to T_{CM} in lymphoid tissues than in peripheral tissues. Moreover, to assess the role of microenvironmental factors on CD8 T cell subsets, we used a transgenic mouse model and investigated the influence of different inflammatory conditions on memory differentiation *in vivo*. Our study demonstrates that memory conversion occurs at similar rates after viral (LCMV) and bacterial (LMgp33) infection, and thus is not altered by different types of inflammation.

Results

CD8 Memory T Cell Subsets

After the resolution of an acute LCMV infection in mice, virus-specific CD62L^{lo} and CD62L^{hi} CD8 T cell populations are found within the host, representing the two memory subsets of T_{EM} (CD62L^{lo}) and T_{CM} (CD62L^{hi}) (Figure 4.1, (a); Figure 3.2).

$T_{EM} \rightarrow T_{CM}$ Conversion of Different Antigen-specific T Cell Populations

We have previously demonstrated that D^{b}-gp33-specific memory T cells convert from T_{EM} to T_{CM} over time (Chapter 3). To examine how the rate of $T_{EM} \rightarrow T_{CM}$ conversion compared for different epitope-specific CD8 T cell populations induced during the same infection, we tracked the reversion of CD62L^{lo} T_{EM} to CD62L^{hi} T_{CM} for three LCMV-specific CD8 T cell populations longitudinally in the blood.

Using wild-type B6 mice, we tracked CD62L expression levels on CD8 T cells specific for the immunodominant epitopes of LCMV, D^{b}-np396, D^{b}-gp33, and D^{b}-gp276 (Figure 4.1, (b)). Before infection (day 0), all naïve CD8 T cells (CD44^{lo}) were CD62L^{hi}. At the peak of expansion (day 8 after infection), 98 to 99% of LCMV-specific CD8 T cells (CD44^{hi}) were CD62L^{lo}, resembling the phenotype of activated effector cells. Twenty-two days after infection, a small fraction (~ 10%) of the LCMV-specific CD8 T cells had converted to CD62L^{hi} T_{CM} and the proportion of T_{CM} further increased gradually over time. By day 95 post infection, more than half of the T_{EM} of the specific memory populations had become T_{CM}, but during these three months of $T_{EM} \rightarrow T_{CM}$ conversion differences between the three epitope-specific T cell populations became apparent. Among T cells specific for the dominant epitope of LCMV in B6 mice, D^{b}-np396, CD62L^{lo} T_{EM} represented still the major fraction even after four months. More than 50% of the D^{b}-np396-specific memory T cells were T_{EM} on day 135. In contrast, for D^{b}-gp33- and D^{b}-gp276-specific populations T_{CM} already had become the larger subset after 90 days post infection (Figure 4.1, (c)). Plotting the reversion rate for all three LCMV-specific populations over time revealed a clear difference (Figure 4.1, (c) lower right panel). We used the time need for 50% of the CD62L^{lo} T_{EM} populations to convert to CD62L^{hi} T_{CM} as an indicator of conversion rate. The D^{b}-gp33- and D^{b}-gp276-specific T cell population exceeded this threshold after 101 and 99 days respectively, whereas the D^{b}-np396-specific population required 152 days to reach equal partitioning of T_{EM} and T_{CM}.

To confirm that $T_{EM} \rightarrow T_{CM}$ conversion was not a phenomenon observed only in B6 mice, we infected Balb/c mice with LCMV and tracked CD62L reversion in the same fashion. L^{d}-np118-specific T cells in Balb/c mice follow similar conversion pattern, although the rate is slightly different compared to the B6 epitopes (Figure 4.1, (b) and data not shown).

$T_{EM} \rightarrow T_{CM}$ Conversion in Lymphoid and Non-lymphoid Tissues

We next investigated whether the conversion observed in the blood was reflected in other lymphoid tissues and also whether $T_{EM} \rightarrow T_{CM}$ reversion could occur in a non-lymphoid site which is known to contain chiefly T_{EM}, the liver.

To address this question, we infected B6 mice with LCMV and assessed CD62L conversion for the three epitope-specific T cell populations in lymphoid (spleen, lymph nodes, bone marrow) and non-lymphoid (liver) tissues over time after the clearance of antigen. Our findings demonstrate that $T_{EM} \rightarrow T_{CM}$ differentiation occurs for all three epitope-specific populations in all tissues that were investigated, lymphoid and non-lymphoid (Figure 4.2, (a)). Interestingly, we found that the conversion rate was different in each tissue. In the spleen, lymph nodes, and bone marrow, memory conversion was more rapid over the first 120 days compared to the liver (Figure 4.2, (b)). The fastest initial conversion rates were found in the lymph nodes, followed by bone marrow and then spleen. For example, 50% of the D^b-gp33-specific CD8 T cells became $CD62L^{hi}$ T_{CM} after 20 days in the lymph nodes and after 70 and 90 days in the bone marrow and spleen, respectively. The conversion rates of LCMV-specific CD8 T cells in the spleen closely resembled those observed in the blood (compare Figure 4.1, (b) and 4.2, (b) and data not shown). In the liver, T_{EM} conversion occurred slowly but constantly over time (Figure 4.2, (a)). Moreover, consistent with our previous findings from blood borne memory T cells, D^b-gp33- and D^b-gp276-specific T_{EM} always converted faster in the tissues than did their D^b-np396-specific counterparts. After approximately 500 days post infection, most of the LCMV-specific CD8 T cells found in the different tissues had become T_{CM}.

Together, our findings demonstrate that the $T_{EM} \rightarrow T_{CM}$ conversion phenomenon can be observed in all tissues and that conversion pattern are characteristically different, depending on tissue and antigen-specificity.

Figure 4.1 (a)

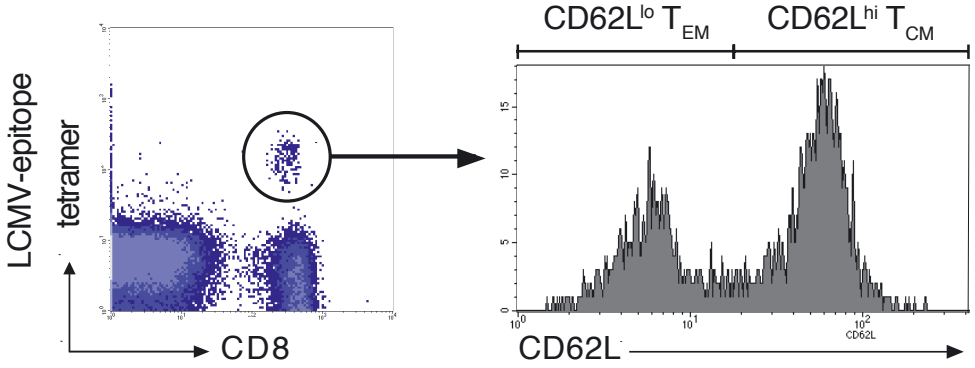


Figure 4.1: Memory Conversion of Different Antigen-specific T Cell Populations.
(a) In LCMV-immune mice, virus-specific $CD62L^{lo}$ and $CD62L^{hi}$ CD8 T cell populations are found, representing the two memory subsets of T_{EM} ($CD62L^{lo}$) and T_{CM} ($CD62L^{hi}$)

Figure 4.1 (b)

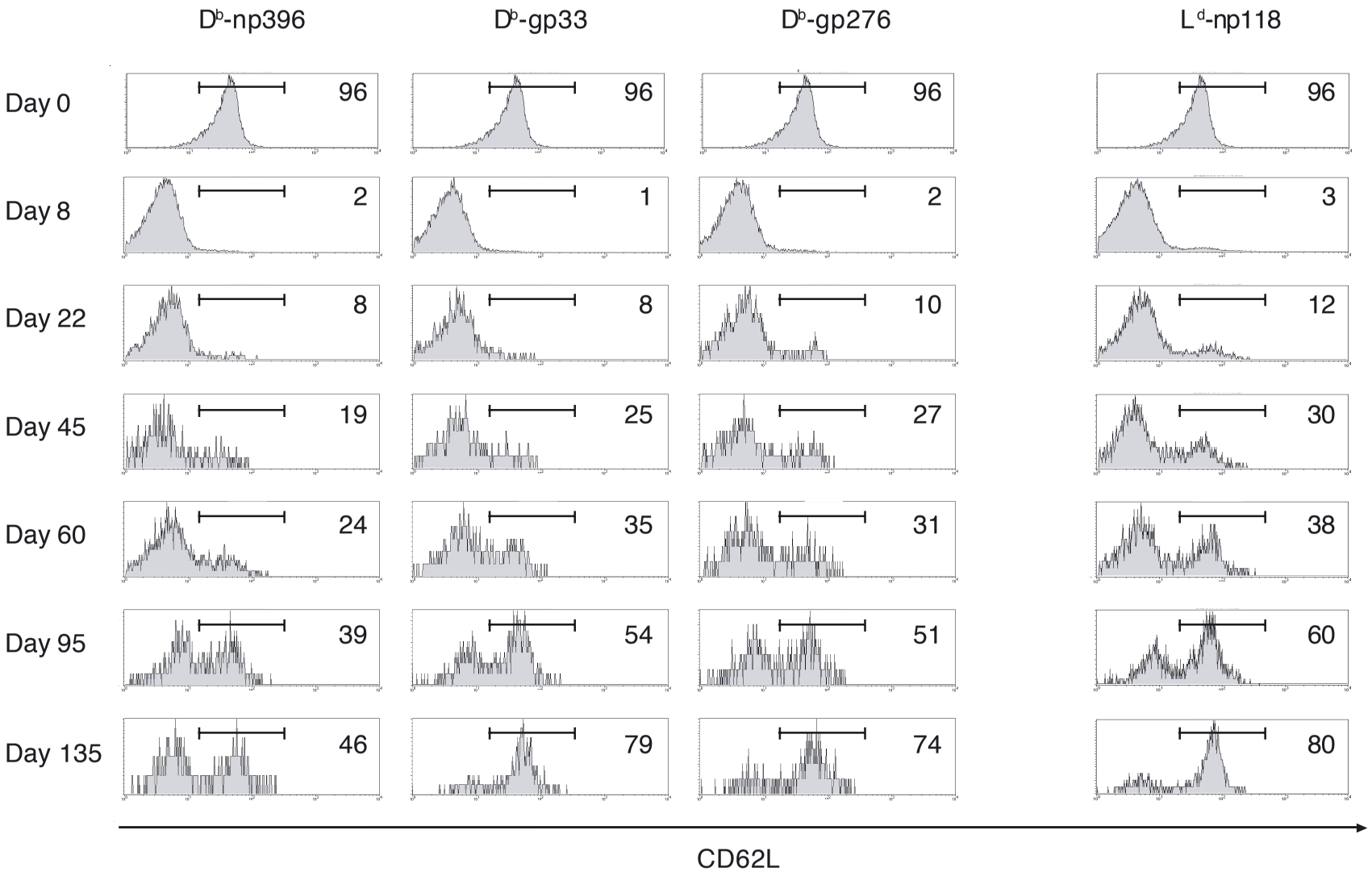

Db-np396
Db-gp33
Db-gp276
Ld-np118
Day 0
Day 8
Day 22
Day 45
Day 60
Day 95
Day 135
96
96
96
96
2
1
2
3
8
8
10
12
19
25
27
30
24
35
31
38
39
54
51
60
46
79
74
80
CD62L

Figure 4.1 (c)

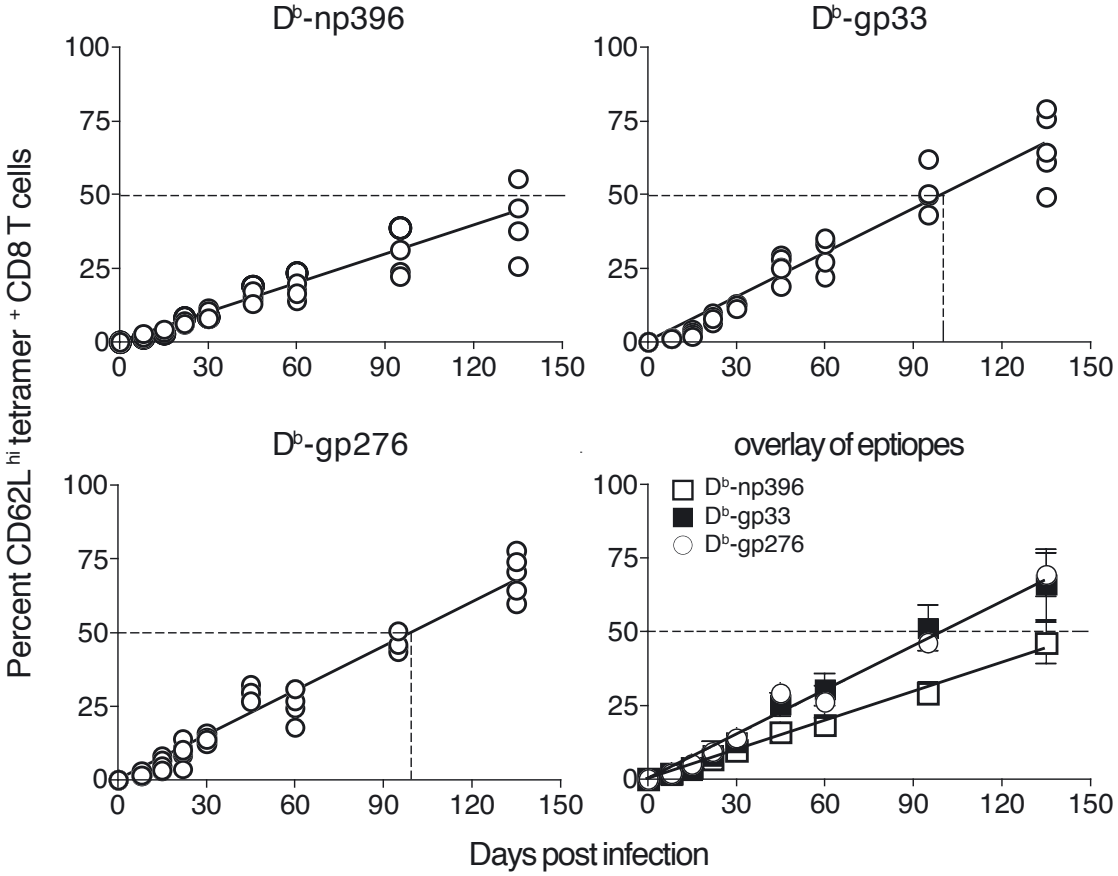


Figure 4.1 (d)

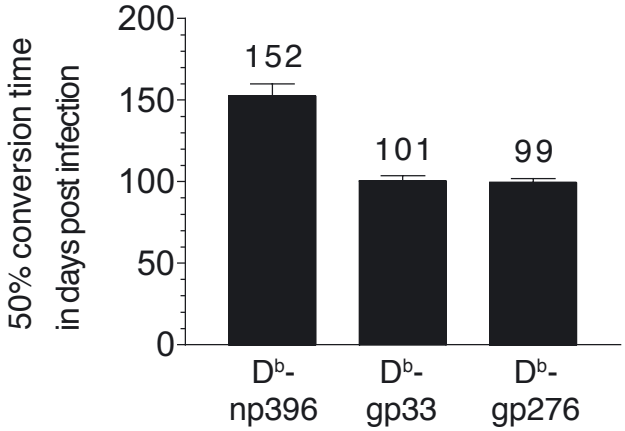


Figure 4.1: Memory Conversion of Different Antigen-specific T Cell Populations.
(b) (previous page) $T_{EM} \rightarrow T_{CM}$ conversion of CD8 T cell populations specific for D^b-np396, D^b-gp33, D^b-gp276 (all B6), and L^d-np118 (Balb/c) in the blood shown for one animal representative of a group of six. Shown numbers indicate percent of $CD62L^{hi}$ T cells. (c) Conversion rates of specific T cell populations in the blood of B6 mice (n=6). The overlay demonstrates the slow conversion rate of D^b-np396-specific CD8 T cells. (d) Time need to 50% conversion as indicator for $T_{EM} \rightarrow T_{CM}$ conversion rates (B6 mice, n=6).

Figure 4.2 (a)

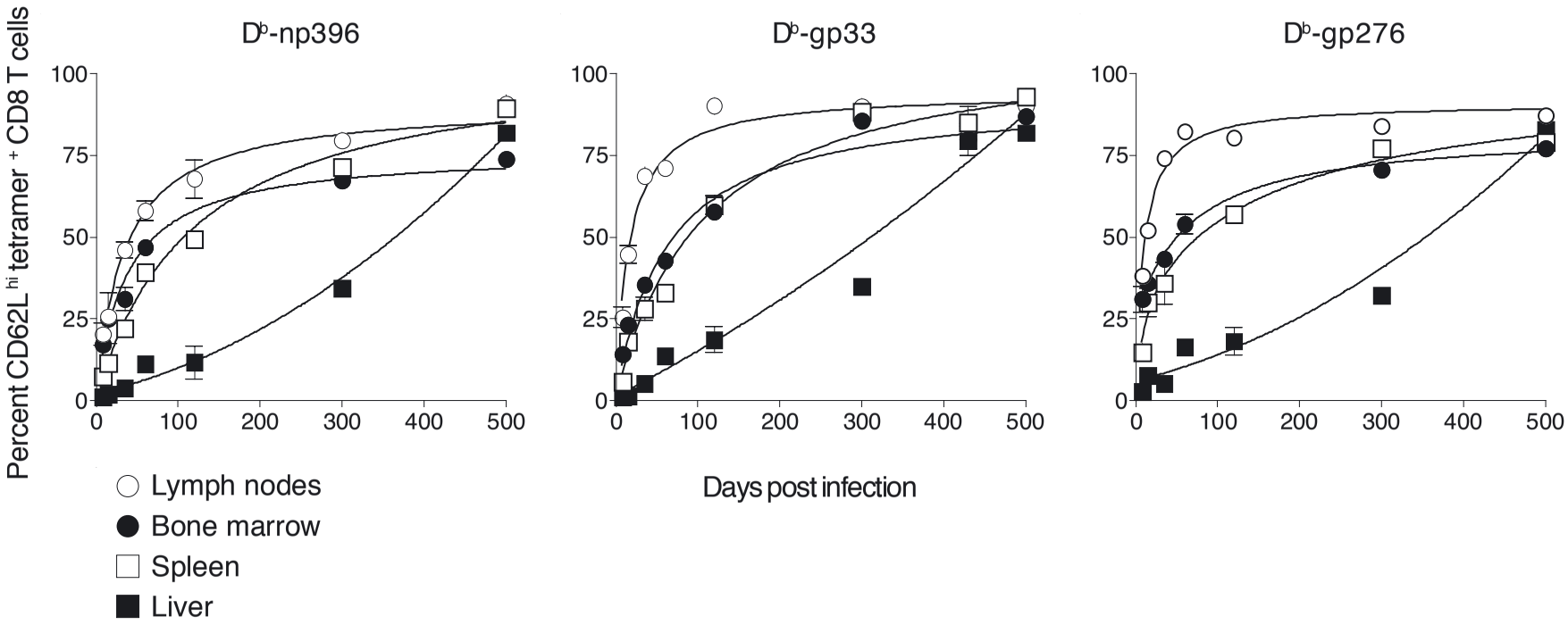
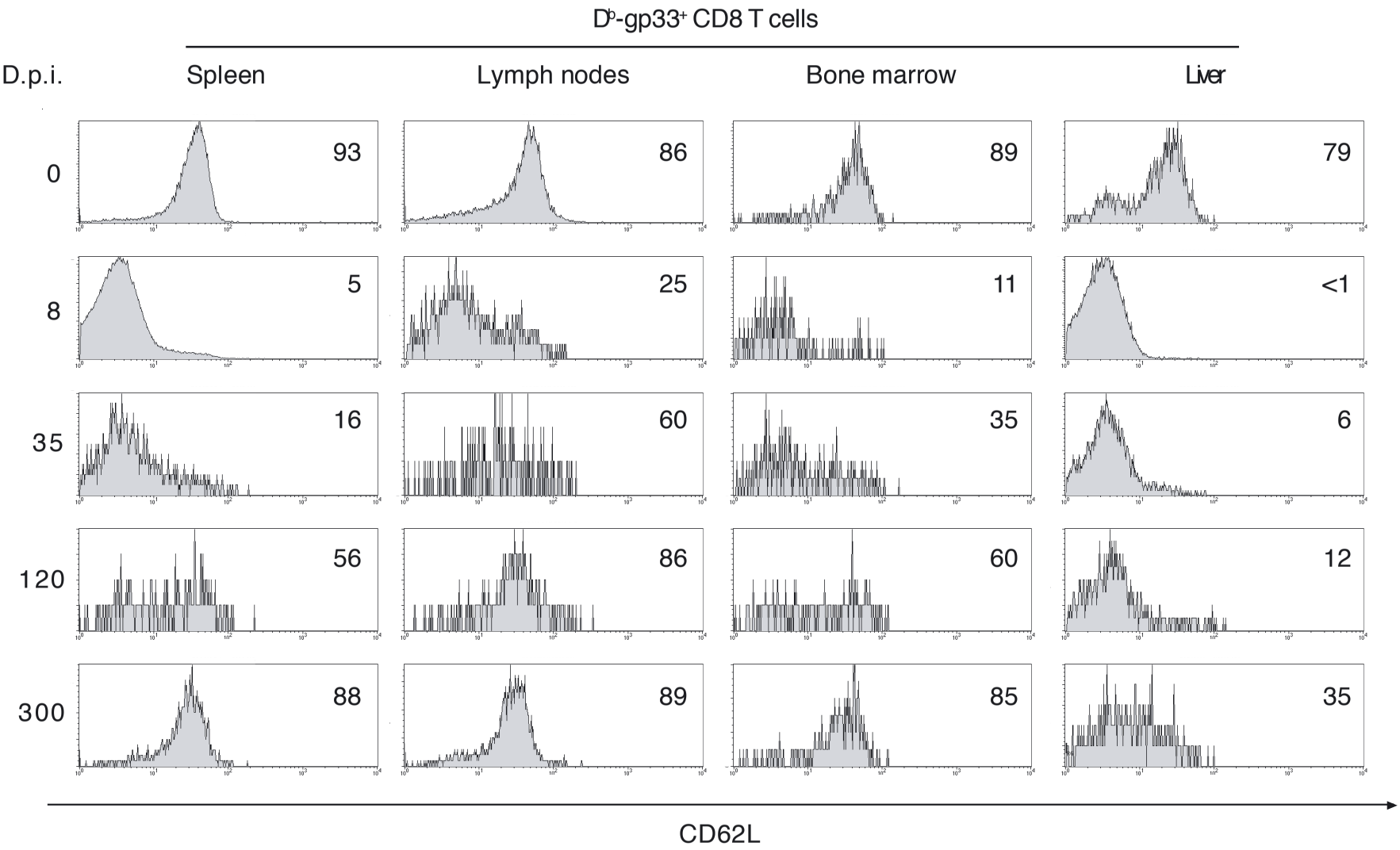


Figure 4.2: $T_{EM} \rightarrow T_{CM}$ Conversion in Lymphoid and Non-lymphoid Tissues.

(a) Memory conversion of D^b-np396-, D^b-gp33-, and D^b-gp276-specific CD8 T cells in lymphoid (lymph nodes, bone marrow, and spleen) and non-lymphoid tissues (liver). CD8 T cells were obtained seperately from the tissues of six individual B6 mice at each indicated time point (n=6; except for day 300 and 500, here n=2). (b) (next page) Comparison of $T_{EM} \rightarrow T_{CM}$ conversion of D^b-gp33-specific CD8 T cells in different tisssues. Numbers shown indicate percent of $CD62L^{hi}$ T cells found in the concerning tissue at the given time. CD62L levels in day 0 histograms (naive animals) are obtained by gating on $CD8^+CD44^{lo}$ cells.

Figure 4.2 (b)


Db-gp33+ CD8 T cells
D.p.i.
Spleen
Lymph nodes
Bone marrow
Liver
0
8
35
120
300
93
86
89
79
5
25
11
<1
16
60
35
6
56
86
60
12
88
89
85
35
CD62L

$T_{EM} \rightarrow T_{CM}$ Conversion after Viral and Bacterial Infection

To further address the question whether those preceedingly observed tissue-dependent conversion patterns were unique for LCMV infection, or whether infection with an other pathogen induces a diverse or comparable pattern of memory differentiation, we compared viral and bacterial infection. Using the previously introduced P14 transgenic system (Chapter 3), we infected one group of P14 chimeras with LCMV and additionally another group with a high dose (6×10^4 c.f.u. i.v.) of the recombinant strain of *Listeria monocytogenes*, which expresses the D^b-gp33 epitope of LCMV (LMgp33). Subsequently, $T_{EM} \rightarrow T_{CM}$ conversion in the tissues was examined as described beforehand.

Interestingly, we found that memory conversion occurred at almost equal rates in all tissues, independent of the type of infection (Figure 4.3). Moreover, the observed conversion pattern itself also closely resembled those of LCMV-infected wild-type animals. Thus, the programmed $T_{EM} \rightarrow T_{CM}$ conversion that we observed in the blood and the different tissues following LCMV infection also occurs following an infection with a distinct pathogen, the bacterium *Listeria monocytogenes*.

$T_{EM} \rightarrow T_{CM}$ Conversion Results in an Absolute Increase of T_{CM}

After the peak of the immune response to LCMV, 90 to 95% of the activated effector cells undergo apoptosis. Nevertheless, the end result is a net increase in the numbers of virus-specific CD8 T cells, which enter the memory pool and are maintained homeostatically in the absence of antigen at constant numbers (Ahmed and Gray, 1996; Becker *et al.*, 2002; Murali-Krishna *et al.*, 1998). This notion rises the question, whether the described $T_{EM} \rightarrow T_{CM}$ conversion, which results in a relative increase of the T_{CM} subset over time, may also change the absolute number of memory CD8 T cells.

To address this question, we looked at absolute memory CD8 T cell numbers in LCMV (Table 4.1) and LMgp33 (Table 4.2) infected P14 mice. Total numbers demonstrate that after viral and bacterial infection of P14 mice, the number of T_{CM} in all tissues increases constantly and absolutely over time. Consistently, T_{EM} decrease in absolute numbers and contribute less and less to the memory pool the farther time proceeds beyond clearance of infection (Figure 4.4 and data not shown). Our data demonstrate that $T_{EM} \rightarrow T_{CM}$ differentiation does not change the absolute number of the memory pool. Memory differentiation results in a relative and in an absolute increase of the central memory subset over time, which is accomplished at the expense of shrinking numbers of effector memory T cells.

Figure 4.3

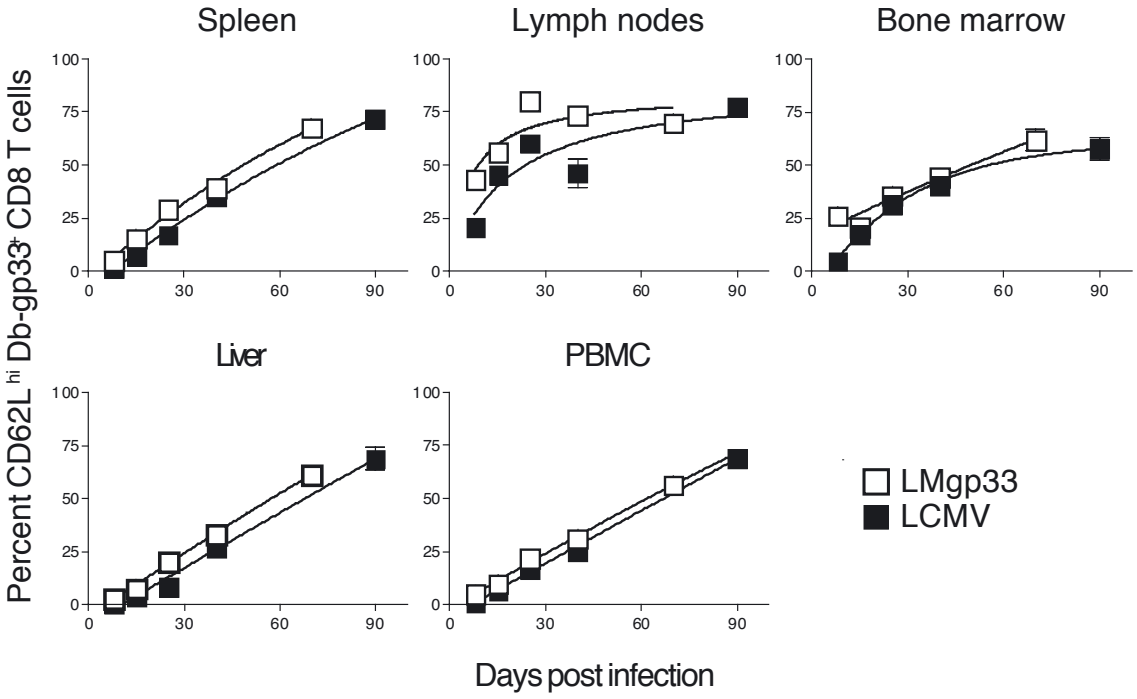


Figure 4.3: $T_{EM} \rightarrow T_{CM}$ Conversion after Viral and Bacterial Infection.
P14 chimeras were infected with either LCMV (2 x 10^5 p. f. u. intravenously, *n*=3) or with a high dose (6 x 10^4 c.f.u. intravenously, *n*=3) of a recombinant strain of *Listeria monocytogenes*, which expresses the D^b-gp33 epitope of LCMV (LMgp33). Subsequently, $T_{EM} \rightarrow T_{CM}$ conversion in the tissues was examined.

Figure 4.4

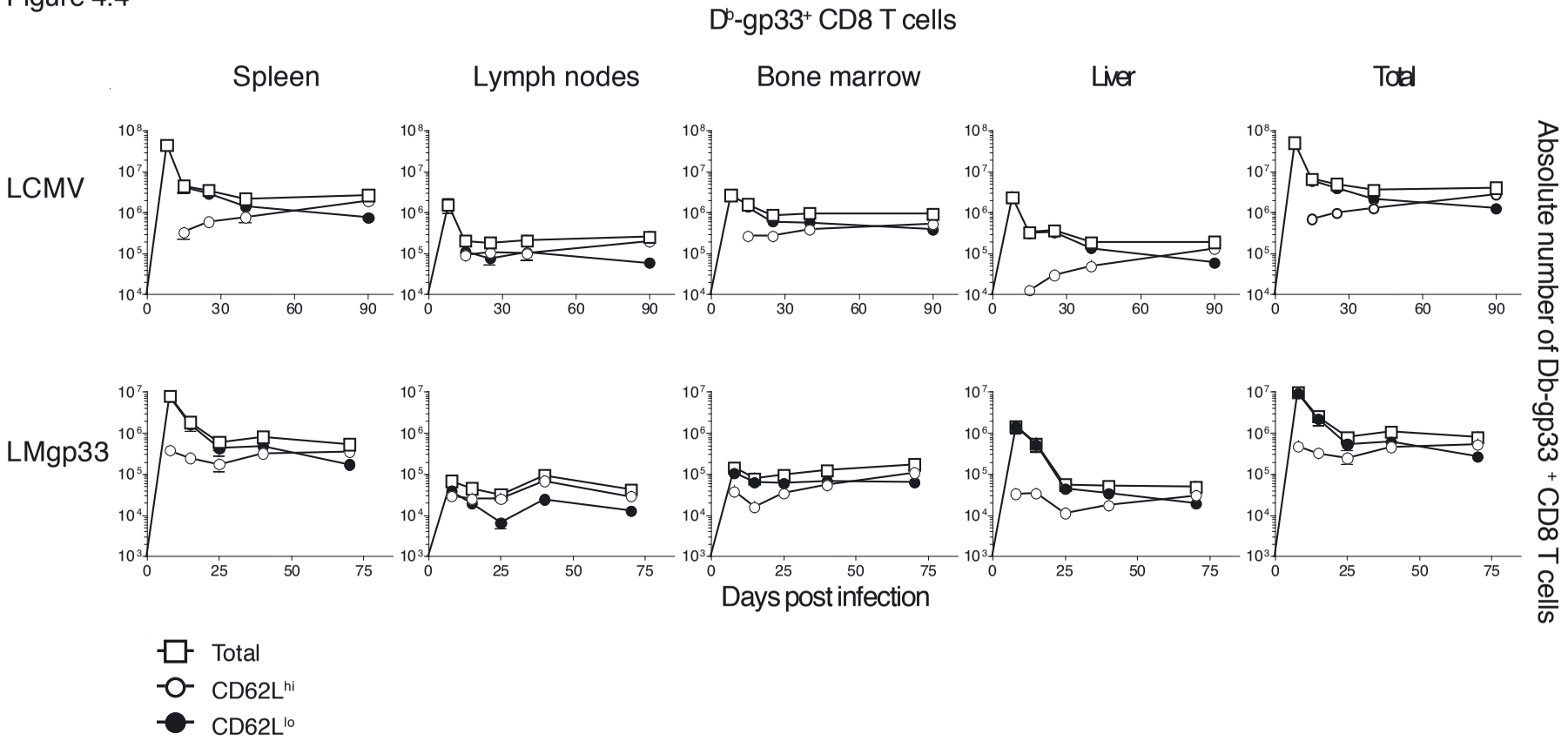


Figure 4.4: $T_{EM} \rightarrow T_{CM}$ Conversion Results in an Absolute Increase of T_{CM}.

D^b-gp33-specific CD8 T cells from P14 chimeras infected with either LCMV (*n*=3) or LMgp33 (*n*=3) were isolated from the tissues (spleen, lymph nodes, bone marrow, and liver). $CD62L^{lo}$ T_{EM} and $CD62L^{hi}$ T_{CM} were counted and absoulte numbers plotted over time. To obtain an estimate of the total number of memory T cells present during $T_{EM} \rightarrow T_{CM}$ conversion in an anmimal after resolved infection, absolute numbers from the single tissues were summed up (rightmost panel).

Table 4.1: Absolute Numbers of Epitope-specific CD8 T Cells afterViral Iinfection.

Total numbers[a] of D^b-gp33-specific T cells expressing CD62L after infection with LCMV

	Spleen		Bone Marrow[e]		Lymph Nodes[f]		Liver		Total		
d.p.i.[b]	hi[c]	lo[d]	hi	lo	hi	lo	hi	lo	hi	lo	hi + lo
8	68.5	4400.0	14.0	260.0	32.3	120.0	1.1	240.0	120	5020	5140
15	33.1	420.0	26.6	140.0	9.3	11.3	1.3	32.3	70	600	670
25	60.8	300.0	27.0	60.9	10.9	7.8	3.0	33.9	100	400	500
40	77.8	140.0	39.1	58.2	10.2	10.9	5.0	13.7	130	220	360
90	190.0	76.9	54.3	39.3	20.1	5.9	13.2	6.2	280	130	410

[a] total cell numbers : [10^4 cells]
[b] days post infection with LCMV
[c] $CD62L^{hi}$
[d] $CD62L^{lo}$
[e] calculated based on cell counts obtained from left and right femur
[f] inguinal, axillary, cervical and submandibulary lymph nodes

Table 4.2: Absolute Numbers of Epitope-specific CD8 T Cells after Bacterial Infection.

Total numbers[a] of D^b-gp33-specific T cells expressing CD62L after infection with LMgp33

	Spleen		Bone Marrow[e]		Lymph Nodes[f]		Liver		Total		
d.p.i.[b]	hi[c]	lo[d]	hi	lo	hi	lo	hi	lo	hi	lo	hi + lo
8	381.5	7800	38.2	107.1	29.6	29.6	33.6	1400	500	9400	9900
15	250.8	1600	16.0	63.2	25.7	25.7	34.4	528.7	300	2300	2600
25	175.9	428.5	34.8	60.9	25.7	25.7	11.3	45.0	260	540	800
40	322.5	489.6	54.8	68.8	69.0	69.0	17.6	34.7	400	600	1000
90	360.8	173.6	109.8	64.8	29.0	29.0	30.4	19.5	530	270	800

[a] total cell numbers : [10^3 cells]
[b] days post infection with LCMV
[c] $CD62L^{hi}$
[d] $CD62L^{lo}$
[e] calculated based on cell counts obtained from left and right femur
[f] inguinal, axillary, cervical and submandibulary lymph nodes

Discussion

The investigation of developmental pathways of memory T cells is of considerable interest because the generation of long-term CD8 T cell memory is an important goal of vaccination. It is critical to understand how T cell memory is formed in order to identify the signals and mechanisms that initiate and drive the differentiation of T cells into powerful memory cells, which can provide protection from infectious diseases or even cancer.

Thus, there has been considerable interest in deciphering the developmental pathways of memory T cells and many differentiation models have been proposed (Hamann *et al.*, 1997; Jacob and Baltimore, 1999; Kaech and Ahmed, 2001; Kaech *et al.*, 2002; Lauvau *et al.*, 2001; Manjunath *et al.*, 2001; Opferman *et al.*, 1999; Sallusto and Lanzavecchia, 2001; Tomiyama *et al.*, 2002; van Stipdonk *et al.*, 2001).

We have shown previously that memory T cell formation follows a linear differentiation pathway and proposed a model, which also incorporates the effector memory (T_{EM}) and central memory (T_{CM}) T cells subsets (Chapter 3), (Kaech and Ahmed, 2001). In this model, T_{EM} represent an intermediate stage in memory development, which gives directly rise to T_{CM}. Therefore, this model does not define T_{EM} and T_{CM} as independent subsets as do other models (Iezzi *et al.*, 2001; Lanzavecchia and Sallusto, 2000; Manjunath *et al.*, 1999; Sallusto *et al.*, 1999). Rather, T_{EM} and T_{CM} are part of a differentiation continuum, which ends with the formation of T_{CM}. Our model also implies that memory formation occurs gradually over time after the clearance of acute LCMV infection. Furthermore, we have shown before that the rate at which a specific T cell population converts from T_{EM} to T_{CM} can vary depending on the nature of the immunization and that this conversion is programmed during the initial period of encounter with antigen *in vivo* (Chapter 3). We found that high dose immunization resulted in slow $T_{EM} \rightarrow T_{CM}$ conversion over several month, whereas low dose infection triggered a more rapid $T_{EM} \rightarrow T_{CM}$ differentiation program. Thus, the rate at which $T_{EM} \rightarrow T_{CM}$ conversion occurs is not constant, but is imprinted during effector generation and varies depending on the magnitude of the initial stimulation (Chapter 3).

Although our results suggest a clear dependence of antigen amount and conversion rate, many other factors may influence the $T_{EM} \rightarrow T_{CM}$ differentiation program. On a per cell basis for example, the amount of signal induced by specific antigen could trigger different programs and may depend on:

1) the efficacy of antigen processing and epitope-presentation (Chen *et al.*, 2001; Gallimore *et al.*, 1998);
2) the affinity and avidity of MHC molecules for the immunogenic epitope (Wong and Pamer, 2003; Yewdell and Bennink, 1999);
3) the concentration/abundance of antigen–MHC complexes, which determines the rate of T cell receptor (TCR) triggering (Valitutti and Lanzavecchia, 1997);
4) the concentrations of costimulatory molecules, which determine the extent of signal amplification (Viola *et al.*, 1999);
5) the duration and frequency of the interaction between T cells and APC, which determines for how long the antigenic signal is engaged (Iezzi *et al.*, 1998; Lanzavecchia and Sallusto, 2002); and
6) the cytokines and other soluble factors produced by tissue cells and cells of the innate and specific immune system in response to a pathogen (Iezzi *et al.*,1999; Manjunath *et al.*, 2001; Richter *et al.*, 1999).

In the present study we examined whether CD8 T cell populations specific for the LCMV-epitopes D^b-np396, D^b-gp33 and D^b-gp276 induced during the same infection revert at identical rates or whether these different epitopes trigger different conversion programs. Indeed, our findings demonstrate that different epitope-specific T cell populations convert at different rates. Interestingly, memory CD8 T cells specific for the D^b-np396 epitope differentiated at a markedly slower rate into T_{CM} compared to D^b-gp33- and D^b-gp276-specific T cells, which converted at almost identical rates. It was recently shown that CD8 effector T cells more efficiently recognize the D^b-np396 epitope than the epitopes D^b-gp33 and D^b-gp276, although quantitation of the peptide epitopes on a per cell basis suggested a different hierarchy (Gallimore *et al.*, 1998). Finally, the MHC class I tetramer-aided reevaluation of the magnitude of specific CD8 T cell responses to acute LCMV infection revealed a new immunobiologically coherent hierarchy among the virus epitopes: the D^b-np396-specific immune response is dominant over the responses to the D^b-gp33 and D^b-gp276 epitopes (Murali-Krishna *et al.*, 1998). In addition, our group showed recently that this hierarchy is also maintained during the early phase in chronic LCMV infection (Wherry *et al.*, 2003). Intriguingly and in contrast to acute LCMV infection, the immune response to dominant viral epitopes here falls victim to selfprotective regulatory and escape mechanisms that result in deletion and exhaustion of specific T cells. This phenomenon provides an excellent explanation why the epitope hierarchy becomes screwed during the course of chronic infection (Wherry *et al.*, 2003). Together, our findings suggest that the described hierarchy among antigen-specific T cell

populations is inversely reflected in the $T_{EM} \rightarrow T_{CM}$ conversion rate of those cells, which means that the most immunodominant epitope triggers the slowest conversion program and *vice versa.*

We were further interested in the question whether $T_{EM} \rightarrow T_{CM}$ conversion takes place in locations and sites other than the blood. In preceeding experiments, we have demonstrated that after the resolution of acute LCMV infection both, T_{EM} and T_{CM} are present in lymphoid and peripheral/non-lymphoid tissues (Chapter 3). We found that $T_{EM} \rightarrow T_{CM}$ conversion occurs in all tissues and that the hierarchy in conversion rates observed among the three LCMV-specific T cell populations in the blood also applies for the different tissues: D^b-np396-specific T_{EM} reverted consistently slower to T_{CM} than did D^b-gp33- and D^b-gp276-specific T_{EM}. Interestingly, we found a separate hierarchy also existing among the different tissues: T_{EM} reverted the slowest in non-lymphoid tissues, here the liver, whereas conversion in lymphoid tissues occurred at much faster rates. Remarkably, within the lymphoid tissues, T_{EM} present in the lymph nodes differentiated more quickly into T_{CM} than those in the bone marrow and the spleen.

For the hierarchy of $T_{EM} \rightarrow T_{CM}$ conversion rates among the different tissues, the influence of the local microenvironment may play a modifying role on the conversion program since the virus is cleared from all tissues by day 8 to 9 post infection (Chapter 3 and data not show). The tissues are composed of different parenchymal and stromal cells, which may produce different sets of cytokines or other soluble factors. Unknown receptor–ligand interactions or other mechanisms between the memory T cells and tissue cells may also represent considerable modulators of the $T_{EM} \rightarrow T_{CM}$ differentiation program. We also need to take into consideration that redistribution processes may influence the pattern of memory conversion, because a switch in the phenotype accompanies $T_{EM} \rightarrow T_{CM}$ conversion: $CCR7^{-}CD62L^{lo}$ T_{EM} differentiate into $CCR7^{+}CD62L^{hi}$ T_{CM}. Both, CCR7 and CD62L mediate homing of T cells to the lymph nodes (reviewed in Chapter 3), and thus bias migration pattern of T_{CM} towards lymph nodes. This may contribute to the finding that $T_{EM} \rightarrow T_{CM}$ conversion occurs the fastest in lymph nodes, because circulating T_{CM} will preferentially home to this lymphoid tissue.

T_{EM} home preferentially to peripheral tissues (Chapter 3) and have been shown to persist for an extended period of time at these sites (Sallusto *et al.*, 1999; Reinhardt *et al.*, 2001; Masopust *et al.*, 2001). There, reencounter of antigen may occur first and thus local/ tissue resident T_{EM} can provide a potent first line of specific defense. On the other hand, T_{CM} home to the lymph nodes and the other lymphoid tissues where antigen might be more efficiently presented and where they can proliferate vigorously in response to antigen. Therefore, the finding that $T_{EM} \rightarrow T_{CM}$ conversion occurs very slowly in peripheral tissues is in concordance with this notion. Furthermore, it has been shown that CD8

T cells specific for the D^b-np396-epitope control LCMV infection more efficiently than D^b-gp33- and D^b-gp276-specific cells (Gallimore *et al.*, 1998). D^b-np396-specific T_{EM} therefore may represent a suitable population to protect the host locally at peripheral sites from small amounts of pathogen. Synergistically, T_{EM} in the periphery are secured and supported by some local T_{CM}, which may be additionally recruited into the response if antigen exposure exceeds the immediate protective capacity of T_{EM}.

In this study we also demonstrated that $T_{EM} \rightarrow T_{CM}$ conversion is not an unique phenomenon of specific T cells of B6 mice. Memory conversion is also found in different mouse strains. For example, L^d-np118-specifc CD8 T cells from BALB/c mice convert T_{EM} to T_{CM} a similar rates (Figure 4.1, (b) and data not shown). Furthermore, TCR-transgenic T cells from P14 mice execute a comparable differentiation program. Interestingly, our data show that P14 T_{EM} convert with slightly faster rates in all the tissues than their wild-type counterparts; but also in the P14 system, the hierarchy between the tissues is maintained as it is in wild-type animals. The accelerated conversion in the P14 chimera system might be due to more rapid clearance of antigen owing to an elevated precursor frequency of naïve D^b-gp33-specific CD8 T cells (approximately 7.5×10^3 to 1.0×10^4 in P14 *versus* 1.0×10^2 in B6) (Blattman *et al.*, 2002).

We applied these findings to examine whether $T_{EM} \rightarrow T_{CM}$ conversion depends on the type of infection and inflammation. We infected P14 chimeras with either LCMV or LMgp33 and assessed $T_{EM} \rightarrow T_{CM}$ conversion rates of CD8 T cells induced by a viral *versus* bacterial infection. Our results demonstrate that D^b-gp33-specific T_{EM} generated during LMgp33 infection convert in both lymphoid and non-lymphoid tissues to T_{CM} at almost identical rates as do LCMV primed T_{EM}. Thus, $T_{EM} \rightarrow T_{CM}$ differentiation appears to be independent to the type of infection and diverse inflammatory conditions. The distinct cytokine profiles that might be induced during viral and bacterial infection seem not to affect the nature of the triggered memory conversion program. The minimal difference observed in $T_{EM} \rightarrow T_{CM}$ conversion rates might rather be explained by small variances in the transferred and available amount of antigen during the initial phase of infection. Therefore, if the amount of antigen is equal during the initial phase, other priming factors such as cytokines or even costimulation may play a minor role in the induction of the conversion program.

We have shown that conversion rates are distinct for different LCMV-specific memory populations and that the rate of $T_{EM} \rightarrow T_{CM}$ conversion varies in different tissue. Furthermore, we demonstrated that these conversion patterns are independent of the type of infection that initiates the T cell differentiation. But importantly, our findings also demonstrate that under all conditions the proportion of T_{CM} within a specific memory population increases continuously over time following acute infection.

Although we found no evidence for T_{CM} converting to T_{EM} after the clearance of viral or bacterial infection (Chapter 3), we cannot exclude that in some locations such as the intestinal mucosa or in response to certain cytokines an antigen-independent $T_{CM} \rightarrow T_{EM}$ or even $T_{CM} \rightarrow$ E (effector) reversion may occur. However, since approximately after 500 days post infection greater than 90% (at day 120 ~70% $CD62L^{hi}$ equaled ~200,000 total T_{CM}) of all LCMV-specific memory T cells are T_{CM}, antigen-independent $T_{CM} \rightarrow T_{EM}$ conversion, if it occurs, is very likely minimal.

In summary, our findings further support the idea that $T_{EM} \rightarrow T_{CM}$ conversion is programmed during initial antigen encounter. Our results are consistent with the concept that the program is triggered mainly by the amount of antigen: Firstly, distinct inflammatory conditions induced by different pathogens, which may influence priming factors such as receptor-mediated costimulation and signal-modulation by cytokines, do not change $T_{EM} \rightarrow T_{CM}$ conversion rates. Secondly, the conversion rate inversely correlates with the immunodominance of an epitope-specific T cell population, and therefore additionally supports this notion. Moreover, conversion rates are different in lymphoid and non-lymphoid tissues. T_{EM} convert much faster to T_{CM} in lymphoid tissues than in the liver. Thus, T_{EM} can be found at peripheral sites for an extended period of time compared to lymphoid tissues. The different homing and recirculation properties of T_{EM} and T_{CM} might accentuate this observation. In this way, modulation and tuning of the programmed $T_{EM} \rightarrow T_{CM}$ conversion rates by antigen and tissue factors may represent a mechanism of the immune system to further optimize protective immunity conferred by different memory T cell populations.

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5

Molecular Profiling of Memory CD8 T Cell Subsets

Molecular Profiling of CD8 T Cell Subsets

Several recent studies have examined the molecular mechanisms involved in memory T cell development by examining the gene expression profile of those cells. The pattern of gene expression changes as T cells progress from the naïve stage through an effector stage toward memory cell development. The memory population is comprised of two subsets, effector memory T cells (T_{EM}) and central memory T cells (T_{CM}). Both T_{EM} and T_{CM} are endowed with distinct properties, but T_{CM} have a greater capacity to persist *in vivo* and are more efficient in mediating protective immunity. T_{EM} developmentally resemble an intermediate memory stage and directly give rise to the T_{CM} population. These subsets are phenotypically and functionally distinct, but whether this is due to transcriptional and/or post-transcriptional mechanisms is not clear. In this study, in order to better understand the emerged differences between T_{EM} and T_{CM}, we now examine genes that are differentially expressed in these subsets and attempt to correlate their molecular profiles with their distinct functions and memory properties. The two major differences between T_{EM} and T_{CM} are migration pattern and proliferative capacity. Here, using DNA microarray techniques both are also confirmed on a molecular basis and are strikingly mirrored in the expression profile of correlating genes.

Introduction

The encounter of antigen represents a frequent event for the immune system but always constitutes a great challenge as well: Upon infection with an unknown pathogen or after immunization with a new vaccine, the immune system is compelled to mount a sufficient response. Briefly, a successful response of the specific arm of the immune system, comprised of B and T cells, is characterized by the elimination of the antigen and the subsequent formation of immunological memory (Ahmed and Gray, 1996). Naïve T cells specific for a certain antigen exist at low frequencies in a host (Blattman *et al.*, 2002). The encounter of antigen activates those T cells and results in massive clonal expansion, acquisition of effector functions, elimination of the pathogen, and resolution of the infection. Subsequently, the effector population is markedly reduced during an apoptosis-mediated contraction phase to maintain T cell homeostasis but some T cells survive and persist at now higher frequencies as long-lived memory T cells, endowed with enhanced effector functions (Ahmed and Gray, 1996; Dutton *et al.*, 1998; Murali-Krishna *et al.*, 1998; Sprent and Surh, 2001; Sprent and Tough, 2001). In concert with B cell-mediated antibody responses, these memory T cells form the basis for protective immunity against reinfection and disease (Ahmed and Gray, 1996).

There is substantial evidence that T cells only require a brief initial exposure to antigen to trigger a unique developmental program that universally drives and regulates their differentiation into effector and memory T cells (Kaech and Ahmed, 2001; van Stipdonk *et al.*, 2001; Wong and Pamer, 2001). The course of this programmed differentiation could be modulated by additional stimuli such as costimulatory molecules and cytokines in order to accentuate certain functional characteristics and properties, but the key feature of this developmental program is that its execution occurs gradual over time and independent of any further antigenic stimulation (Kaech and Ahmed, 2001; van Stipdonk *et al.*, 2001; Wong and Pamer, 2001).

Recent studies indicate that one important factor that can affect the differentiation process and thus might regulate functional qualities of effector and memory T cells, is the strength and duration of initial antigen exposure (Gett *et al.*, 2003; Lanzavecchia and Sallusto, 2002). Indeed, we showed in our preceding work (Chapter 3) that upon T cell activation the amount of initial antigen exposure determines the rate at which single steps of the same differentiation program are executed, and therefore how fast an effector T cell achieves memory status: a strong initial antigenic stimulus imprinted a differentiation program that occurred over several months, whereas a lower amount of priming antigen resulted in a more rapid differentiation.

Recently, based on work with human peripheral blood mononuclear cells *in vitro*, two functionally distinct subsets of memory CD4 and CD8 T cells had been introduced (Sallusto *et al.*, 1999b). Effector memory T cells (T_{EM}) and central memory T cells (T_{CM})were identified based on the expression levels of the lymph node homing receptor molecules CCR7 and CD62L. $CCR7^{-}CD62L^{lo}$ T_{EM} were reported to be chiefly present in peripheral and non-lymphoid tissues and would rapidly respond to antigen by producing effector molecules, whereas $CCR7^{+}CD62L^{hi}$ T_{CM} would reside exclusively in the lymph nodes and lymphoid tissues and would inferiorly respond to antigen but were capable to replenish the T_{EM} pool in the periphery (Lanzavecchia and Sallusto, 2000; Sallusto *et al.*, 1999b). As a consequence of these distinct functions and phenotypes of the two memory T cell subsets, a divergent differentiation model was proposed, suggesting T_{EM} and T_{CM} as separate lineages that arise separately during the activation phase of the immune response.

However, we have previously shown in mice that $CCR7^{-}CD62L^{lo}$ T_{EM} and $CCR7^{+}CD62L^{hi}$ T_{CM} do not differ in effector function (Chapter 3). We found that memory CD8 T_{EM} and T_{CM} both were equally efficient in producing effector cytokines or becoming cytotoxic killer cells after restimulation with antigen. A recent study confirmed these findings also in humans (Ravkov *et al.*, 2003). Interestingly, besides the beforehand mentioned equal effector functions, T_{CM} demonstrated significantly superior mediation of protective immunity and persistence *in vivo* due to their increased proliferative capacity. Moreover, our preceding work demonstrates that the differentiation of CD8 T cells follows a programmed linear developmental pathway and that memory cells directly descend from effector cells (naïve → effector → memory). According to our model, the subsets of T_{EM} and T_{CM} do not arise from separate lineages but are part of a developmental continuum along this linear pathway (Chapter 3). Furthermore, following this model T_{EM} developmentally resemble an intermediate memory stage, which directly gives rise to T_{CM} that represent the true memory population (Chapter 3).

In light of these findings, the generation of T_{CM} should be the goal of any vaccination approach. Moreover, an acceleration or regulation of the $T_{EM} \rightarrow T_{CM}$ conversion could represent a promising goal of new therapeutic approaches to many acute and chronic viral infections. Indeed, many efforts are already undertaken to discover the pathways and mechanisms, which appoint naïve T cells toward memory differentiation. Several recent studies have examined the molecular mechanisms involved in memory T cell development (Hathcock *et al.*, 2003; Jacob and Baltimore, 1999; Liu *et al.*, 2001; Slifka *et al.*, 1999; Teague *et al.*, 1999). For example, the gene expression patterns of naïve, effector, and memory CD8 T cells have been previously compared in a molecular profiling study and uncovered a variety of genes, which are differentially expressed dur-

ing the progression of T cells through the different stages (Kaech *et al*., 2002). This study did not incorporate the two memory CD8 T cell subsets but compared a memory population comprised of both, T_{EM} and T_{CM} with naïve and effector T cells.

In consideration of the heterogeneity of the memory pool and many remarkable differences of T_{EM} and T_{CM} in the mediation of protective immunity, we were interested in the gene expression profiles of these subsets because it is not clear, whether these phenotypic and functional differences are due to transcriptional and/or post-transcriptional mechanisms. In order to better understand the differences between T_{EM} and T_{CM}, we investigated genes that were differentially expressed in these subsets. T_{EM} and T_{CM} subsets were generated in B6 mice following an acute LCMV infection. During the memory phase, we isolated RNA from FACS-purified subsets for comparative analysis on DNA microarrays. Containing and analyzing approximately 8700 murine gene sequences, the DNA microarray study revealed 27 genes out of broad spectrum of functional categories, including surface receptors, translation and protein synthesis machinery, energy metabolism, and signaling molecules. Additionally, more than 50 unassigned genes and expressed sequence tags (EST) were differentially expressed. Reflecting the functions of the differentially expressed genes and their protein products, we successful to correlate the transcriptional state with the functional properties of T_{EM} and T_{CM}. For example, the two major differences between these subsets are migration pattern and proliferative capacity (Chapter 3 and 4) and both were confirmed on the molecular basis and were strikingly mirrored in the differential expression of a variety of correlating genes.

Results

Purification of Memory T Cell Subsets

LCMV-immune P14 chimeric mice were used to analyze the differences in gene expression between T_{EM} and T_{CM} in 3 independent expriments. Sixty to ninety days after LCMV infection (2 x 10^5 p.f.u. i.p.), splenocytes were isolated, purified and subsequently enriched for $CD8^+$ cells using positive magnetic separation. T_{EM} and T_{CM} were then isolated by FACS-sorting $CD8^+D^b$-$gp33^+CD62L^{lo}$ and $CD8^+D^b$-$gp33^+CD62L^{hi}$ cells (Figure 5.1). The purity of the subsets ranged between 94 to 97% and 92 to 94% for T_{EM} and T_{CM}, respectively. Total RNA of T_{EM} and T_{CM} was isolated and reversely transcribed to cDNA. Subsequently, transcription with a T7 RNA polymerase was used to amplify cRNA from the cDNA. The synthesized cRNA was then converted to fluorescently labeled cDNA, which was subjected to DNA microarray hybridization and analysis (see also Chapter 2 and Figure 5.2).

Gene Expression Profile of Memory T Cell Subsets

To analyze gene expression pattern of memory T cell subsets, we compared T_{EM} and T_{CM} on DNA microarrays from Incyte Genomics, containing approximately 8700 murine gene sequences. The relative abundance of specific transcripts from each subset was quantified and a differential expression ratio ($\Delta\overline{E}[CD62L^{hi}/CD62L^{lo}]$) was calculated (Figure 5.2). $\Delta\overline{E}[CD62L^{hi}/CD62L^{lo}]$ is given as a ratio for expression in T_{CM} compared to T_{EM} expression levels ($CD62L^{hi}$ over $CD62L^{lo}$). This means, T_{EM} expression levels are used for reference. Therefore, $\Delta\overline{E} > 0$ indicates a relative upregulation of the gene of interest in T_{CM} compared to T_{EM}, and $\Delta\overline{E} < 0$ a downregulation, respectively. Genes that had been previously identified or were highly similar to known genes were putatively assigned to functional categories (Figure 5.3 and Table 5.1). An expanded version of Table 5.1 that includes remaining expressed sequence tags (ESTs) and unassigned genes is found in Table 5.2.

Differentially Expressed Genes in Memory CD8 T Cell Subsets

In this section, only a brief description of the differentially expressed genes in memory CD8 T cell subsets and the known function of their products is given. Implications for the distinct functional properties of T_{EM} and T_{CM} are discussed in the subsequent section.

Figure 5.1

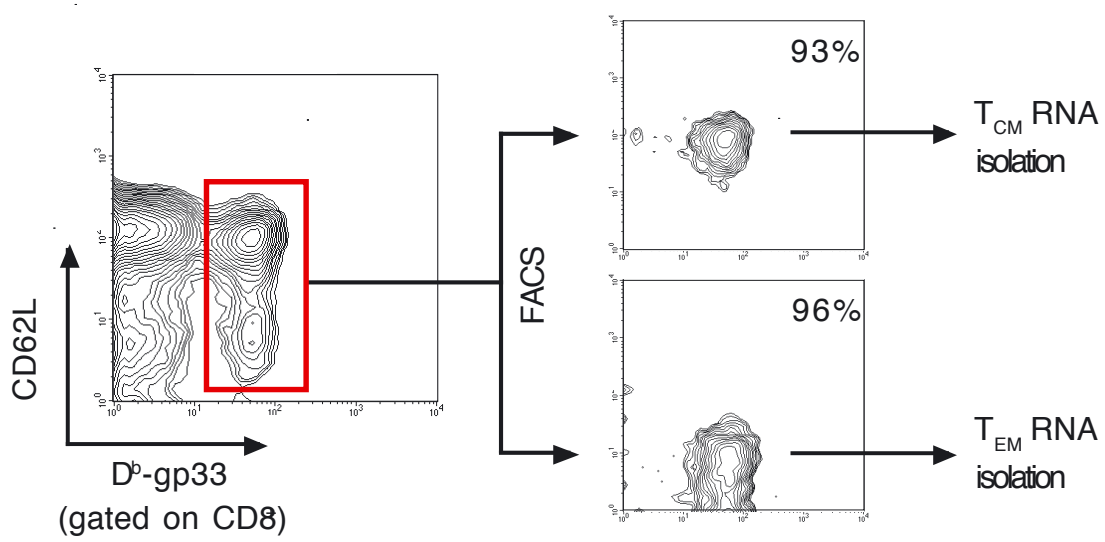

Figure 5.1: Isolation of Memory T Cells Subset RNA.
Splenocytes of LCMV-immune P14 chimeric mice were isolated, purified and enriched for $CD8^+$ cells using positive magnetic separation. Obtained cell fractions were further subjected to FACS. T_{EM} and T_{CM} were isolated sorting either $CD8^+D^b$-$gp33^+CD62L^{lo}$ or $CD8^+D^b$-$gp33^+CD62L^{hi}$ cells. One representative experiment out of three is shown. The purity of the subsets ranged between 94 to 97% and 92 to 94% for T_{EM} and T_{CM}, respectively. Total RNA of sorted T_{EM} and T_{CM} was isolated, processed to cRNA and subjected to DNA microarray hybridization and analysis.

Figure 5.2

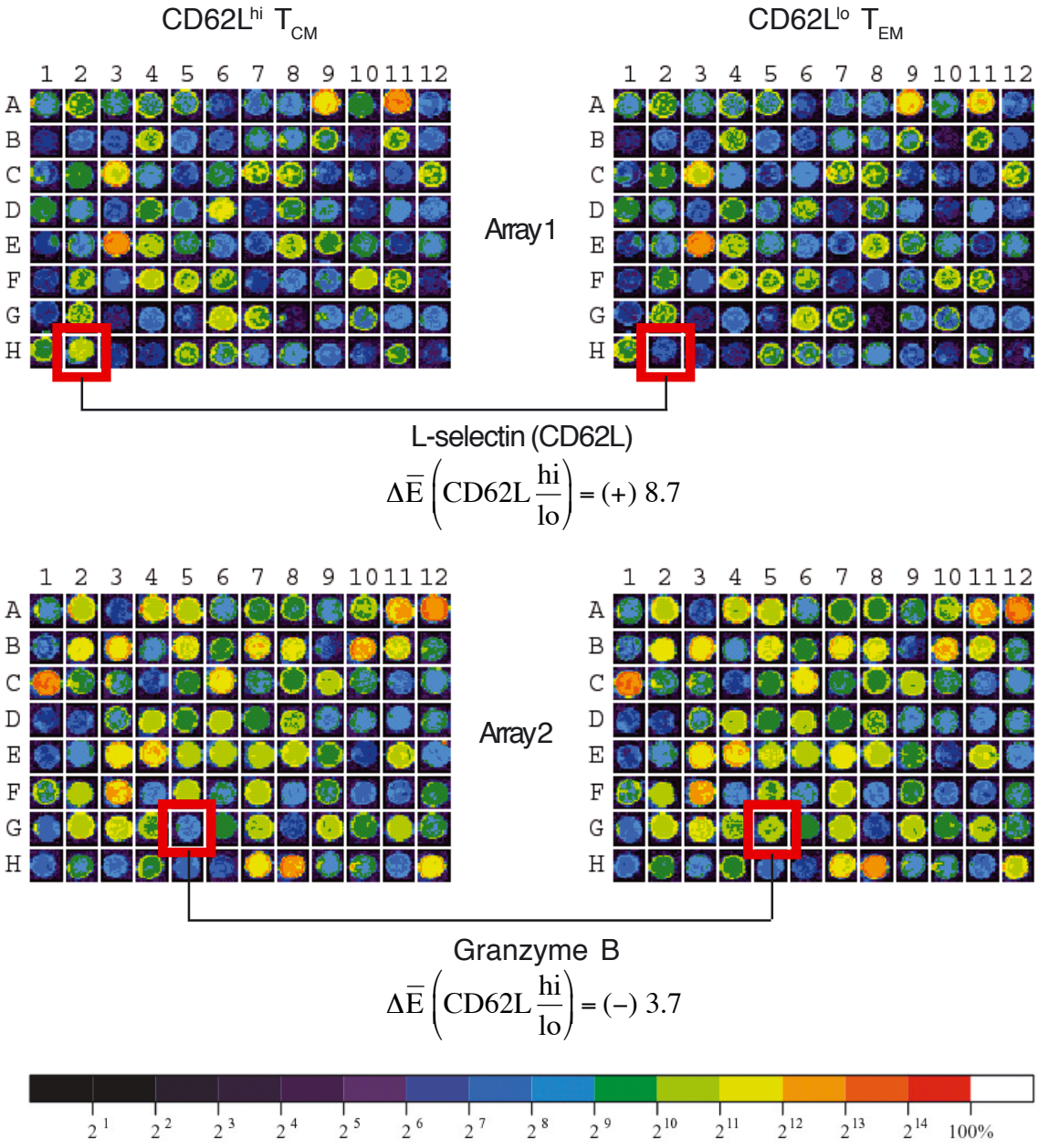

Figure 5.2: DNA Microarray Hybridization.

cRNA of T_{CM} was reverse transcribed to cDNA using fluorescent Cy3-labeled nucleotides. T_{EM} cDNA contained fluorescent Cy5-labeled nucleotides. Subsequently, the cDNA was hybridized on mouse GEM-1 DNA microarrays. Microarrays were then scanned with a fluorescence reader and images were acquired at 535 nm for Cy3 and at 625 nm for Cy5. An image analysis algorithm (GEMTools software, Incyte Genomics) was used to quantify signal and background intensity for each target element. The ratio of the two corrected signal intensities was calculated and used as the differential expression ratio ($\Delta\bar{E}$[$CD62L^{hi}$/$CD62L^{lo}$]) for this specific gene in the genuine two mRNA samples.

Figure 5.3

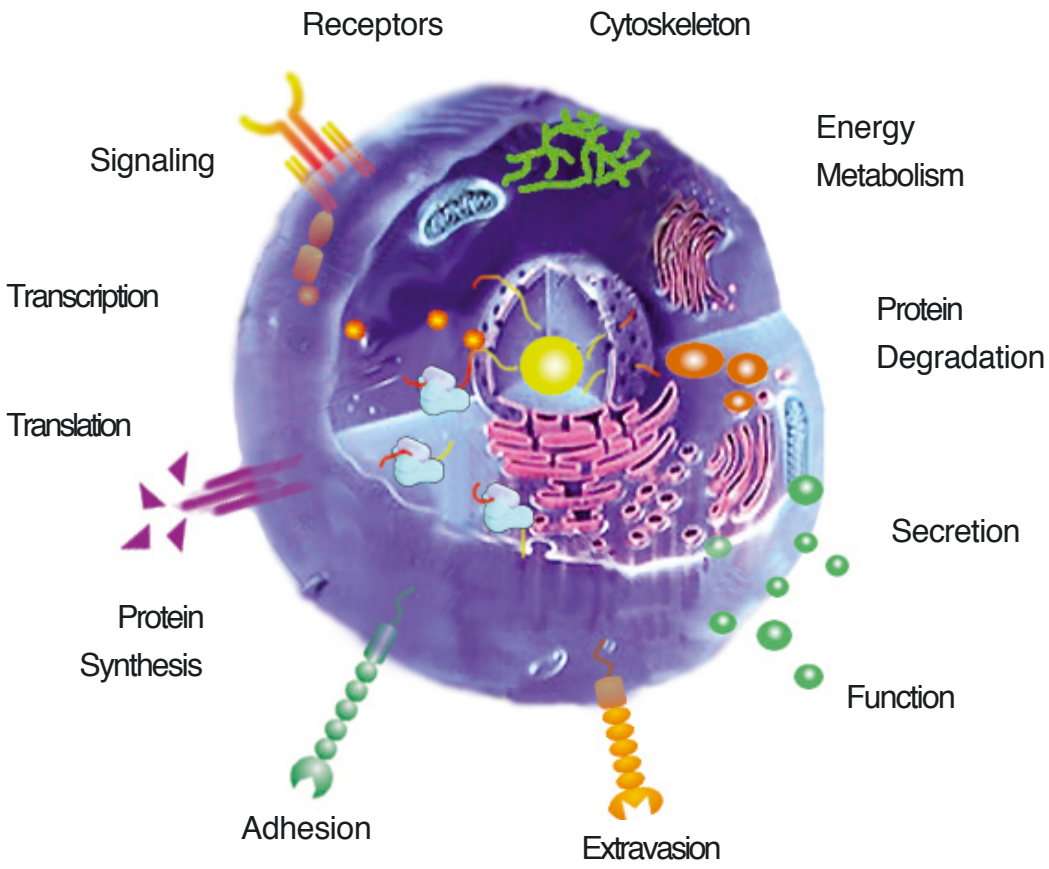

Figure 5.3: Categories of Differentially Expressed Genes.
Differentially expressed genes in the two memory T cell subsets were assigned to functional categories. The molecular profiling of T_{EM} and T_{CM} revealed that differences between these subsets are not confined to a single functional unit. Rather, differences in the whole cellular apparatus are observed.

Table 5.1: Differentially Expressed Genes in T_{CM} compared to T_{EM}.

Gene Category *(Up-regulation)*	$\Delta\overline{E}\left(\text{CD62L}\frac{\text{hi}}{\text{lo}}\right)$ **> 0**
Receptor / Signaling	
CD62L	8.7
Toll-like Receptor 1	2.2
Guanine Nucleotide Binding Protein β2	1.5
Translation / Protein Synthesis	
Ribosomal Protein L3	1.7
Ribosomal Protein L23	1.6
Similar to Elongation Factor-1 γ	1.6
Similar to Small Nucleolar RNA	1.5
Ribosomal Protein L4	1.5
Elongation Factor 1-β	1.5
Miscellaneous	
Dynein Heavy Chain 11	1.6
Methyltransferase-like 1	1.5
Myelocytomatosis oncogene	1.5
Translocase of Inner Mitochondrial Membrane	1.5

Gene Category *(Down-regulation)*	$\Delta\overline{E}\left(\text{CD62L}\frac{\text{hi}}{\text{lo}}\right)$ **< 0**
Function	
Granzyme B	3.7
Macrophage Inflammatory Protein 1β	1.7
Stromal Cell Derived (B Cell) Growth Factor	1.5
Adhesion / Extravasation	
Galectin 3	2.0
Neuropilin-1 Precursor	1.5
L1 (Ig-domain-containing) Adhesion Molecule	1.5
Enzymes	
Esterase 1	1.7
Similar to Trans-1,2-Dihydroreductase	1.7
Signaling	
Cytokine Inducible SH2-containing Protein	1.6
FK506 Binding Protein 7	1.6
ATPase, calcium transporting	1.6
Similar to Chloride Channel	1.6
Receptors	
GP49B	1.6
IgM	1.5

Table 5.2: Extended Version of Table 5.1 including ESTs.

Gene Name	Accession Number	$\Delta E\left(\text{CD62L}\frac{\text{hi}}{\text{lo}}\right)$			$\Delta\overline{E}\left(\text{CD62L}\frac{\text{hi}}{\text{lo}}\right)$
		ΔE_1	ΔE_2	ΔE_3	
Selectin, lymphocyte	AA183698	6.8	7.8	11.4	**8.7**
Toll-like receptor 1	AA177549	2.0	2.3	2.3	**2.2**
Gene rich cluster, C8 gene	W98889	1.1	1.0	3.3	**1.8**
Ribosomal protein L3	AA108363	1.6	1.5	2.0	**1.7**
Ubiquitin conjugating enzyme 7 interacting protein 4	AI605675	1.6	1.5	1.7	**1.6**
Ribosomal protein L23	AA220584	1.4	1.4	2.0	**1.6**
DNA segment, Chr 5, Wayne State University 111 (Plac 8, placenta-specific 8)	AA245029	1.1	1.2	2.5	**1.6**
Dynein, axon, heavy chain 11	AA172519	1.5	1.7	1.5	**1.6**
Elongation factor 1-γ (EF-1γ, eEF-1Bγ, lysosomal acid lipase 1)	AA240994	1.6	1.5	1.6	**1.6**
ESTs	W08673	1.8	1.5	1.3	**1.5**
ESTs	W65070	1.4	1.5	1.7	**1.5**
Methyltransferase-like 1 (*S. cerevisiae*)	AA052208	1.7	1.4	1.5	**1.5**
Ribosomal protein L4, cytosolic (cyclin ania-6α)	AA245452	1.5	1.4	1.7	**1.5**
Eukaryotic translation elongation factor 1-β homolog	AA268148	1.4	1.4	1.8	**1.5**
40S ribosomal protein S5 (ribosomal protein S5)	AA275884	1.3	1.3	2.0	**1.5**
Guanine nucleotide binding protein, β2, related sequence 1	AA048915	1.4	1.3	1.8	**1.5**
Myelocytomatosis oncogene	AA009268	1.5	1.5	1.4	**1.5**
Translocase of inner mitochondrial membrane 8, homolog α (yeast)	W11535	1.5	1.3	1.6	**1.5**
60S ribosomal protein L15 (ubiquitin conjugating enzyme 2ε)	AA068842	1.3	1.3	1.8	**1.5**
Laminin receptor 1 (P40-8, functional)	W75686	1.2	1.3	1.9	**1.5**
Nascent polypeptide-associated complex α polypeptide	AA230684	1.3	1.3	1.8	**1.5**
60S ribosomal protein L22	AA174807	1.2	1.3	1.9	**1.5**
Ribosomal protein S5	AA240279	1.3	1.2	1.9	**1.5**
Hypothetical protein MNCb-5680	AA080443	-1.2	-1.4	-1.8	**-1.5**
L1 cell adhesion molecule	AA288224	-1.4	-1.5	-1.5	**-1.5**

Table 5.2: Extended Version of Table 5.1 including ESTs.

Gene Name	Accession Number	$\Delta E\left(\text{CD62L}\frac{\text{hi}}{\text{lo}}\right)$			$\Delta\overline{E}\left(\text{CD62L}\frac{\text{hi}}{\text{lo}}\right)$
		ΔE_1	ΔE_2	ΔE_3	
Ngg1 interacting factor 3-like 1 (*S. pombe*)	AA177264	-1.3	-1.5	-1.6	**-1.5**
Stromal cell derived factor 1	AA068750	-1.2	-1.5	-1.7	**-1.5**
ESTs	AA208292	-1.2	-1.6	-1.6	**-1.5**
Chromodomain protein, Y chromosome-like	AA080272	-1.2	-1.6	-1.6	**-1.5**
Mannosyl-oligosaccharide 1,2-α-mannosidase	AA014889	-1.1	-1.6	-1.7	**-1.5**
Bmp2-induced gene	AA200279	-1.6	-1.4	-1.5	**-1.5**
ESTs, Weakly similar to AF119384_1 putative CAMP protein	AA036517	-1.6	-1.5	-1.5	**-1.5**
Neuropilin-1 precursor (A5 protein)	AA432934	-1.5	-1.5	-1.6	**-1.5**
ESTs, Weakly similar to Δ-6 fatty acid desaturase	AA068575	-1.3	-1.5	-1.8	**-1.5**
Chloride channel 4 protein	AA097957	-1.3	-1.5	-1.8	**-1.5**
EST, weakly similar to dimethylarginine dimethylaminohydrolase 2	AA241295	-1.3	-1.5	-1.9	**-1.6**
ESTs, Moderately similar to molybdopterin synthase sulfurylase (*H.sapiens*)	W99918	-1.1	-1.8	-1.8	**-1.6**
ATPase, Ca^{++} transporting, cardiac muscle, fast twitch 1	W34420	-1.7	-1.6	-1.5	**-1.6**
Glycoprotein 49 B	AA423373	-1.6	-1.9	-1.3	**-1.6**
FK506 binding protein 7 (23 kDa)	AA242149	-1.3	-1.6	-1.9	**-1.6**
Nel-like 2 homolog (chicken)	W18484	-1.1	-1.8	-2.0	**-1.6**
Cytokine inducible SH2-containing protein	AI893893	-1.6	-1.8	-1.5	**-1.6**
ESTs, highly similar to trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	AA080332	-1.3	-2.0	-1.7	**-1.7**
Macrophage inflammatory protein 1β (CCL4, small inducible cytokine A4)	AA178155	-1.7	-1.8	-1.6	**-1.7**
Lysosomal acid lipase 1	AA178361	-1.1	-1.9	-2.1	**-1.7**
Carboxylesterase precursor (esterase 1)	AA403730	-1.4	-1.7	-2.1	**-1.7**
Dickkopf homolog 3 (*Xenopus laevis*)	AA073904	-1.5	-1.8	-2.1	**-1.8**
Lectin, galactose binding, soluble 3 (galectin 3)	AA403841	-1.9	-2.1	-2.1	**-2.0**
Granzyme B	AA183327	-4.0	-4.1	-3.1	**-3.7**

Relative Upregulation in T_{CM} / Relative Downregulation in T_{EM}

L-selectin (CD62L): consistent with the differences in the CD62L phenotype of T_{CM} ($CD62L^{hi}$) and T_{EM} ($CD62L^{lo}$), CD62L is the most differential expressed gene in these subsets ($\Delta\overline{E}[CD62L^{hi}/CD62L^{lo}] = +8.7$). CD62L mediates leukocyte rolling on vascular endothelium at sites of inflammation and migration of naïve lymphocytes to peripheral lymph nodes (Bradley *et al.*, 1994; Dunon *et al.*, 1996; Mackay *et al.*, 1996; Rigby and Dailey, 2000; Stein *et al.*, 1999; Symon *et al.*, 1999; Tedder *et al.*, 1995). Reduced expression impairs the ability to migrate to lymph nodes (Bradley *et al.*, 1998; Lefrancois and Masopust, 2002; Steeber *et al.*, 1996; Warnock, 1998). Furthermore, the expression of CD62L also regulates in part the migration of effector CTL, effector memory and central memory T cells (see Chapter 3 and 4), (Cerwenka *et al.*, 1999; Masopust *et al.*, 2001; Sallusto *et al.*, 1999b; Tripp *et al.*, 1997; Tussey *et al.*, 2000; Weninger *et al.*, 2001).

Toll-like Receptor 1 (TLR-1): TLR-1 is a member of the Toll-like receptor (TLR) family, which is a component of the innate antimicrobial response in mammals (Medzhitov and Janeway, 1997; Medzhitov *et al.*, 1997). TLR-1 associates with TLR-2 and signals through a domain closely related to that of IL-1 receptors, resulting in NF-κB activation and the secretion of IL-5 and TNF-α (Takeuchi *et al.*, 2002). TLR-1/2 complexes recognize conserved products of microbial metabolism, such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids, and other components of microbial cell walls (Poltorak *et al.*, 1998; Takeuchi *et al.*, 1999; Takeuchi *et al.*, 2002). TLR-1 and TLR-2 are coexpressed on cells of the innate immune system, including macrophages and dendritic cells (Ochoa *et al.*, 2003; Wyllie *et al.*, 2000). Here, it is also expressed on memory T cell subsets, in T_{CM} two-fold higher than in T_{EM}.

Guanine Nucleotide Binding Protein β2 (GNBP-β2): the expanding family of guanine nucleotide binding proteins is involved in many signaling and transduction pathways. In T cells, GNBP have been shown to be associated with chemokine receptors and the TCR–CD3 complex (Bacon *et al.*, 1995; Ohmura *et al.*, 1992). GNBP-β2 is a member of the stimulatory $G_{(s)}$-group, which triggers a signaling pathway leading to transient increase in cytosolic ionized calcium (Ca^{+2}-influx) (reviewed in (Abbas *et al.*, 2000; Janeway *et al.*, 2001; Leo and Schraven, 2001)). In phytohemagglutinin-stimulated T cells, IL-2 and IL-4 may augment the level of steady-state GNBP-β2 mRNA (Shan *et al.*, 1994). It may also mediate LPS-stimulated arachidonic acid metabolism in peritoneal macrophages (Coffee *et al.*, 1990). GNBP-β2 is 1.5-fold overexpressed in T_{CM}.

Ribosomal Proteins L3, L4, L23 (RP-L3/L4/L23): the highly conserved RP-L3 is vital for the function of the ribosome and has been shown to initiate or at least to participate in the early steps of the ribosomal assembly, where it binds with high affinity to 23S rRNA (Avliyakulov *et al.*, 2000; Nowotny and Nierhaus, 1982). RP-L3 is involved in the formation of the peptidyltransferase center and is essential for its catalytic activity (Franceschi and Nierhaus, 1990; Green and Noller, 1997; Hampl *et al.*, 1981; Khaitovich *et al.*, 1999). Additionally, RP-L3 has been shown to possess extraribosomal functions, such as stimulation of helicase activity in *Escherichia coli* or binding to yeast adenylyl cyclase-associated protein (Soultanas *et al.*, 1998; Yanagihara *et al.*, 1997). RP-L4 also resides near the peptidyltransferase center and might together with rRNA, PR-L2 and PR-L3 actively participate in catalysis of peptide bond formation (Hampl *et al.*, 1981; Schulze and Nierhaus, 1982). RP-L4 in particular is known to play a crucial role in the assembly of 50S units (Nierhaus, 1991). It further might be involved in the regulation of a multiple ribosome exit system, facilitating cotranslational processing of nascent proteins (Gabashvili *et al.*, 2001). RP-L4 as well has been shown to have extraribosomal functions involved in the expression of ribosomal components (Trifa and Lerbs-Mache, 2000; Worbs *et al.*, 2000; Yates and Nomura, 1980). RP-L23 is located at the exit of the peptide tunnel in the ribosome (Kramer *et al.*, 2002). It provides an essential docking site for chaperones on the ribosomes and thus links directly protein synthesis with chaperone-assisted protein folding (Bukau *et al.*, 2000; Hartl and Hayer-Hartl, 2002). The ribosomal proteins L3, L4 and L23 are 50 to 70% higher expressed in T_{CM}.

Elongation Factor 1 Subunit β, Subunit γ (EF-1β/γ): the Elongation Factor 1 (EF-1) consists of four subunits, EF-1α, EF-1β, EF-1γ, and EF-1δ, which induce efficient transfer of aminoacyl-tRNA to 80S ribosomes (Janssen and Moller, 1988; van Damme *et al.*, 1991). GTP-activated EF-1α (EF-$1\alpha_{(GTP)}$) acts as the carrier of the aminoacyl-tRNA on its way to the ribosome (Negrutskii and Deutscher, 1991). Aminoacyl-tRNA is released to the ribosome under hydrolysis of GTP (Negrutskii and Deutscher, 1991). The EF1-βγδ -complex colocalizes with the endoplasmatic reticulum (Sanders *et al.*, 1996). It recycles inactive EF-$1\alpha_{(GDP)}$ to EF-$1\alpha_{(GTP)}$. EF-1γ has also been reported to be a major substrate for the maturation promoting factor MPF (Tokumoto *et al.*, 2002). EF-1β and EF-1δ are 50 to 60% higher expressed in T_{CM}.

Dynein Heavy Chain 11: dynein heavy chain 11 is one subunits of the cytoplasmic dynein. Cytoplasmic dynein is a microtubule motor that uses dynactin as an accessory complex to perform various *in vivo* functions including vesicle transport, spindle assembly, and nuclear distribution as well as flagellar and ciliary motility (Han *et al.*, 2001;

Karki and Holzbaur, 1999; Neesen *et al.*, 2001; Wubbolts *et al.*, 1999). Importantly, it might be essentially involved in the dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing (Kuhn and Poenie, 2002). T_{CM} have 1.6-fold increased mRNA levels compared to T_{EM}.

Myelocytomatosis Oncogene (myc): the myelocytomatosis oncogene myc acts as a transcription factor which binds to specific DNA sequences in regions that control genes involved in growth, proliferation, and maturation (Alberts, 1994; Babior, 1994). Overexpression of myc results in the transformation of cells of the myelomonocytic lineage and a wide panel of other cell types by either blocking or maintaining differentiation, depending on the cell type (Fauquet *et al.*, 1990). It can induce myelocytomatosis (monocytes), kidney and liver carcinomas (parenchymal cells), myogenic tumors (myoblasts) or lymphoid leukemia of T and B cell origin (Fauquet *et al.*, 1990; Roy-Burman *et al.*, 1983; Symonds *et al.*, 1986). In non-transformed T cells, myc is induced by IL-2 receptor-mediated signal transduction via the JAK/STAT pathway, promoting lymphocyte growth and proliferation (Asao *et al.*, 1994; Erickson *et al.*, 1999; Iritani *et al.*, 2002; Lord *et al.*, 2000; Matikainen *et al.*, 1999). In conjunction wiht this signaling pathway it has also been shown to protect the T cell from apoptosis (Lauder *et al.*, 2001). The myc oncogene is 50% higher expressed in T_{CM}.

Translocase of Inner Mitochondrial Membrane Subunit 8 (Tim-8): the inner membrane of mitochondria houses two multi-subunit protein complexes that each handles special subsets of mitochondrial proteins on their way to their final destination (Rehling *et al.*, 2003). According to their primary function, these two complexes have been termed the pre-sequence translocase (TIM-23) and the protein insertion complex (TIM-22) (reviewed in (Rehling *et al.*, 2003)). Tim-8 forms a complex with the Tim-13 subunit (Tim-8/13 complex) in the intermembrane space (Koehler *et al.*, 1999). It appears that the Tim-8/13 complex binds to TIM23 when it reaches into the intermembrane space. Tim-8/13 prevents backsliding of the protein out of the translocase of outer mitochondrial membrane (TOM) complex into the cytosol under conditions of reduced membrane potential (Paschen *et al.*, 2000). Tim-8 is 50% overexpressed in T_{CM}.

Relative Downregulation in T_{CM} / Relative Upregulation in T_{EM}

Granzyme B: granzyme B is one member of the granzyme family (reviewed in (Barry and Bleackley, 2002; Russell and Ley, 2002). It has been shown recently to enter target cells by receptor-mediated endocytosis (Motyka *et al.*, 2000). The mannose-6-phosphate receptor was indentified to bind and internalize granzyme B, but perforin is required for the release of granzyme B into the cytoplasm of target cells (Froelich *et al.*, 1996; Motyka *et al.*, 2000; Pinkoski *et al.*, 1998). The substrate for granzyme B is a member of the caspase family (Darmon *et al.*, 1995). Members of the caspase family are crucial for apoptotic cell death, and they require activation by cleavage. The cleavage of target cell caspases by granzyme B results in the activation of the cellular apoptotic cascade (Atkinson *et al.*, 1998). Granzyme B RNA levels are 3.7-fold elevated in T_{EM} compared to T_{CM}.

Macrophage Inflammatory Protein 1β (MIP-1β): MIP-1β is a member of the CC-chemokine family. It is a small molecular mass protein produced by tissue cells as well as by leukocytes and cells of the monocyte-macrophage system (Baggiolini, 1998; Furie and Randolph, 1995). Chemokines are considered to play an important role in the induction and maintenance of leukocytic infiltrates at the site of inflammation (Baggiolini, 1998). MIP-1β binds CCR5, which is expressed on memory T cells and is coupled to signal transduction (Fukada *et al.*, 2002; Kaech *et al.*, 2002a; Lee *et al.*, 1999; Sallusto *et al.*, 1999a). TNF-mediated signaling causes enhanced secretion of MIP-1β by T cells, leading to subsequent autocrine suppression of CCR5 surface expression (Hornung *et al.*, 2000; Kamin-Lewis *et al.*, 2001). MIP-1β is 70% overexpressed in T_{EM}.

Stromal Cell Derived Growth Factor (SDF-1): Mesothelial cells constitutively produce SDF-1 in embryos as well as in adults (Coulomb-L'Hermin *et al.*, 1999; Foussat *et al.*, 2001; Zlotnik and Yoshie, 2000). SDF-1 is a CXC-chemokine that potently attracts T and pre-B cells as well as dendritic cells, and has an effect on T cell rolling and tight adhesion to activated endothelial cells (Bleul *et al.*, 1996b; D'Apuzzo *et al.*, 1997; Kantele *et al.*, 2000; Sozzani *et al.*, 1997). It exerts its chemoattractive and activating functions upon binding to its G protein-coupled receptor CXCR4, which is expressed on B cells, CD4 T cells and CD8 T cells (Bleul *et al.*, 1996a; Bleul *et al.*, 1997; Forster *et al.*, 1998; Oberlin *et al.*, 1996). SDF-1 also is a costimulatory factor for CD4 T cell activation (Nanki and Lipsky, 2000). Most recently, SDF-1/CXCR4 interactions have been implicated in the accumulation of T cells within the inflamed synovia of rheumatoid arthritis, suggesting SDF-1 as a key regulator of local inflammation (Nanki *et al.*, 2000). Our data show that it is expressed in memory CD8 T cells as well, 50% higher in T_{EM} than in T_{CM}.

Galectin-3: Galectin-3 is a member of the growing family of β-galactoside-binding regulatory animal lectins (Cortegano *et al.*, 1998; Yang *et al.*, 1996). It is expressed mainly in tumor cells, macrophages, epithelial cells, fibroblasts and activated T cells and binds to matrix glycoproteins such as laminin, fibronectin, 90K/Mac-2 binding protein and CEA (Rabinovich *et al.*, 2002). It is localized mainly in the cytoplasm, but in activated and/or proliferating cells, a significant amount of galectin-3 can also be detected in the nucleus, on the cell surface or in the extracellular compartment (reviewed in (Rabinovich *et al.*, 2002)). Functionally, galectin-3 acts as an amplifier of the inflammatory cascade (Liu, 2000). It further influences in concert with other galectins cell survival, intracellular signaling, cell growth and proliferation, chemotaxis, cytokine secretion, and migration (Akahani *et al.*, 1997; Blaser *et al.*, 1998; Dagher *et al.*, 1995; Joo *et al.*, 2001; Matarrese *et al.*, 2000; Sacchettini *et al.*, 2001; Yang *et al.*, 1996; Yu *et al.*, 2002). Galectin-3 is two-fold upregulated in T_{EM}.

Neuropilin-1 Precursor (NRP-1): Neuropilin-1 is a non-tyrosine kinase transmembrane protein, which was originally identified as a receptor for the semaphorin family of secreted polypeptides, implicated in axonal guidance and neuronal patterning (Committee, 1999; He and Tessier-Lavigne, 1997; Kolodkin *et al.*, 1997; Liu and Strittmatter, 2001). It also binds vascular endothelial growth factor (VEGF) (Gluzman-Poltorak *et al.*, 2000; Gluzman-Poltorak *et al.*, 2001; Soker *et al.*, 1998) and is involved in the regulation of angiogenesis and vasculogenesis (Miao and Klagsbrun, 2000; Soker, 2001). In the normal mouse embryo, NRP-1 is expressed on endothelial cells and the surrounding mesenchymal cells (Kitsukawa *et al.*, 1995) but unlike other VEGF receptors, NRP-1 is also expressed in many other cell types and tissues (Banerjee *et al.*, 2000; Ding *et al.*, 2000; Soker *et al.*, 1998). Recently, NRP-1 has been found to be expressed on both, naïve T cells and mature dendritic cells and was implicated to function as an additional receptor in naïve T cell–dendritic cell interactions (Tordjman *et al.*, 2002; Tordjman *et al.*, 2003). Our data suggest that NRP-1 is expressed on memory T cells, too. T_{EM} are endowed with 50% higher NRP-1 RNA levels than T_{CM}.

L1 Adhesion Molecule (L1): L1 is a member of the immunoglobulin superfamily (Brummendorf *et al.*, 1998; Hortsch, 2000). In the central nervous system, L1 is expressed only by postmitotic neurons and mainly on non-myelinated axons, whereas in the peripheral nervous system it is expressed on neurons as well as on non-myelinating Schwann cells (Kalus *et al.*, 2003). L1 is involved in neuronal migration, neurite outgrowth, and myelination as well as in axon guidance, fasciculation, and regeneration (Brummendorf *et al.*, 1998; Castellani *et al.*, 2000; Zhang *et al.*, 2000). Furthermore, it

enhances cell survival and synaptic plasticity (Chen *et al.*, 1999; Luthi *et al.*, 1996). Interestingly, expression of L1 has also been demonstrated in the hematopoietic system, including lymphocytes of the bone marrow, spleen, thymus, and in the blood (Ebeling *et al.*, 1996; Kowitz *et al.*, 1992). Beside a homotypic L1-L1 interaction, it can also bind to the VLA-5 integrin, which is mainly expressed on endothelial cells and thus was suggested to play a role in lymphocyte adhesion and migration (Duczmal *et al.*, 1997; Ebeling *et al.*, 1996; Hubbe *et al.*, 1993). Furthermore, L1 was demonstrated to be involved in the T cell–dendritic cell interaction, functioning as a costimulatory molecule in T cell activation (Balaian *et al.*, 2000). Memory CD8 T cells also express L1. It is 1.5-fold overexpressed in T_{EM}.

Cytokine Inducible Src Homology 2-containing Protein (CIS): CIS was the first identified member of a family of cytokine inducible genes, which encode proteins that can modulate cytokine signaling, called suppressors of cytokine signaling (SOCS) (Starr *et al.*, 1997; Yoshimura *et al.*, 1995). CIS/SOCS proteins are capable of binding to activated cytokine receptor complexes via their src homology (SH)-2 domain (Yoshimura *et al.*, 1995). Therefore, they can interfere with the binding of cytoplasmic effector molecules to their receptor, inhibit the catalytic activity of JAK tyrosine kinases, and target receptor complexes to the proteasome (Endo *et al.*, 1997; Krebs and Hilton, 2000; Leonard and O'Shea, 1998; Matsumoto *et al.*, 1997; Matsumoto *et al.*, 1999; Ram and Waxman, 1999; Yasukawa *et al.*, 2000; Yoshimura *et al.*, 1995). The CIS family members have been shown to function as feedback inhibitors attenuating the response of cytokines – such as IL-2, IL-3 and erythropoietin – mainly through inhibition of the signal transducer and activator of transcription (STAT) protein 5 in a multitude of cell types (Masuhara *et al.*, 1997; Matsumoto *et al.*, 1997; Matsumoto *et al.*, 1999; Starr *et al.*, 1997; Tonko-Geymayer *et al.*, 2002; Yasukawa *et al.*, 2000). Interestingly, in CD4 T cells CIS was found to be an immediate early gene induced by TCR stimulation (Li *et al.*, 2000). Contrary to its inhibitory effect on the STAT5 signaling pathway, CIS apparently promotes TCR-induced T cell activation and increases the activity of mitogen-activated protein kinases (MAPK) and transcription factors (Chen *et al.*, 2003; Li *et al.*, 2000). We found that CIS is 1.7-fold upregulated in T_{EM}.

FK506 Binding Protein 7 (FKBP-7): FK506 binding proteins belong to the family of immunophilins (Bierer *et al.*, 1990). These intracellular proteins are defined by their ability to mediate the pharmacological actions of immunosuppressant drugs such as FK506 (Tacrolimus) and rapamycin inside the cell (Dumont, 2000; Gothel and Marahiel, 1999; Marks, 1996). The main cytoplasmic FKBP isoform is FKBP-12, which – after it

formed a complex with FK506 – binds to and inhibits the phosphatase calcineurin (Friedman and Weissman, 1991; Fruman *et al*., 1994; Liu *et al*., 1991). This complex prevents the translocation of nuclear factor of activated T cells (NFAT)-1 to the nucleus, leading to the inhibition of the transcription of the IL-2 gene (Schreiber, 1992; Schreiber and Crabtree, 1992). In addition, immunophilin–FK506 complexes block the JNK and p38 MAPK during T cell activation and also inhibit IL-2 synthesis via these pathways (Matsuda *et al*., 2000). Furthermore, the degranulation of CTL is impaired by the FKBP-FK506 mediated inhibition of calcineurin (Dutz *et al*., 1993). The FKBP-7 is expressed in memory T cells subsets and is 60% upregulated in T_{EM} compared to T_{CM}.

Glycoprotein 49B (GP49B): The protein GP49B is a member of the C2 family of the immunoglobulin (Ig) superfamily and is expressed on the surface of mouse mast cells, macrophages, and natural killer cells (Arm *et al*., 1991; Castells *et al*., 1994; Katz *et al*., 1989; Rojo *et al*., 1997; Wang *et al*., 1997). The cytoplasmic domain of GP49B contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Katz *et al*., 1996). GP49B1 binds the integrin αvβ3, which is expressed on a wide variety of cells (Castells *et al*., 2001). This interaction between GP49B1 and αvβ3 was shown to inhibit IgE-mediated release of β-hexosaminidase from secretory granules and the generation of leukotriene C4 in mast cell activation (Castells *et al*., 2001; Katz *et al*., 1996; Lu-Kuo *et al*., 1999). Memory T cells express GP49B as well. It is 1.6-fold higher expressed in T_{EM} than in T_{CM}.

Discussion

Memory T cells are qualitatively distinct from naïve cells. The anamnestic (secondary) response to antigen mediated by memory T cells is more rapid and more aggressive than the primary response. This enhanced response is reflected in a quicker control of infection and elimination of pathogen. Intensive research is undertaken to reveal the physiologic basis for this phenomenon by directly comparing naïve and effector T cells with memory T cells. The findings of many studies suggest that a concert of certain characteristics and specific properties of memory T cells might collectively explain how T cell memory mediates recall responses and confers long-term protective immunity. For example, as a consequence of clonal expansion during the primary response, the precursor frequency of antigen-specific T cells is substantially increased in immune individuals (Busch *et al.*, 1998; Hou *et al.*, 1994; Murali-Krishna *et al.*, 1998; Whitmire *et al.*, 1998). These cells are maintained at stable numbers for a long time due to slow but continuous homeostatic proliferation (Homann *et al.*, 2001; Murali-Krishna *et al.*, 1998). Cytokines such as IL-2, IL-7 and IL-15 are important factors that regulate this antigen-independent proliferation of memory CD8 T cells (Becker *et al.*, 2002; Goldrath *et al.*, 2002; Jameson, 2002; Schluns *et al.*, 2000; Weng *et al.*, 2002). Upon reencounter of antigen, memory cells are more easily activated, respond to lower amounts of antigen, require less stringent costimulatory signals, and produce more diverse cytokines as compared to naïve cells (Bachmann *et al.*, 1999; Dutton *et al.*, 1998; London *et al.*, 2000; Mullbacher and Flynn, 1996; Pihlgren *et al.*, 1996; Swain, 1994; Swain *et al.*, 2002; Tanchot *et al.*, 1997). Memory CD8 T cells also express a different pattern of surface molecules, which are involved in cell adhesion and chemotaxis, allowing memory T cells to home to lymphoid tissues as well as to extravasate into non-lymphoid tissues and mucosal sites (see Chapter 3 and 4), (Dutton *et al.*, 1998; Masopust *et al.*, 2001; Moser and Loetscher, 2001; Sallusto *et al.*, 1999b; Weninger *et al.*, 2001). Moreover, as naïve T cells differentiate into memory cells, their gene expression profile is reprogrammed by changes in chromatin structure and in the profile of active transcription factors (Agarwal and Rao, 1998). Recently, our group compared the gene expression profile of memory CD8 T cells to naïve T cells to better understand all these functional differences (Kaech *et al.*, 2002a). This study revealed sets of genes and potential pathways that may be important for the generation of memory cell phenotypes.

Unfortunately, the T cell memory is not a uniform compartment. Rather, it is heterogeneous and the observed differences may not apply equally for each subpopulation. Basically, the memory CD8 T cell pool can be divided into two subsets with distinct functions and properties (Chapter 3). In the present study we examined the gene expression profiles of these subsets – effector memory T_{EM} and central memory T_{CM}.

One major difference in T_{EM} and T_{CM} is migration pattern (see Chapter 3 and 4), (Masopust *et al.*, 2001; Moser and Loetscher, 2001; Sallusto *et al.*, 1999b). Indeed, molecular profiling revealed differential expression of genes, which are involved in adhesion, extravasation, migration and chemotaxis.

We found consistent with the $CD62L^{lo}$ and $CD62L^{hi}$ phenotype of T_{EM} and T_{CM} respectively, that CD62L is the most differential regulated gene in these subsets. T_{CM} are endowed with almost 9-fold higher L-selectin levels than T_{EM}, what pivotally influences the migration pattern of these memory CD8 T cells subsets: $CD62L^{lo}$ T_{EM} predominantly extravasate into mucosal sites and non-lymphoid-tissues such as the liver and the lungs, whereas $CD62L^{hi}$ T_{CM} efficiently home to lymph nodes and the bone marrow (Chapter 3 and 4), (Lefrancois and Masopust, 2002; Masopust *et al.*, 2001; Ostler *et al.*, 2001; Sallusto *et al.*, 1999b). Nevertheless, beside these distinct homing preferences, the migration pattern is not absolute and both subsets can be found in each tissue (Chapter 3 and 4). Both populations easily access the spleen and the blood circulation. Thus, T_{EM} may play a major role in surveying peripheral tissues where microbial infections are generally initiated, providing a potent line of defense against reinfections (Masopust *et al.*, 2001; Ostler *et al.*, 2001). Conversely, T_{CM} are specialized to reside in lymph nodes and thus may encounter activated dendritic cells from infected sites more efficiently (Banchereau and Steinman, 1998; Sallusto *et al.*, 1999b). As a consequence of their high proliferative capacity, a huge wave of secondary effectors is generated upon antigen reencounter, which are subsequently released (with a $CD62L^{lo}$ phenotype) to the circulation to combat the infection (Chapter 3). This illustrates how essential different CD62L expression levels could be for the modulation/regulation of protective immunity by altering the *in vivo* trafficking of memory T cells. CD62L expression can be regulated by both transcriptional and post-transcriptional mechanisms such as proteolytic cleavage after TCR activation (Chao *et al.*, 1997). CD62L mRNA levels are substantially lower in effector T cells compared to naïve T cells, indicating that beside proteolytic cleavage transcriptional repression of CD62L occurs *in vivo* as well (Kaech *et al.*, 2002a). But the CD62L locus is not permanently silenced. CD62L transcription increases with the maturation of the T cell from the effector state towards the memory state (Kaech *et al.*, 2002a). In the memory state, the reexpression of CD62L on the cell surface continues as T_{EM} convert to T_{CM}, whereas the transcription of the CD62L gene has reached a steady state. This finding strongly supports our proposed model of linear memory differentiation.

Beside CD62L several other genes involved in cell migration, adhesion and chemotaxis were differentially expressed. This includes the membrane receptor molecules galectin-3, L1 adhesion molecule and neuropilin-1 as well as the secretory proteins mac-

rophage inhibitory protein-1β (MIP-1β) and stromal cell derived growth factor-1 (SDF-1). Interestingly, these five genes were upregulated in the $CD62L^{lo}$ T_{EM}. This might represent a mechanism for T_{EM} to maintain and diversify their migration pattern independent of CD62L.

For example, galectin-3 promotes adhesion of neutrophils to laminin (Kuwabara and Liu, 1996), and tumor cells were found to utilize this lectin for homotypic cell–cell, cell–endothelium and cell–matrix interactions (Glinsky *et al.*, 2000; Inohara *et al.*, 1996). T_{EM} might use galectin-3 to facilitate adhesion to the endothelium and the extracellular matrix (ECM) at sites of inflammation as well as in lymph nodes.

L1 adhesion molecule (L1) was originally recognized as a neural adhesion molecule shown to be involved in neuron migration (Lindner *et al.*, 1983). Recently, it was identified as a cellular ligand for the fibronectin receptor α5β1 in the mouse and the αvβ3 integrin in humans (Ebeling *et al.*, 1996; Ruppert *et al.*, 1995). On mouse leukocytes, L1 was found to play a role in the binding to endothelial cells (Ebeling *et al.*, 1996; Hubbe *et al.*, 1993; Kowitz *et al.*, 1992). Additionally, shed from the cell surface L1 can be deposited in the ECM, mediating adhesion through homotypic L1–L1 interaction (Martini *et al.*, 1988; Montgomery *et al.*, 1996). Moreover, αvβ3 integrin ligation was suggested to promote cell motility and migration (Leavesley *et al.*, 1992). These findings may suggest that T_{EM} could increase adhesion to and migration through the endothelium and/or the ECM using high L1 levels.

Another neuronal marker might be involved in the modulation of T_{EM} migration: neuropilin-1 was first described as a receptor for the family of chemotropic semaphorins, which mediate axon guidance (He and Tessier-Lavigne, 1997; Kolodkin *et al.*, 1997; Kolodkin *et al.*, 1993). The neuropilin–semaphorin interaction induces structural changes to the cytoskeleton and might thus be involved in driving the axon to its target location (Kolodkin, 1998; Takahashi and Strittmatter, 2001). T_{EM} might employ a similar neuropilin-mediated chemotactic mechanism to rearrange their cytoskeleton during the process of extravasation and subsequent migration to inflamed tissues.

The chemokines MIP-1β and SDF-1 are known to be potent chemotactic factors for T cells, modulating rolling, adhesion, diapedesis, and activation (reviewed in (Baggiolini, 1998)). MIP-1β is produced by tissue cells as well as by leukocytes and cells of the monocyte-macrophage system (Baggiolini, 1998; Furie and Randolph, 1995), whereas SDF-1 is mainly secreted by stromal cells (Nagasawa *et al.*, 1996; Nagasawa *et al.*, 1994). MIP-1β was recently found to be also produced and secreted by effector and memory CD4 and CD8 T cells, while the production of SDF-1 by T cells has not been reported yet (Dorner *et al.*, 2003; Hamann *et al.*, 1997; Kamin-Lewis *et al.*, 2001; Sallusto *et al.*, 1999a). Autocrine TNF-α-induced secretion of MIP-1β was dem-

onstrated to suppress the expression of its receptor CCR5 in T cells (Hornung *et al.*, 2000). One the one hand, the paracrine secretion of MIP-1β by T_{EM} at the site of infection could recruit more effector cells to the location, whereas on the other hand the autocrine action of MIP-1β might be necessary for the T_{EM} to switch chemokine receptor expression to overcome the initial migration stimulus in order to leave the site of inflammation and to migrate toward other signals (Hecht *et al.*, 2003; Hornung *et al.*, 2000; Sallusto *et al.*, 1999a). Similar mechanisms might be proposed for the action of T_{EM}-secreted SDF-1, too. Paracrine secretion of SDF-1 could attract a differently composed set of effector cells (including B cells) to the site of inflammation (Dunussi-Joannopoulos *et al.*, 2002; Wright *et al.*, 2002). For example, SDF-1 was found to act as costimulator of CD4 T cells and to enhance IL-2 production by CD4 memory T cells – which is little produced in T_{EM} (Chapter 3) – (Nanki *et al.*, 2000; Nanki and Lipsky, 2000; Nanki and Lipsky, 2001; Suzuki *et al.*, 2001). This could represent a mechanism how T_{EM} modulate their migration and the composition of effector cells in inflamed tissues to optimize the microinvironment for an efficient clearance of pathogen.

Some of these molecules might not only play a role in migration. Galectin-3, neuropilin-1 and L1 were also suggested to play an important role in the interaction of T cells and dendritic cells. For example, a possible costimulatory role in T cell activation was reported for L1(Balaian *et al.*, 2000), and neuropilin-1 might be essential for the formation of the immunologic synapse between T cells and antigen presenting cells (Tordjman *et al.*, 2002). Enhanced galectin-3-mediated binding of T cells to dendritic cells was demonstrated after triggering L-selectin (Swarte *et al.*, 1998). Even tough T_{EM} express low levels of CD62L, together these mechanisms might constitute a possible way for T_{EM} to interact with dendritic cells (DC) in peripheral tissues more effectively, since it is assumed that optimal DC–T cell interaction occurs mainly in the lymph nodes (Banchereau and Steinman, 1998).

We found also that several genes involved in cell activation, signal transduction, and transcription are differentially expressed in the memory T cells subsets. In T_{EM}, the cytokine inducible src homology 2-containing (CIS) protein and FK506 binding protein 7 (FKBP) were elevated, whereas in T_{CM} the levels of myc oncogene, guanine nucleotide binding protein β2 and the toll-like receptor 1were increased.

Both, CIS and FKBP can interfere with the IL-2 pathway. CIS can impair signaling through the IL-2 receptor (Matsumoto *et al.*, 1997; Starr *et al.*, 1997; Yasukawa *et al.*, 2000), whereas FKBP7 alone or in association with FK506 or other molecules could possibly block the transcription and translation of the IL-2 gene (Matsuda *et al.*, 2000; Schreiber, 1992; Schreiber and Crabtree, 1992). We have shown previously (Chapter 3) that T_{EM} produce less IL-2 than T_{CM}. It might be possible that the increased expression of

CIS and FKBP7 contribute to this phenomenon. Moreover, IL-2 is the major cytokine that mediates proliferation in antigen-stimulated T cells (Cheng and Greenberg, 2002; Cousens *et al.*, 1995). T_{CM} express high levels of myc, which is induced by IL-2 receptor mediated signaling via the JAK/STAT pathway and promotes T cell growth and proliferation (Asao *et al.*, 1994; Erickson *et al.*, 1999; Iritani *et al.*, 2002; Lord *et al.*, 2000; Matikainen *et al.*, 1999). Additionally, activation and proliferation of T_{CM} could be further enhanced by the increased expression of guanine binding proteins and toll-like receptors. Guanine binding proteins function as important adapter molecules in a multitude of signal transduction pathways and have been found to be associated with the TCR and other activating receptors for T cells (Cantrell, 1994; Shan *et al.*, 1994; Stanners *et al.*, 1995). Toll-like receptors, originally thought to be exclusively expressed on cells of the innate immune system and APC, were recently discovered on B and T cells, too (Hornung *et al.*, 2002). Triggering of the TLR by conserved pathogen-specific molecular patterns such as LPS and CpG oligodeoxynucleotides (ODN) for example activates macrophages and dendritic cells, resulting in the production and secretion of modulatory cytokines such as IL-12 (reviewed in (Trinchieri, 2003), (O'Neill and Dinarello, 2000; Schnare *et al.*, 2001)). Interestingly, some recent studies suggest that TLR might exert direct activating and costimulatory effects on T cells (Bendigs *et al.*, 1999; Iho *et al.*, 1999; Myers *et al.*, 2003). Therefore, T_{CM} might be more easily activated; possibly directly by signals from the innate system and even in the absence of APC. Altogether, these findings could collective account for the increased antigen-driven proliferative capacity of T_{CM} compared to T_{EM} (Chapter 3).

Whether those mechanisms could also contribute to the differences in homeostatic proliferative capacity observed in memory subsets remains to be investigated (Chapter 3). In contrast to the strong proliferation of T_{CM} upon reencounter of antigen, it was shown that division and survival of CD8 memory T cells under homeostatic/antigen-free conditions is promoted by IL-7 and IL-15 (Becker *et al.*, 2002; Schluns *et al.*, 2000) and could be further markedly increased by the inhibition of IL-2 (Ku *et al.*, 2000). But for survival and homeostasis of the memory subsets other mechanisms might play an important role, too. For example, bcl2-expression is significantly increased in memory T cells compared to naïve and effector T cells (Grayson *et al.*, 2000; Kaech *et al.*, 2002a). Interestingly, bcl-2-levels detected by intracellular staining assays are not equally high in both memory subsets. T_{CM} are endowed with higher bcl-2-levels (data not shown), but relative upregulation of galectin-3 in T_{EM}, which has a significant sequence similarity with bcl-2 (Yang *et al.*, 1996), might help to compensate for this difference in this subset.

Consistent with the higher proliferative capacity of T_{CM} – antigen-driven and homeostatic (see Chapter 3) – we found also a battery of genes involved in translation and protein synthesis to be expressed at higher levels in this subset. This includes the ribosomal proteins L3, L4 and L23 as well as the elongation factor 1 subunits β and γ. Proliferating cells require an active protein synthesis machinery to prepare for division. Thus, it is not surprising that protein synthesis and translation factors in T cells were found to be regulated by signals received trough CD3 and CD28 (Kleijn and Proud, 2002; Miyamoto *et al.*, 2000). In addition, certain components of the translation and protein synthesis apparatus can be induced by cytokines, for example by IL-2 (Sabath *et al.*, 1990).

Although T_{EM} and T_{CM} do differ significantly in their capability to mediate protective immunity due to their different proliferative potentials, we have shown that both subsets are endowed with equal capacities to produce the effector molecules TNF-α, INF-γ, and granzyme B (Chapter 3). Interestingly, granzyme B is upregulated in T_{EM}. However, there is strong evidence that for granzyme B expression levels do not necessarily correlate with protein levels (Chapter 3),(Kaech *et al.*, 2002a). The uncoupling of transcription from translation may signify a general mechanism to simultaneously maintain functional preparedness while preventing the improper release of cytotoxic or other effector molecules (Bachmann *et al.*, 1999; Grayson *et al.*, 2001; Slifka *et al.*, 1999; Veiga-Fernandes *et al.*, 2000). As for T_{CM}, the relatively reduced granzym B mRNA levels might be still above a threshold that is needed for sufficient granzym B protein synthesis. On the other hand, the lower mRNA levels in central memory T cells could also be balanced with more effective translation based on their more potent ribosomal apparatus compared to effector memory T cells.

Taken together, molecular profiling represents a powerful tool to gain insight into cellular function. Although we do not fully understand the phenomenon of immunological memory in all its detail and many questions remain unanswered, the different gene expression profile of the two memory subsets T_{EM} and T_{CM} helped us to successfully correlate the transcriptional state with functional properties. The two major differences between T_{EM} and T_{CM} – migration pattern and proliferative capacity – could be confirmed on a molecular basis and were strikingly mirrored in the expression profile of correlating genes. On the one hand, the dominating genes in T_{CM} very well explained their preferential homing to lymph nodes and secondary lymphoid tissues (CD62L) as well as their superior proliferative response to antigen-related and homeostatic signals (myc, GNBP, ribosomal apparatus, TLR-1). On the other hand, the leading differentially expressed genes in T_{EM} could convincingly support their migration profile to peripheral tissues (MIP-1β, SDF-1, galectin-3, NRP-1, L1 adhesion molecule) and their impaired response to proliferative stimuli (CIS, FKBP7).

Further characterization of gene expression profiles of functional memory CD8 T cells will hopefully aid the discovery of mechanisms that regulate development and maintenance of these cells, which would prove invaluable for optimizing vaccination. Also, delineating when memory CD8 T cells form *in vivo* and acquire a high proliferative capacity has considerable implications for vaccine regimens that involve boosting for efficacy. Our results suggest that vaccine boosters should be separated by a significant length of time to allow generated effector cells to differentiate into central memory cells. Only this way T cells are able to reset their responsiveness and to acquire their full proliferative capacities to optimally mediate protective immunity.

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6

Summary

Summary

Natural and experimental exposure to certain pathogens can induce long-lived or even life-long immunity against recurrent infections with the same organism. This ability of the specific immune response to remember antigenic structures constitutes the essence of immunological memory and remains central to the comprehension of protective immunity. Moreover, it is essential for the development of new vaccination strategies for many current major public health concerns, including many acute and chronic viral infections. Therefore, understanding the mechanisms that generate and maintain immune responses and induce successfully immunological memory during acute and chronic viral infections remains crucial to the prevention and cure of these diseases. The goal of this dissertation is to add new perspectives to the complex phenomenon of immunological memory and protective immunity. The immune system and the immunological memory are entities of high complexity and heterogeneity. Therefore, we focused on memory T cells in this work.

The generation of memory T cells is the result of a successful immune response to intracellular pathogens. Memory T cells persist in a host at increased numbers and in an enhanced functional status, and thus are capable to provide faster and more effective protection against infection than naïve T cells. Recent studies have shown that these memory T cells can be divided into two distinct subsets: effector memory T cells (T_{EM}) and central memory T cells (T_{CM}). $CCR7^{+}CD62L^{hi}$ T_{CM} efficiently home to lymph nodes, whereas $CCR7^{-}CD62L^{lo}$ T_{EM} were reported to be primarily found in the blood, the spleen, and in non-lymphoid tissues. Moreover, remarkable differences in the execution of effector functions were attributed to the two $CD8^{+}$ memory T cells subsets, proposing CD8 T_{EM} being superior in the protection from reinfection. Owing to these differences in function and phenotype, T_{EM} and T_{CM} had been postulated to arise from separate lineages during the activation phase of the immune response.

The accurate delineation of memory T cell differentiation has profound impact on vaccination immunology because it is essential to understand the lineage relationships of the different subsets in order to target the correct population for enhancement of vaccination efficiency. Our data obtained in mice after the infection with lymphocytic choriomeningitis virus (LCMV) are in discordance with widely accepted models of memory CD8 T cell differentiation. Firstly, we found both memory CD8 T cell subsets, T_{EM} and T_{CM}, equally efficient in producing the effector cytokines IFN-γ and TNF-α. Moreover, T_{EM} and T_{CM} displayed also equal cytotoxic function after the reexposure to antigen *ex vivo*. Secondly, we used several different models of infection to address the question of protective capacity of T_{EM} and T_{CM}. Strikingly, and in contrast to the prevailing notion,

we found purified and adoptively transferred T_{CM} to be more efficient in providing protective immunity after challenging the recipient with different infectious agents via various entry routes. We additionally discovered upon reencounter with antigen, that T_{CM} are endowed with a much higher proliferative capacity than T_{EM}, which correlates with the increased production of IL-2 by T_{CM}. Superior protective capacity therefore is very likely the result of increased antigen-driven proliferation and generation of secondary effector T cells. Furthermore, in the absence of antigen both, T_{EM} and T_{CM} underwent homeostatic proliferation, but T_{CM} exhibited a substantially higher turnover rate. Thus, compared to T_{EM}, T_{CM} possess an increased proliferative capacity to antigenic and homeostatic signals and represent the more efficient mediators of protective immunity.

We next investigated the lineage relationship of the two memory subsets. Analyzing the expression levels of CCR7 and CD62L longitudinally for an extended period of time after infection, we found that the proportion of T_{CM} steadily increased in the immune host and became the predominant memory population. This observation suggested that either T_{CM} overgrow T_{EM} after infection or that T_{EM} convert to T_{CM} over time. To verify our presumption that T_{EM} directly give rise to T_{CM}, we used several adoptive transfer approaches and confirmed $T_{EM} \rightarrow T_{CM}$ conversion in the absence of antigen and indepent to proliferation. The results of our study now allow us to propose a model of T cell differentiation that incorporates the recently defined memory CD8 T cell subsets of T_{EM} and T_{CM}. The essence of this model is that T_{EM} are a transitory population representing an intermediate cell type in the effector → memory transition. Thus, according to this model T_{CM} and T_{EM} cells are not separate subsets but are part of a developmental continuum that ends with the formation of T_{CM} cells. We consider T_{CM} being the true memory cells because it is only this subset that exhibits unique characteristics of memory T cells: long-term persistence *in vivo* by homeostatic self-renewal and the ability to rapidly expand upon reexposure to antigen. This model also predicts that memory development is a gradual process and that memory cells only develop several weeks after clearance of the acute infection.

Our study also shows that the rate at which a T cell population converts from T_{EM} to T_{CM} can vary depending upon the nature of the immunization, and that this conversion rate is programmed during the initial period of encounter with antigen *in vivo*. Using infection models that provide a strong initial antigenic stimulus, we observed that $T_{EM} \rightarrow T_{CM}$ conversion occurred slowly over several months, whereas a lower amount of priming antigen resulted in more rapid differentiation of T_{EM} into T_{CM}. Thus, the duration of $T_{EM} \rightarrow T_{CM}$ conversion is not constant, but is imprinted during effector generation and varies depending on the magnitude of the initial stimulation.

Yet, the immune response to LCMV and other pathogens is highly complex and we were further interested in the arising questions, whether the programmed rate of $T_{EM} \rightarrow T_{CM}$ conversion is constant or varies by epitope-specificity, type of infection, or mouse strain. Therefore, we additionally examined in the present study the rate of reversion from T_{EM} to T_{CM} for several different epitope-specific T cell populations induced during the same infection. We discovered an inverse correlation of the epitope hierarchy and the rate of memory conversion. The most immunodominant epitope of LCMV triggered the slowest differentiation program in specific T cells. Furthermore, we compared the reversion rates of these different virus-specific CD8 T cell populations in multiple lymphoid and non-lymphoid tissues. Interestingly, memory conversion occurred in all locations and we recognized a separate hierarchy also existing among these different tissues: T_{EM} reverted the slowest in non-lymphoid tissues, whereas conversion in lymphoid tissues occurred at much faster rates. Remarkably, within the lymphoid tissues, T_{EM} present in the lymph nodes differentiated more quickly into T_{CM} than those in the bone marrow and the spleen.

Moreover, our study demonstrates that conversion occurs at similar rates after different types of infection. T_{EM} generated in the course of bacterial infection convert in both, lymphoid and non-lymphoid tissues to T_{CM} at almost identical rates as do virally primed T_{EM}. Thus, $T_{EM} \rightarrow T_{CM}$ differentiation appears to be independent to the type of infection. The distinct cytokine profiles that might be induced during viral and bacterial infection seem not to affect the nature of the triggered memory conversion program.

In this dissertation we also demonstrate that $T_{EM} \rightarrow T_{CM}$ conversion is not unique for specific T cells of one certain mouse strain. Specifc CD8 T cells from different mouse strains convert T_{EM} to T_{CM} at similar rates. Furthermore, TCR-transgenic T cells from P14 mice also execute a comparable differentiation program.

All these findings further support our idea that $T_{EM} \rightarrow T_{CM}$ conversion is inevitably programmed during initial antigen encounter. Our results are also consistent with the concept that the developmental program is triggered mainly by the amount of antigen: firstly, distinct inflammatory conditions induced by different pathogens do not change $T_{EM} \rightarrow T_{CM}$ conversion rates. Secondly, the finding that the conversion rate inversely correlates with the immunodominance of an epitope-specific T cell population additionally supports this notion. Remarkably, conversion rates are different in lymphoid and non-lymphoid tissues. T_{EM} convert much faster to T_{CM} in lymphoid tissues than in non-lymphoid. The different homing and recirculation properties of T_{EM} and T_{CM} might accentuate this observation, but modulation of the programmed $T_{EM} \rightarrow T_{CM}$ conversion rates by antigen and/or tissue factors may represent an additional mechanism of the immune system to further optimize protective immunity at different sites conferred by memory T cell subsets.

As naïve T cells differentiate into memory cells, their gene expression profile is reprogrammed. Recently, our group compared the gene expression profile of memory CD8 T cells to naïve T cells to better understand all these functional differences. This study did not incorporate the two memory CD8 T cell subsets but compared a memory population comprised of both, T_{EM} and T_{CM} with naïve and effector T cells.

In consideration of the heterogeneity of the memory pool and the many remarkable differences of T_{EM} and T_{CM} in the mediation of protective immunity, we were interested in the gene expression profiles of these subsets because it is not clear, whether these phenotypic and functional differences are due to transcriptional and/or post-transcriptional mechanisms. In order to better understand the differences between T_{EM} and T_{CM}, we investigated genes that were differentially expressed in these subsets using DNA microarray techniques. Containing and analyzing approximately 8700 murine gene sequences, the DNA microarray study revealed 27 genes out of broad spectrum of functional categories, including surface receptors, translation and protein synthesis machinery, energy metabolism, and signaling molecules. Additionally, more than 50 unassigned genes and expressed sequence tags (EST) were differentially expressed. Reflecting the functions of the differentially expressed genes and their protein products, we were able to successfully correlate the transcriptional state with the functional properties of T_{EM} and T_{CM}. The two major differences between T_{EM} and T_{CM} – migration pattern and proliferative capacity – could be confirmed on a molecular basis and were strikingly mirrored in the expression profile of correlating genes. On the one hand, the dominating genes in T_{CM} very well explained their preferential homing to lymph nodes and secondary lymphoid tissues as well as their superior proliferative response to antigen-related and homeostatic signals. On the other hand, the leading differentially expressed genes in T_{EM} could convincingly support their migration profile to peripheral tissues and their impaired response to proliferative stimuli.

Further characterization of gene expression profiles of functional memory CD8 T cells will hopefully aid the discovery of mechanisms that regulate development and maintenance of these cells, which would prove invaluable for optimizing vaccination. Also, the determination of the key points in memory CD8 T cell differentiation that are connected to the acquisition of a high proliferative capacity, has considerable implications for vaccine development. Our results suggest that vaccine boosters should be separated by a significant length of time to allow generated effector cells to fully differentiate into central memory cells. Only this way T cells are able to reset their responsiveness to antigen and to acquire their full proliferative capacities to optimally mediate protective immunity.

Altogether, our results ought to provide new assistance and help to decipher the complexity and heterogeneity of the immunological memory in order to support the design of more potent vaccines and to optimize their administration protocols, and to provide new targets for therapeutic manipulation of the immune system to protect from infectious and tumor diseases.

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Appendix

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APPENDIX

Honors and Prizes

Scholar of the Studienstiftung des deutschen Volkes
Scholar of the Konrad-Adenauer-Stiftung
Scholar of the College of Graduates (Cell Biology in Medicine)
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- First Prize of Baden-Württemberg
- Special Award of the Ministry of Education and Arts

Third prize in mathematics competition "Tag der Mathematik"

Publications, Abstracts, Posters & Talks

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Oral presentation

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European Journal of Cell Biology, 1997, 74: Supplement 47
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Teichgräber, V., Wherry, E. J., and Ahmed, R.
"Conversion Kinetics of Memory CD8 T Cells subsets"
Manuscript in preparation

Teichgräber, V., Moris, A., Müller, M., Schittek, B., Garbe, C., and Rammensee, H. G.
"Melanoma-specific CD8 T Cells in Local and Metastatic Disease"
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Manuscript submitted for publication