Preclinical development of adoptive T-cell immunotherapy for EBV-associated diseases using third-party donors

by

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ABSTRACT

A significant number of patients requiring adoptive T-cell therapy (ATCT) need to resort to third party donors; we aimed to find ways to optimise ATCT from third party donors in EBV-associated diseases.

Firstly, we evaluated the T-cell response to 29 EBV-restricted peptides in a cohort of 100 healthy donors. For each peptide we found at least one high-responding donor. Also, we compared the efficacy of different separation techniques. These results support the setting up of a registry of third party donors, to provide fresh EBV-specific T cells for ATCT.

Secondly, we investigated the mechanisms generating T memory stem cells (T_{SCM}), which are considered most suitable for ATCT. We demonstrated that homeostatic cytokines revert recently differentiated $CD8^+$ memory T cells from cord blood (CB) to cells with a T_N-like phenotype (T_{Nrev}) and T_{SCM}-like characteristics.

Finally, we compared phenotype and function of $CD8^+$ T cells from peripheral blood and CB, after transduction of an EBV-specific TCR. Transduction efficiency, growth kinetics and cytolytic activity were comparable. However, TCR-transduced CB T cells showed less differentiated phenotype, increased multi-cytokine expression, and lacked expression of the senescence marker CD57. These data suggest that survival of engineered T cells *in vivo* is likely to be improved by using cells from CB.

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LIST OF ABBREVIATIONS

7AAD	7-aminoactinomycin D
APC	Antigen presenting cell
ATCT	Adoptive T-cell therapy
BARTs	Bam H1A rightward transcripts
BL	Burkitt lymphoma
CAEBV	Chronic active EBV infection
CAR	Chimeric antigen receptor
CB	Umbilical cord blood
CBMC	Cord blood mononuclear cells
CCR	Chemokine receptor
CD	Cluster differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
CR2	Complement receptor 2
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DC	Dendritic cell
DLBCL	Diffuse large B cell lymphoma
DLI	Donor lymphocyte infusion
DMSO	Dimethyl sulfoxide
DNR	Dominant-negative TGFβ receptor type II
EBER	Epstein-Barr virus-encoded RNA

EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr Virus
ER	Endoplasmic reticulum
FCS	Fetal calf serum
GCSF	Granulocyte colony-stimulating factor
GMCSF	Granulocyte-macrophage colony-stimulating factor
Gp	Glycoprotein
GVHD	Graft versus host disease
HIV	Human immunodeficiency virus
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplant
IFN	Interferon
IL	Interleukin
IM	Infectious mononucleosis
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
MDSC	Myeloid-derived suppressor cell
MoAb	Monoclonal antibody
NHL	Non-Hodgkin lymphoma
NK	Natural killer
NPC	Nasopharyngeal carcinoma
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered solution

PCNSL	Primary central nervous system lymphoma
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	PD-1 ligand 1
PI	Propidium iodide
PTLD	Post-transplant lymphoproliferative disorders
RS	Reed-Sternberg
SOT	Solid organ transplant
TAP	Transporter associated with antigen processing
T_{CM}	Central memory T cells
TCR	T-cell receptor
T_{Eff}	Effector T cells
T_{EM}	Effector memory T cells
T_{EMRA}	$CD45RA^{+}$ effector memory T cells
TGFβ	Tumor growth factor β
T_{MNP}	Memory T cells with naïve phenotype
T_N	Naïve T cells
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cells
T_{SCM}	T memory stem cells

1. INTRODUCTION

1.1. THE EPSTEIN-BARR VIRUS

1.1.1. CLASSIFICATION AND STRUCTURE

The analysis of molecular sequences in different virus strains suggests that Herpesviridae appeared during the Jurassic period, about 200 million years ago (McGeoch et al., 1995). Since then, they have evolved to infect, besides reptiles, also birds and mammals.

Nine different species of Herpesviridae can cause diseases in humans, but the most common pathogens are the Herpes Simplex Virus, the Varicella Zoster Virus, the Cytomegalovirus (CMV), and the Epstein-Barr virus (EBV).

The EBV, also called human herpesvirus 4, belongs to the subfamily Gammaherpesvirinae, genus Lymphocryptovirus, and was first described in 1964 by Michael Epstein, Albert Achong and Yvonne Barr in lymphoblastoid cells from a patient with the endemic form of Burkitt's lymphoma (Epstein et al., 1964). There are two types of EBV: Type 1 is the most common, being present in Europe, America and Asia, and in Africa it coexists with Type 2, which is also present in New Guinea (Zimber U et al., 1986). The two types differ in their nuclear antigens and in their ability to transform infected cells, Type 1 being more effective than Type 2 (Odumade et al., 2011). Several different strains of EBV have been identified so far, the first strain to have the genome sequenced being B95-8 (Baer et al., 1984). Although overall sequence similarities between strains can go down to 95%, the different genomes share common features: they are about 170 kb in length, and code for at least 86 different proteins (Kwok et al., 2012). The best known open reading frames and their products are listed in Table 1.

Table 1.1. Examples of EBV genes and gene products.
(Various authors, 2012)

Open reading frame	Protein		Main proposed function
	Common name	Alternative nomenclature	
Latent genes			
BKRF1	EBNA-1[a]		Plasmid maintenance, DNA replication transcriptional regulation
BYRF1	EBNA-2[a]		*trans*-activation
BERF1	EBNA-3A[a]	EBNA-3	Transcriptional regulation
BERF2	EBNA-3B[a]	EBNA-4	Unknown
BERF3/4	EBNA-3C[a]	EBNA-6	Transcriptional regulation
BWRF1	EBNA-LP[a]	EBNA-5	*trans*-activation
BNLF1	LMP-1[a]		B-cell survival, anti-apoptosis
BNRF1	LMP-2A[a]/2B	TP1/2	Maintenance of latency
BARF0			Not shown to be translated, unknown function
EBER1/2			Non-translated, regulation of innate immunity
Lytic genes			
Immediate early genes			
BZLF1	ZEBRA		*trans*-activation, initiation of lytic cycle
BRLF1			*trans*-activation, initiation of lytic cycle
BI'LF4			*trans*-activation, initiation of lytic cycle
Early genes			
BMRF1			*trans*-activation
BALF2			DNA binding
BALF5			DNA polymerase
BORF2			Ribonucleotide reductase subunit
BARF1			Ribonucleotide reductase subunit
BXLF1			Thymidine kinase
BGLF5			Alkaline exonuclease
BSLF1			Primase
BBLF4			Helicase
BKRF3			Uracil DNA glycosylase
Late genes			
BLLF1	gp350/220		Major envelope glycoprotein
BXLF2	gp85 (gH)		Virus–host envelope fusion
BKRF2	gp25 (gL)		Virus–host envelope fusion
BZLF2	gp42		Virus–host envelope fusion, binds MHC class II
BALF4	gp110 (gB)		Unknown
BDLF3	gp100–150		Unknown
BILF2	gp55–78		Unknown
BCRF1			Viral interleukin-10
BHRF1[ab]			Viral *bcl-2* analogue

Some latent genes are not translated, such as the Epstein-Barr virus-encoded RNA (EBER) 1 and 2, and at least 17 micro-RNAs are also encoded (Cai et al., 2006).

The linear, double stranded DNA is wrapped around an annular-shaped protein core that is contained within a capsid resulting from the assembly of 150 hexameric and 12

pentameric capsomers (Germi et al., 2012). The capsid is inside a protein tegument that is surrounded by a membrane with virus-encoded glycoprotein spikes (Figure 1.1).

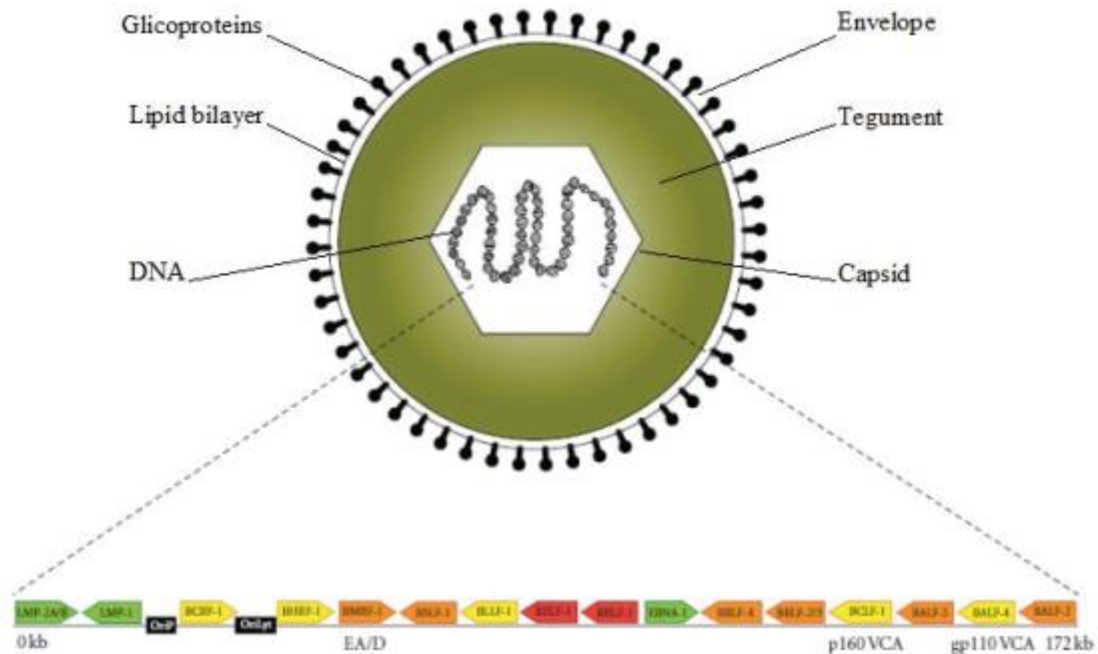

Figure 1.1. Scheme of structure and genome of EBV.
OriP and OriLyt indicate the origins of latent and lytic replication respectively. The location of some genes is shown: in green are latent genes, in yellow, orange and red are lytic ones. The arrow indicates the direction of translation. (Modified from Draborg et al., 2012)

1.1.2. MECHANISMS OF CELL INFECTION

1.1.2.1. VIRUS ENTRY

The virus is primarily spread through saliva, rarely through semen or blood. The main targets of EBV primary infection are initially the epithelial cells of oronasopharynx, and then B lymphocytes, but the virus uses different strategies for attaching to and entering into the two cell types (Figure 1.2).

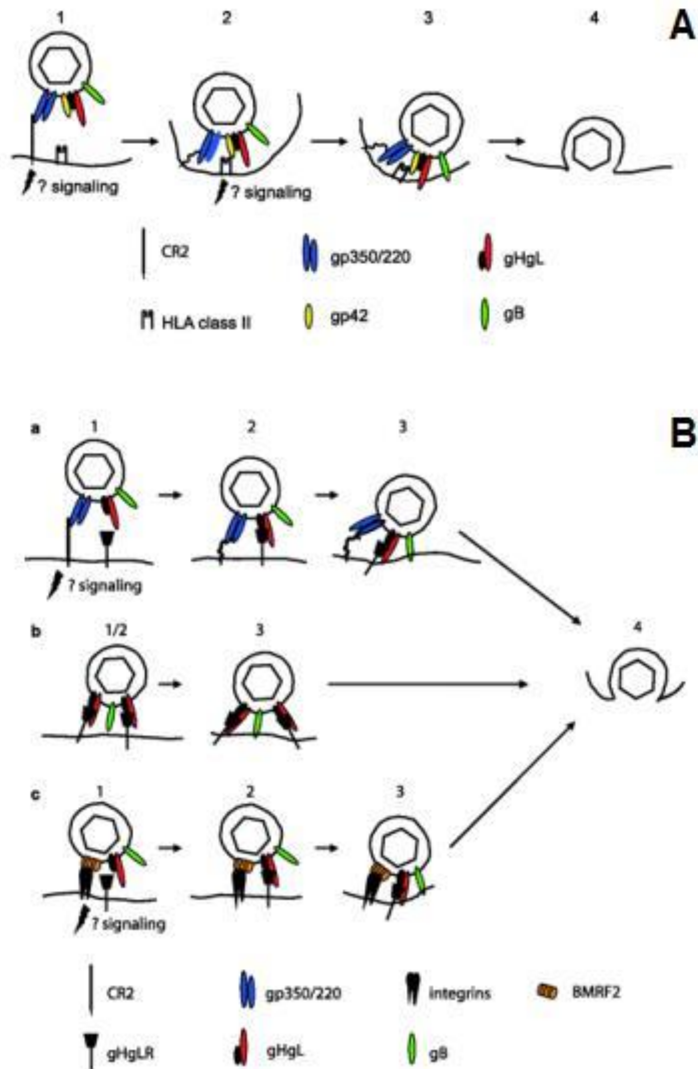

Figure 1.2. Models of EBV entry in human cells.
The different steps that have been hypothesized for virus entry are shown for B lymphocytes (A) and for epithelial cells (B). Initially, binding of a viral ligand to a cellular receptor delivers a phagocytotic signal (1). Other interactions strengthen the bond (2). Further interactions start the fusion process (3). Finally the endosomal membrane fuses with the viral envelope and the capsid enters into the cytosol (4). (Hutt-Fletcher, 2007)

In B lymphocytes, glycoprotein (gp) 350/220 on virus envelope binds to complement receptor 2 (CR2) on cell membrane (Frade R et al., 1985), inducing capping of CR2 and triggering an endocytotic signal (Tanner et al., 1987). Further interactions stabilize the bound and allow the virus to come closer to the cell membrane (Hutt-Fletcher, 2007). These interactions involve binding of viral proteins gHgL, gB and gp42 with the human

leukocyte antigen (HLA) Class II molecules, either HLA-DR, HLA-DQ or HLA-DP, on the cell membrane. The mechanisms of attachment of EBV to epithelial cells are more debated, since it is unclear whether all epithelial cells express CR2, and at what level, moreover they do not express HLA Class II molecules. Interactions of viral proteins BMRF and gHgL with a still unknown receptor and integrins $\beta1$, $\alpha3$, $\alpha5$ and αv on the cell membrane have been suggested (Hutt-Fletcher., 2007; Xiao et al., 2007). Whichever is the underlying mechanism, EBV attachment to a target cell results in fusion of the viral envelope with the endosomal membrane and release of the tegumented capsid into cell cytosol.

Although EBV infects predominantly B lymphocytes and epithelial cells, in some instances it was found to be able to infect also natural killer (NK) cells, T lymphocytes and smooth myocytes (Rickinson et al., 2007). It can also infect monocytes (Savard et al., 2000), albeit this ability seems to be restricted mainly to viruses generated in epithelial cells (Guerreiro-Cacais et al., 2004), and possibly follicular dendritic cells (DCs) (Rickinson et al., 2007).

1.1.2.2. VIRUS REPLICATION, LYTIC AND LATENT PROGRAMMES

The downstream events, leading to entry of the EBV genome into the nucleus, have still to be elucidated, and might only be inferred from comparison with other herpesviridae. Once reached the nucleus, the EBV genome can follow two different replication strategies: it can either start a lytic cycle or remain latent, depending on the transcription pattern involved (Figure 1.3). During the lytic phase viral genes regulating transcription, transduction and coding for structural proteins are active. Immediate early, early and late lytic antigens are sequentially expressed. Transcription from the linear DNA starts in B lymphocytes 10-12 hours after infection, and circularization occurs after 6 more hours

(Arvin et al., 2007), but amplification of the newly produced episomes requires more than a week (Longnecker and Neipel, 2007). The transcription of the immediate early genes leads the host cell to start the S phase of the cell cycle 24-48 hours after infection, the following gene products induce continuous proliferation of the infected B cell, and prevent its apoptosis (Tsurumi et al., 2005).

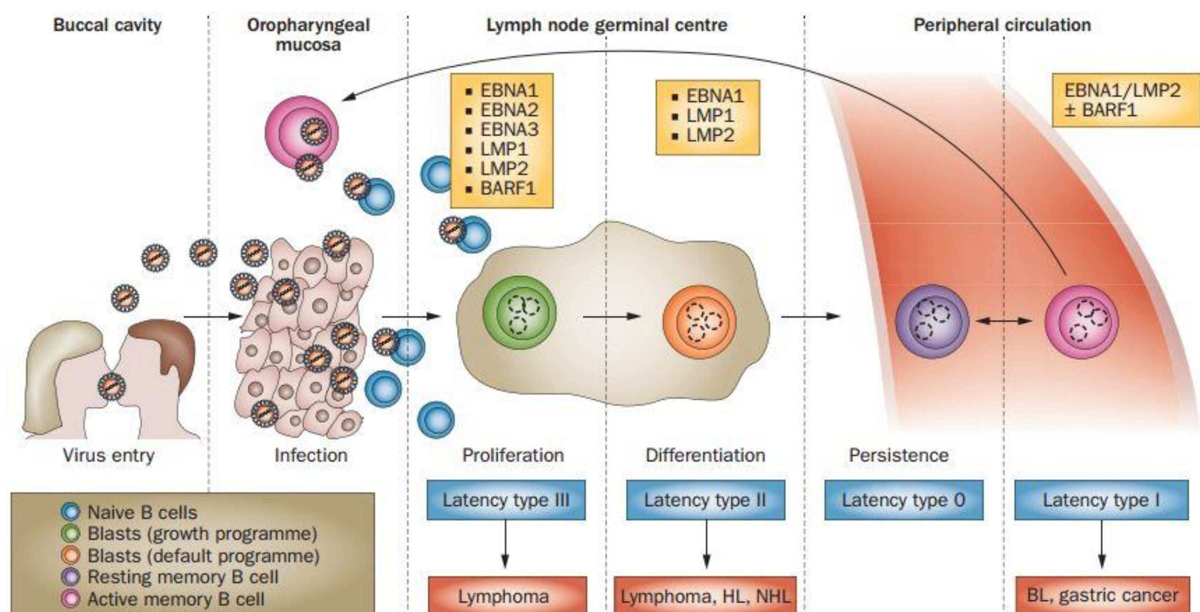

Figure 1.3. The different phases of the EBV life cycle.
During lytic phase all the genes are transcribed, starting a cascade of events that results in EBV genome replication, viral structural protein synthesis, and virion assembly. During latent phase only a limited set of viral gene products are transcribed. (Bollard et al, 2012)

During lytic replication the viral genome is amplified hundreds of times, all the proteins are transcribed, and new virions are assembled and released. This event takes place during acute infections, but virions can be occasionally found in asymptomatic carriers, indicating a sporadic activation of the lytic programme (Tsurumi et al., 2005).

Contrary to the lytic phase, the latent phase comes up with different transcription programmes, from 0 to III, depending on host cell factors. The different programmes activate different promoters, involve the expression of different genes, occur in different subtypes of normal B lymphocytes, and have different effects on the host cell (Table 1.2).

Table 1.2. The different latency programmes in B cells.
(Modified from: Various authors, 2012)

Transcription programme	Gene products expressed	Infected normal B-cell type	Function
Latency 0 (true latency)	EBERs	Peripheral memory	Allow lifetime persistence
Latency I	EBERs, EBNA-1	Dividing peripheral memory	Allow virus in latency programme cells to divide
Latency II (default)	LMP-1, LMP-2A, EBNA-1, EBERs	Germinal centre	Differentiate activated B cell into memory
Latency III (growth)	LMP-1, LMP-2A, LMP-2B, EBNA-1, -2, -3A, -3B, -3C, -LP, EBERs	Naive	Activate B cell

True latency can be found in peripheral blood B lymphocytes after resolution of primary infection. In this state the EBV DNA is circular, packaged with cellular histones, replicates only once during mitosis, and is partitioned in the daughter cells (Thompson and Kurzrock, 2004). At the opposite end, during latency III the host cell proliferation is driven by EBV and all the latency genes are expressed (Werner et al., 2007).

During primary infection lytic replication occurs in the Waldeyer ring, then the newly formed virions infect naïve B cells, inducing the latency III programme (Figure 1.4). The activated B lymphoblasts migrate into lymphoid follicles, where they initiate a germinal center reaction shifting to the latency II programme that provides the cells with signals preventing apoptosis. At this point the infected B lymphocytes return to the

blood stream as resting memory B cells, having turned to the latency 0 programme. Afterward, whenever they proliferate the latency I programme is activated.

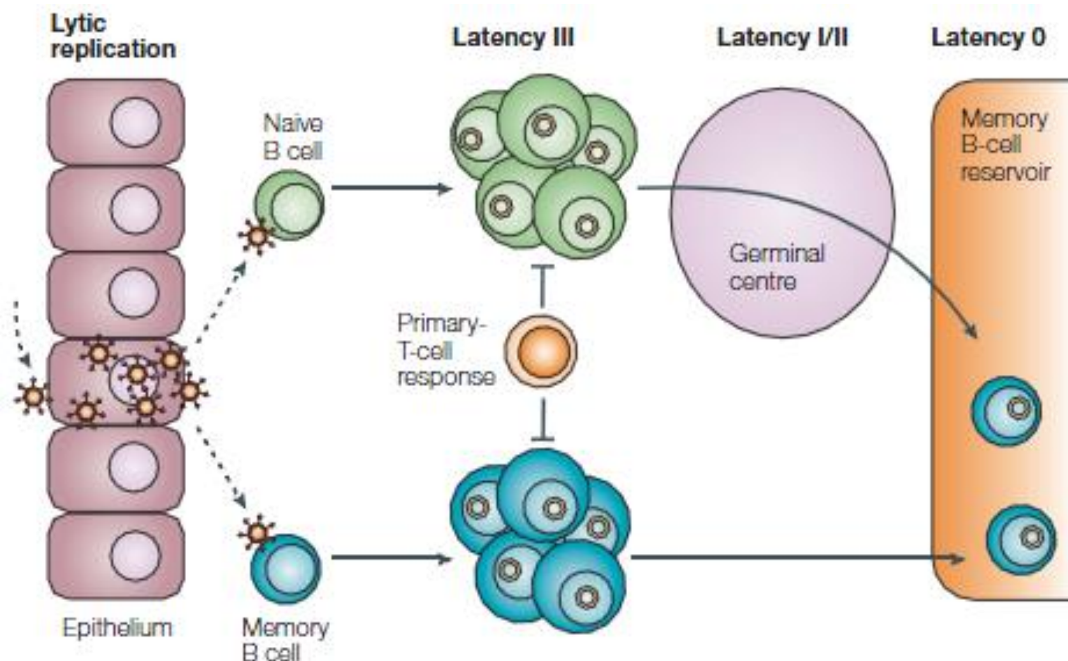

Figure 1.4. The different replication programmes used by EBV during primary infection. (Young and Rickinson, 2004)

The resting memory B lymphocytes are the long term reservoir of the virus when the primary infection is terminated.

In infected cells, EBV circularizes into an episomal form, but integration into the human genome may also occur (Morisette and Flamand, 2010). In a cohort of 104 cases of EBV-associated diseases integration was demonstrated in 11 cases (Ohshima et al., 1998).

1.1.2.3. EBV-DEPENDENT B-CELL TRANSFORMATION

The EBV is able to transform resting B lymphocytes mainly through the coordinate action of EBER, latent membrane protein (LMP), and Epstein-Barr nuclear antigen

(EBNA) molecules. EBERs and EBNA-1 effectively prevent apoptosis (Komano et al., 1998; Kennedy et al., 2003), in addition LMP1 and LMP2A provide the cell with survival signals (Maier et al., 2006; Thorley-Lawson, 2001). Moreover, EBERs induce overexpression of IL-10, which has a growth factor-like effect on B lymphocytes (Kitagawa et al., 2000). EBNA-2 and EBNA-LP induce shift from G0 to G1 phase via activation of Cyclin 2A (Sinclair et al., 1994), whilst EBNA3C supports T cell cycle (Maruo et al., 2006). The immortalized lymphoblastoid cells that are generated *in vitro* by EBV infection of B lymphocytes express the latency III programme (Young and Rickinson, 2004).

1.1.3. EBV-ASSOCIATED DISEASES

Primary infection generally occurs in the oral cavity through infected saliva (Thorley-Lawson, 2001). The incubation period ranges between 30 and 50 days, and the full-blown disease may show different degrees of severity. It has been estimated that 95 % of adult people worldwide are EBV-positive, in many cases unknowingly since infection is in most cases asymptomatic or causes mild, flu-like symptoms. This circumstance occurs predominantly in children, whilst people having primary infection during or after the second decade more likely develop infectious mononucleosis (IM), also called glandular fever in the UK. In individuals with severe immune suppression, primary EBV infection may pose a serious threat (Luzuriaga and Sullivan, 2010) and develop into fuminant haemophagocyic lymphohistiocytosis with multiorgan failure.

The EBV can immortalize B lymphocytes *in vitro* and transform human cell *in vivo*, due to the ability to induce proliferation and to inhibit apoptosis. Not surprisingly, expression of EBV antigens is associated with several hematological malignancies,

epithelial carcinomas and mesenchymal tumors, that as a whole account for about 1 % of the malignancies occurring in humans (Parkin, 2006).

1.1.3.1. CLINICALLY RELEVANT EBV PRIMARY INFECTIONS

1.1.3.1.1. Infectious Mononucleosis

Usually the presenting symptoms include pharyngitis, malaise, fatigue, fever, lymphadenopathy and splenomegaly (Odumade etal., 2011; Hurt and Tammaro, 2007). Blood analyses show lymphocytosis with atypical lymphocytes (Figure 1.5). Diagnosis is routinely made by heterophile antibody test.

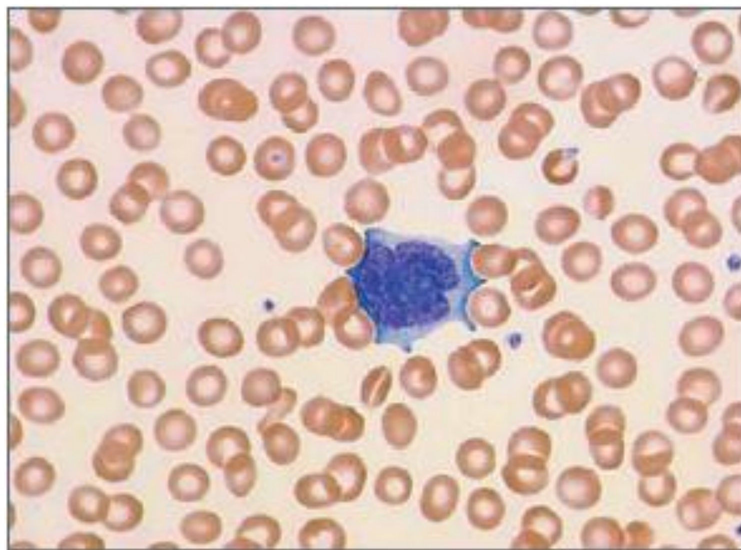

Figure 1.5. Peripheral blood smear of a case of IM, showing an atypical lymphocyte. Wright-Giemsa stain (Luzuriaga and Sullivan, 2010)

Hematological complications are frequent but generally mild; severe complications occur in about 5 % of the cases and involve splenic rupture, upper airway obstruction and neurologic complications. In rare cases primary EBV infection triggers an acquired

hemophagocytic lymphohistiocytosis, often lethal over a few weeks. Nevertheless, most of the IM patients recover spontaneously in 2-3 months, albeit fatigue may last longer (Luzuriaga and Sullivan, 2010).

1.1.3.1.2. Chronic active and fulminant EBV infections

The term chronic active EBV infection (CAEBV) indicates the persistence of primary infection in immunocompetent individuals for at least 6 months, often accompanied by life-threatening complications. These include interstitial pneumonia, hepatic failure, gastrointestinal perforation, hematophagocytosis, disseminated intravascular coagulopathy, pancytopenia (Fox et al., 2011; Cohen et al., 2011; Sonke et al., 2008). Serological analysis shows high titers of IgG antibodies against both capsid and early antigens (Fox et al., 2011). In the Western hemisphere the major cell targets are B lymphocytes, while in the other hemisphere are T lympocytes and NK cells (Fox et al., 2011). CAEBV is often associated with underlying immunosupression syndromes but is rare in itself, and overall mortality ranges between 42 % and 65 % (Cohen et al., 2011; Kimura et al., 2003).

The X-linked lymphoproliferative disease is a rare immune depression syndrome characterized by mutations in SH2D1A or XIAP genes (Coffey et al., 19987; Rigaud et al., 2006), that code for molecules involved in interactions between B- and T cells, and in regulating apoptosis, respectively (Ma et al., 2007; Wilkinson et al., 2004). The individuals with X-linked lymphoproliferative disease are highly susceptible to develop a fulminant form of primary EBV infection (Purtilo et al., 1974).

Due to the poor response to therapy in CAEBV and fulminant EBV infection, unconventional treatments are sought after and attention has focused on improving the

anti-EBV immune response through hematopoietic stem cell transplant (HSCT) or adoptive cell immunotherapy (Cohen at al., 2011; Fox et al., 2011).

1.1.3.1.3. EBV reactivation

Reactivation of EBV infection occurs after allogeneic HSCT or solid organ transplant (SOT), and is more frequent after unrelatred transplants and anti-thymocyte globulin or anti-lymphocyte globulin treatment (Hornef et al., 1995; Clave et al., 2004). Recipients of HSCT from a CB or EBV seronegative donor are at higher risk of EBV reactivation.

Reactivation is associated with reduction in immunosuppression and onset of post-transplant EBV-associated lymphomas. Rituximab, an anti cluster differentiation (CD) 20 monoclonal antibody (MoAb), used to pre-empt transformation to PTLD, decreases but not abrogates the incidence of this complication (Blaes et al., 2010).

1.1.3.2. EBV-ASSOCIATED HEMATOLOGICAL MALIGNANCIES

1.1.3.2.1. Hodgkin lymphoma

The Hodgkin lymphoma (HL) tissue is characterized by a few malignant Reed-Sternberg (RS) cells surrounded by a number of tumor-infiltrating normal cells (Figure 1.6).

The RS cells belong to the B cell lineage and, having somatic mutations in the V region of the immunoglobulin genes, probably originate form germinal centre or post-germinal centre B cells (Kapatai and Murray, 2007). However, RS cells do not express functional cell surface immunoglobulins, and in the majority of cases they do not express classical B-cell markers such as CD19 or CD20.

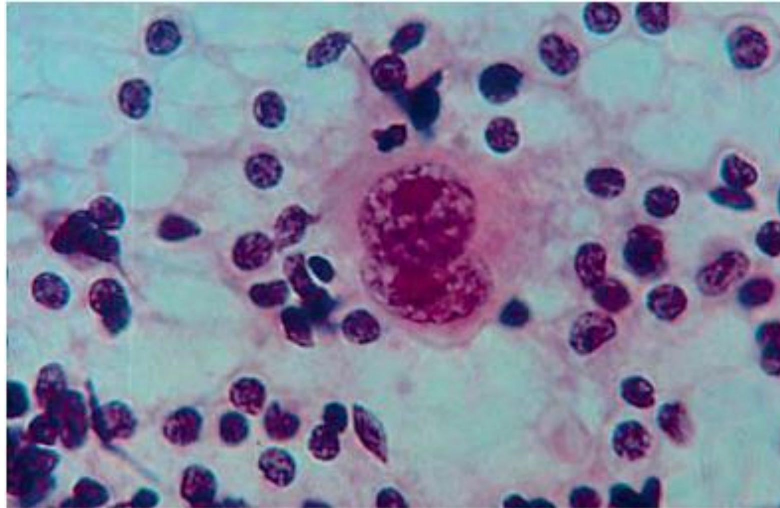

Figure 1.6. A Reed-Sternberg cell, typical of HL, surrounded by lymphocytes. Papanicolau stain. (Michelow et al, 2012).

The HL is divided into two major types: the classical HL, further divided into four histological subtypes, nodular sclerosing, lymphocyte rich, mixed cellularity and lymphocyte depleted, the latter being very rare and the nodular sclerosing the most frequent. The association with EBV expression is related to the type, being high in the mixed cellularity, intermediate in nodular sclerosis and rare in lymphocyte rich (Pallesen et al., 1991), but also to geographical and socio-economic factors: it is lower in Western hemisphere and it is higher in underdeveloped countries, ranging from 50 % of classical HL in Germany to 94 % in Peru (Herbst et al., 1992; Chang et al., 1993). The possibility that EBV plays a role in the pathogenesis of the classical form of HL is confirmed by the fact that the relative risk of developing HL is 2 to 5 times higher in individuals having had IM (Gutenshon and Cole, 1980). The EBV-positive RS cells have a latency II pattern of expression, expressing EBNA-1, EBER 1 and 2, LMP1 and 2, and the Bam H1A rightward transcripts (BARTs) (Kapatai and Murray, 2007).

1.1.3.2.2. Burkitt lymphoma

There are three forms of Burkitt lymphoma (BL): the endemic, the sporadic, and the human immunodeficiency virus (HIV)-associated form (Table 1.3).

While the two latter forms are variably associated with EBV expression, EBV is present in almost all the cases of endemic BL. High rates of association with EBV are also found in some geographical areas, such as Egypt and Brasil, where BL is not endemic but is nevertheless more frequent than in USA or Europe (Anwar et al., 1995; Klumb et al. 2004).

Table 1.3. Characteristics of the different forms of BL. (Brady et al, 2007).

	Endemic	Sporadic	HIV-associated
Distribution	Equatorial belt of Africa and Papua New Guinea	Worldwide	Worldwide
EBV association	98%	5–10%	30–40%
Co-factors	EBV, malaria infection	–	HIV infection
Incidence	5–10/100 000	0.01/100 000	Variable
MYC breakpoint	Often >1 kb upstream from 1st coding exon	Exon 1/intron 1 of *MYC* gene	Exon 1/intron 1 of *MYC* gene
Ig breakpoint	Joining (J) region, switch (S)μ in some cases	Sμ, Sα or J region	Sμ region
Progenitor cell	GC, late GC or memory B cell	GC B cell	GC, late GC or memory B cell
Frequent site of occurrence	Most frequently jaw. Abdomen, kidneys and ovaries may also be involved	Most frequently abdomen. Kidneys, bone marrow and ovaries may also be involved	Lymph nodes, abdomen, bone marrow, CNS

Although BL is quite uncommon in western countries, the endemic form accounts for 3 out of 4 malignancies in children in equatorial Africa. Plasmodium falciparum or HIV infection increase the risk of developing BL, possibly due to the suppression of the T-cell immune response induced by these pathogens (God and Haque, 2010). Mortality at 5 years is related to patient's age, ranging in the USA from about 25 % in children to about 75 % in elderly (Mbulaiteye et al., 2010)

BL is originated by the translocation of the Myc oncogene into one the V(D)J region of immunoglobulin genes on chromosomes 14, 2 or 22. Tumor cells in BL express B cell markers such as CD19, CD20 and CD22, and express immunoglobulins at the cell surface, generally IgM (Brady et al., 2007; Weils et al., 1982). In general they also show

features of germinal centre centroblasts, expressing CD10 and B-cell lymphoma 6 (BCL6), and having undergone hypermutation (Blum et al., 2004; Chapman et al., 1998). However, there is evidence of intraclonal heterogeneity, and a possible origin of BL also from memory B cells has been suggested (Brady et al., 2007). Diversity is in part related to the EBV status: EBV-negative BLs show lower mutation rates and no sign of antigen selection (Bellan et al., 2005). In the EBV-positive BL cells, generally only the EBNA-1 and the EBER 1 and 2 are expressed (Brady et al., 20076).

1.1.3.2.3. Post-transplant lymphoproliferative disorders

The term post-transplant lymphoproliferative disorders (PTLD) encompasses several lymphomatous diseases, ranging from myeloma to B-cell or T-cell lymphoma, arising in patients having received HSCT or SOT. The most common histology seen is that of Diffuse Large B-Cell Lymphoma (DLBCL) The onset of the disease is related to therapeutic immune suppression. The incidence of PTLD is variable, being about 4 % in HSCT and 1.2 % in SOT (Sundin et al., 2006; Dharnidharka et al., 2002). In the latter case the incidence is related to the type of organ transplant, being highest in small bowel and in thoracic organ transplantation and lowest in kidney transplantion (Gottshalk et al., 2005; Trappe et al., 2012). The frequency of EBV expression in B-cell PTLD is about 80 %, a little less in T-cell lymphomas (Michelow et al., 2012). The EBV-positive PTLD occurs mainly in children, develops earlier, and has a better prognosis than the EBV-negative one (Michelow et al., 2012).

Noteworthy, a study performed on PTLD after HSCT showed that the disease arises mainly in patients having received a T-cell depleted preparation or a treatment with anti-thymocyte immunoglobulins (van Esser et al., 2001). Immune suppressive treatment for GVHD is also a major risk factor (Barker et al., 2001). Complete resolution of EBV-

positive PTLD can be achieved through reduction of the immune suppression (Shaffer et al., 2010). Taken together, these data support the concept that PTLD needs a severely depressed immune environment in order to emerge. In the United Kingdom, where alentuzumab, an anti CD52 MoAb, is commonly used for T cell depletion, PTLD is less frequent, probably due to the concomitant eradication of B cells by the drug.

The timing of emergence of PTLD differs between HSCT and SOT, and this might have an impact on a hypothetical adoptive T-cell therapy (ATCT). The median onset of PTLD is at day 78 after HSCT (Sundin et al., 2006), i.e. when the patient is still severely immune suppressed. Instead, the median onset of PTLD after SOT is 48 months (Evens et al., 2010), when patients are on maintenance immunosuppression. Cases occurring after 10 years post-SOT account for more than 10 %.

The pattern of latent gene expression in PTLD is the most extensive, categorised as latency III, consisting of EBERs, LMP1 and 2, and all the EBNA proteins (Rickinson and Moss, 1997; Michelow et al., 2012), but also some lytic antigens are expressed, mainly BZLF1 (Montone et al., 1996; Oertel et al., 2002). More than 50 % of patients respond to the anti CD20 MoAb rituximab, however relapse occurs frequently, also due to downregulation of CD20 expression by the tumour cells (Moosman et al., 2010).

1.1.3.2.4. Diffuse large B cell lymphoma of the elderly

The diffuse large B cell lymphoma (DLBCL) of the elderly is a subtype of DLBCL occurring in people over 50 years of age and without immunodeficiency or history of lymphoma, and is characterized by rapid progression and poor response to therapy. Despite the name, a disease with the characteristics of the DLBCL of the elderly can also be found in young individuals (Castillo et al., 2016). As it is rare, little is known of it. Tumour cells derive from post-germinal centre B cells, express CD19, and frequently

also CD20 and CD30. The latency patter is unclear, some claiming that EBV positive cells display the latency III type (Castillo et al., 2011), while others suggesting that most of the tumours express LMP1 and 1 out of 3 also EBNA-2 (Adam et al., 2011).

1.1.3.2.5. Primary central nervous system lymphoma

The primary central nervous system lymphoma (PCNSL) is in general associated to HIV infection. In these cases the presence of EBV can be detected in 90 % of patients, whilst in immunocompetent patients EBV is generally absent (Fine and Mayer, 1003). Tumour cells express the B-cell markers CD19, CD20 and CD22. LMP1 and EBER 1 are expressed in almost all the EBV-positive PCNSLs, and EBNA-1 only in half of them (Guterman et al., 1996). Other studies indicate a broader pattern of latent gene expression, particularly in those associated with HIV (Michelow et al., 2012).

1.1.3.2.6. Lymphomatoid granulomatosis

This is a very rare extranodal lymphoproliferative disease with extensive angiodestruction, almost invariably associated with EBV expression. In lymphomatoid granulomatosis, EBV-positive tumor cells are of B lineage and have a pattern of latent gene expression of type III (Dunleavy et al., 2012).

1.1.3.2.7. Extranodal T- and NK-cell lymphomas

Although B-cells are the preferential target of EBV, the virus can infect and transform also NK cells, NK/T cells and T lymphocytes, either $CD4^+$ or $CD8^+$ ones. Actually, EBV is associated with a number of non-B lymphomas, such as immunoblastic T-cell lymphoma, cutaneous T-cell lymphoma, aggressive NK cell leukemia/lymphoma and nasal NK/T cell lymphoma (Carbone et al., 2008). The latter, like the nasopharyngeal

carcinoma, is more common among orientals, in particular Chinese. EBV is present in almost all of these tumors (Dunleavy et al., 2012). Tumour cells from extranodal T- and NK-cell lymphomas express CD3, CD56, CD2, granzyme B and perforin, but not CD4 and CD16, and only occasionally CD8 (Michelow et al., 2012; Fox et al., 2011). The latency pattern is of either type I or type II, with predominance of the latter type (Michelow et al., 2012; Dunleavy et al., 2012; Fox et al., 2011). Interestingly, in this disease LMP2 is expressed mainly in a truncated form (Fox et al., 2010).

1.1.3.2.8. Other lymphomas

EBV expression can occasionally be found in the other lymphoproliferetive diseases.

In the rare plasmablastic lymphoma, EBV expression was described in cases associated with HIV infections (Ferrazzo et al., 2007).

In the primary effusion lymphoma the association with EBV is variable (Michelow et al., 2012); in the positive cases tumor cells express EBNA-1 and low levels of LMP1 and LMP2 (Horenstein et al., 1997).

A few cases of secondary B-cell lymphomas have been described in patients with angioimmunoblastic T-cell lymphoma. They are EBV-positive large B-cell lymphomas, often with extranodal localization, and are supposed to arise as result of the severe immune suppression associated with the T-cell lymphoma (Yang et al., 2012; Smeltzer et al., 2012).

1.1.3.3. EBV-ASSOCIATED SOLID TUMORS

1.1.3.3.1. Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a rare tumor in western countries, but is very common in south-eastern China, being the 3rd most common cancer in men and, to a

lesser extent, among Inuits (Yu and Yuan, 2002). It is an aggressive epithelial carcinoma originating from nasopharyngeal mucosa: tumour cells are positive for cytokeratins and do not express CD45. Three histological types have been identified: the keratinizing squamous cell carcinoma, the non-keratinizing squamous cell carcinoma, and the basaloid squamous cell carcinoma. The former type does not express EBV antigens; in the other types expression is close to 100 % but is restricted to EBNA-1, LMP2, EBERs and BARTs, LMP1 being variably expressed (Michelov et al., 2012; Dawson et al., 2012).

The fact that NPC is mainly found amongst Chinese people suggests other aetiological factors in addition to EBV. There is some evidence of genetic predisposition; the tumour often runs within families and certain human leukocyte antigen (HLA) antigens seem to predispose to the disease such as HLA B46. Environmental factors have also been blamed, in particular it was observed that the disease is found in regions with high consumption of preserved salty fish. Although over 70% of the patients can be cured by radiotherapy if they present in early stage, late stage disease and refractory disease have poor prognosis.

1.1.3.3.2. Lymphoepithelial-like gastric carcinoma

This tumour accounts for about 10 % of gastric carcinomas (Chen et al., 2012). As compared to gastric adenocarcinoma, the lymphoepithelial-like gastric carcinoma affects the upper portion of the stomach, arises in younger patients, has a better prognosis, and EBV positivity is much higher, 80 % instead of 16 % (Deyrup, 2008; Delecluse et al., 2007). EBV-positive gastric carcinomas express EBNA-1, EBERs, BARTs, and in 40 % of the cases LMP2A (Iisaza et al., 2012). It is of note that also lytic gene transcripts have

been found in EBV-positive gastric cancer samples, albeit immunohistochemistry has failed to detect lytic antigens (Tang et al., 2012; Osato and Imai, 1996).

1.1.3.3.3. EBV-associated smooth muscle tumors

These mesenchymal tumors are quite rare, and are frequently multifocal but metastases have not been described, so surgery is the first-line treatment. The pattern of latent gene expression is unclear, the cases investigated being a few and the results obtained contradictory (Deyrup, 2008).

1.1.3.3.4. Follicular dendritic cell tumor

The origin of this rare, low-grade tumor generally affecting young people is debated, whether hematopoietic or mesenchymal (Deyrup, 2006). Tumor cells express CD21, CD35, vimentin and fascin, and show variable expression of CD45. EBV is present in 30 % of tumors (Michelov et al., 2012). Since LMP1 is a common finding in EBV-positive cases (Cheuk et al., 2001), the latency should be of type II or III, but further studies on larger cohorts are required.

1.1.3.4. OTHER DISEASES POSSIBLY ASSOCIATED WITH EBV

Whilst the association of EBV with the diseases mentioned above is widely accepted, the association with other diseases is controversial.

EBV DNA has been found with some frequency by the highly sensitive polymerase chain reaction (PCR) in breast cancer samples, however the detection of viral gene transcripts or proteins has been occasional and has raised a number questions; it seems

that EBV infection of mammary cells is rare, and the cells containing the viral genome in breast tumour are very few (Huang et al., 2003).

Some evidences, albeit still inconclusive, have been found for a role of EBV in the pathogenesis of systemic lupus erythematosus, multiple sclerosis and myocardial infarction (Draborg et al., 20102; Tselis, 2012; Binkley et al., 2013).

1.2. T LYMPHOCYTES

1.2.1. T-CELL DEVELOPMENT AND DIFFERENTIATION

T cells develop in the foetus from common lymphoid progenitors committed for T cell differentiation, which migrate from bone marrow to the thymus. There they undergo processes of selection and maturation that lead thymocytes to become mature naïve T lymphocytes (T_N), which enter the bloodstream. Upon antigen encounter with the cognate antigen, T_N progressively differentiate into memory T lymphocytes.

1.2.1.1. THYMIC MATURATION

T-cell precursors entering the thymus do not express most of the markers characteristics of mature circulating T lymphocytes, including both CD4 and CD8 molecules (Hernandez et al., 2010). These markers will be expressed following the activation of a proliferation and differentiation programme triggered by the interaction with thymic stromal cells. The first molecule of the T cell receptor (TCR) complex to be expressed by double negative T cells is CD3, afterward the complex process of rearranging the TCR genes starts (Murphy et al., 2011). A failure in generating a functional TCR leads to cell apoptosis, instead a successful rearrangement of either γ and δ or α and β genes results in generation of γδ or αβ T cells, respectively. During the later steps of TCR gene rearrangement the αβ T cells express both the CD4 and CD8 molecules, becoming double positive T cells. At this point the surviving thymocytes are selected on the basis of their affinity for the self HLA Class I or Class II molecules loaded with self peptides. T cells with a TCR that does not recognize self HLA/self peptide complexes undergo apoptosis by neglect. T cells expressing a TCR with high avidity for self HLA/self

peptide complexes are negatively selected, and in this way the vast majority of potentially autoreacting T cells is deleted. Finally, T cells expressing a TCR with weak avidity for self HLA/self peptide complexes receive survival signals, for which they are positively selected (Hernandetz et al., 2010).

However, some cells expressing a TCR with high affinity for a self peptide escape negative selection and become thymic-derived regulatory T cells (T_{regs}) (Jordan et al., 2001). These cells do not proliferate and do not secrete cytokines when challenged with the cognate antigen but suppress the proliferation of normal T cells and contribute to the maintenance of peripheral tholerance.

Double positive T cells undergoing positive selection retain the expression of the sole CD8 or CD4 molecule, depending on whether their TCR interacts with Class I or Class II HLA molecules respectively, becoming single positive, mature T lymphocytes. Due to failure in rearranging TCR genes or to inappropriate interaction with self HLA/self peptide complexes, 98 % of T cells developing in thymus die off, and only 2 % exit thymus as mature T lymphocytes (Murphy et al., 2011).

In humans the thymus is fully developed at birth and the rate of T lymphocyte production remains high up to puberty; afterward the organ shrinks, and production of T lymphocyte progressively drops, albeit generation of mature T lymphocytes occurs at low rate for the rest of the life (Murphy et al., 2011).

1.2.1.2. The role of Interleukin-7 in thymus

The orderly sequence of events leading to the generation of mature T lymphocytes is orchestrated through cell to cell signals exchanged between T cells and thymic stromal cells, and through cytokines present in the extracellular milieu. In this regard a crucial role is played by interleukin (IL)-7, secreted by non-hematopoietic thymic epithelial

cells (Hong et al., 2012). In particular IL-7 induces proliferation and differentiation of both T-cell precursors and double negative T cells (Varas et al., 2009; Yarilin and Beliakov, 2004), increases the rearrangement of α and β TCR genes (Okamoto et al., 2002), and promotes, with IL-15, the specification of $CD8^+$ T lymphocytes (McCaughtry et al., 2012).

1.2.2. MATURE T LYMPHOCYTES

Following antigenic stimulation T_N start a differentiative programme that results in the cells progressively losing their replicative potential while acquiring increasing effector functions.

1.2.2.1. NAÏVE T LYMPHOCYTES

Mature T lymphocytes egressing from the thymus are classified as naïve (T_N), in that they have not yet encountered the antigen recognized by the TCR they express. T_N are phenotypically characterized by the co-expression of chemokine receptor (CCR) 7, CD45RA and CD62L (Figure 1.7).

A small percentage in this subset is represented by recent thymic emigrants, young T cells which differ from classical T_N cells both phenotypically and functionally (Berkley et al., 2013).

T_N lymphocytes continuously shuttle between lymphoid tissues via blood and lymphatic vessels. In doing so the $CD8^+$ T lymphocytes come in contact with the complexes between peptides and HLA Class I molecules expressed by all the nucleated cells, and the $CD4^+$ T lymphocytes come in contact with the complexes between peptides and HLA Class II molecules expressed by antigen presenting cells (APCs), mainly DCs. The

encounter of a T_N lymphocyte with the cognate antigen starts a differentiation programme that leads the cell to expand clonally and to acquire a different phenotype and new functions characteristic of memory T lymphocytes.

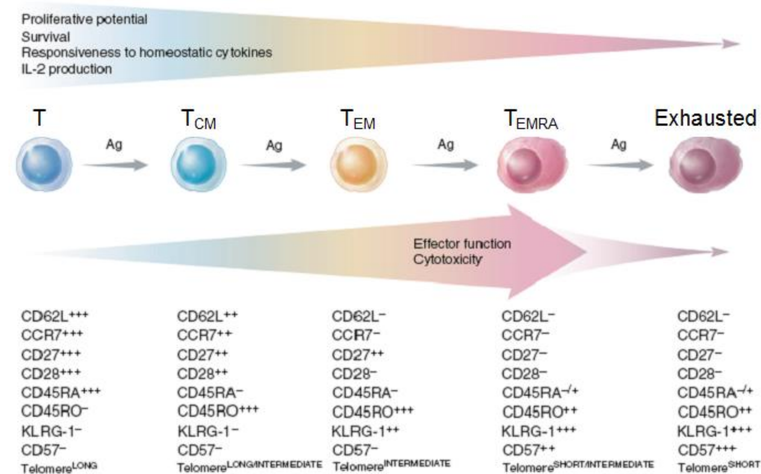

Figure 1.7. Antigenic stimulations induce progressive differentiation of mature T lymphocytes. The differentiative steps are characterized by change in phenotype, progressive loss of proliferative potential and increase in effector functions. After prolonged antigen stimulations, T lymphocytes become exhausted, non functional and eventually die. (Klebanoff et al., 2006)

1.2.2.2. MEMORY T LYMPHOCYTES

1.2.2.2.1. Memory T lymphocyte subsets

The initial antigenic stimulation leads T_N lymphocytes to become central memory T (T_{CM}) lymphocytes, lose the CD45RA expression and acquire the expression of the RO isoform (Figure 1.8). Following further antigenic stimulation, T_{CM} become effector memory T (T_{EM}) and lose the expression of CD62L and CCR7, which targets T lymphocyte homing to T-cell areas of lymph nodes, becoming able to infiltrate tissues and inflammatory sites (Klebanoff et al., 2006). The final step of differentiation results,

after more antigenic stimulation, in T_{EM} lymphocytes becoming CD45RA-positive (T_{EMRA}).

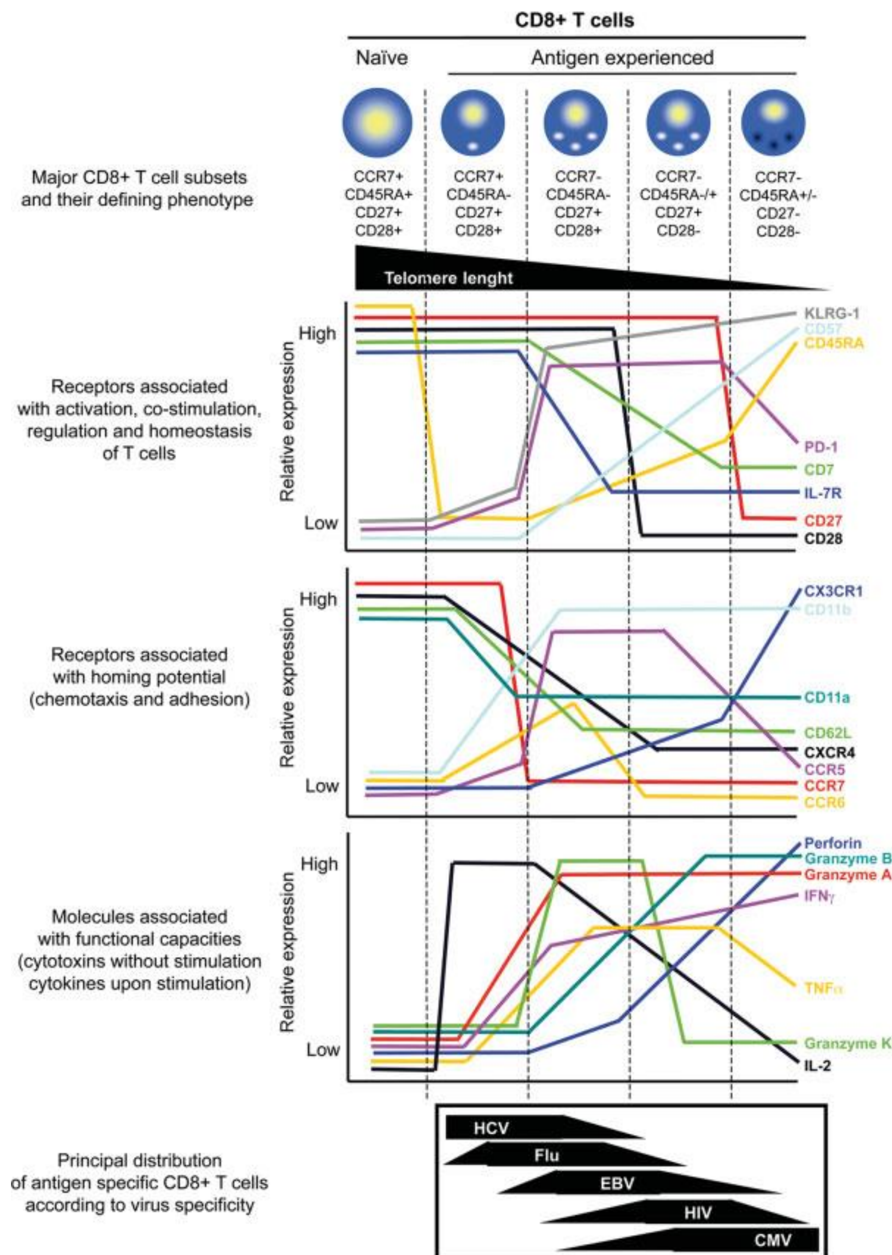

Figure 1.8. Changes in membrane and intracellular markers associated with CD8$^+$ **T lymphocyte differentiation.** Repeated antigenic stimulation leads CD8$^+$ T lymphocytes to shift their phenotype, function and homing characteristics. The response profile to some viruses is shown. HCV: hepatitis C virus, Flu: influenza virus, CMV: cytomegalovirus. The authors have split T_{EMRA} into two cell subsets on the basis of CD27 expression. (Appay et al., 2008)

During the differentiation of CD8$^+$ T lymphocytes from T_N to T_{EMRA} the different subsets progressively lose their capacity to proliferate and secrete IL-2 and acquire the ability to release IFNγ and to exert cytotoxicity, due to the newly acquired ability to express perforin and granzymes, and to degranulate. At the same time telomeres undergo progressive shortening (Klebanoff et al., 2006; Appay et al., 2008). Other changes in phenotype and function take place during CD8$^+$ T-cell differentiation, involving in particular membrane proteins regulating cell homing, activation and homeostasis.

Similar changes occur during differentiation of CD4$^+$ T lymphocytes (Figure 1.9), although these phenomena are better known for CD8$^+$ T lymphocytes than for CD4$^+$ ones (Appay et al., 2008).

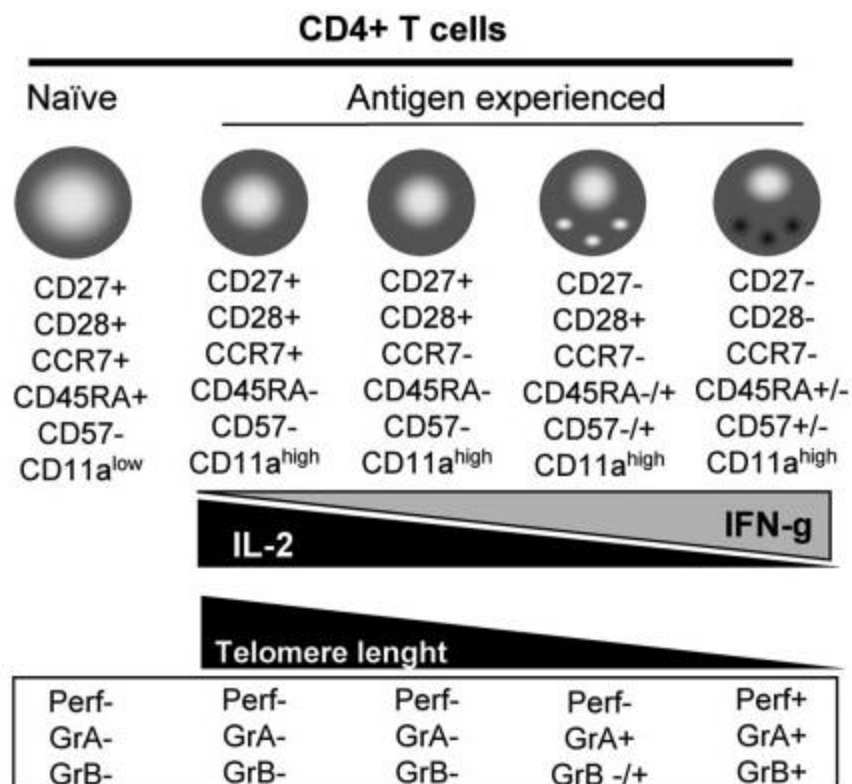

Figure 1.9. Changes in the expression of membrane and intracellular markers associated with CD4$^+$ **T lymphocyte differentiation.** Repeated antigen stimulationleads CD4$^+$ T lymphocytes to modify their phenotype and functions. Perf: perforin, GrA: granzyme A, GrB: granzyme B. (Appay et al., 2008)

Altough most of the markers follow the same trend during memory cell differentiation in $CD4^+$ and $CD8^+$ T cells, some differ in the two cell subsets, like CD11a.

It has to be noted that the phenomenon of T lymphocyte differentiation is more a continuum than a progression through discontinuous steps, therefore any categorization into subsets is a bit factitious. The most widely used classification is based on the expression of two markers, CCR7 and CD45RA, but also other categorizations have been proposed, for instance by further dividing the T_{EMRA} subset into $CD27^+$ and $CD27^-$ (Appay et al., 2008).

In the peripheral blood of immune individuals the $CD8^+$ T lymphocytes directed against different pathogenic viruses have different subset profiles, the response against latent EBV infection involving predominantly the T_{EM} and early T_{EMRA} subsets ((Figure 1.8).

1.2.2.2.2. Memory T cells expressing a naïve phenotype

Two subsets of phenotypically naïve $CD8^+$ T cells with stem cell features and memory traits that have enhanced differentiative and proliferative potential have been recently described.

T-memory stem cells (T_{SCM}) have been proposed to constitute the precursor memory T-cell pool, which lies between T_N and T_{CM} in the differentiation pathway (Gattinoni et al., 2011). Despite displaying some phenotypic features in common with T_N cells, such as co-expression of CD45RA and CCR7, and showing ability of self-renewal, T_{SCM} exhibit functional characteristics of antigen-experienced cells. These cells are supposed to originate from naïve cells, but upon antigenic stimulation they rapidly expand and differentiate into memory subsets (Figure 1.10) but also retain the capacity to self-renew. For these reasons T_{SCM} are currently regarded as the best candidates for ATCT

(Restifo et al., 2012). They represent a small percentage of circulating T_N (Gattinoni et al., 2011).

The recently described memory T cells with naïve phenotype (T_{MNP}) represent a subset of antigen-experienced $CD8^+$ T cells with naïve phenotype, high proliferative potential and polyfunctional capability (Pulko et al., 2016), but it is unclear how they are related to T_{SCM} and how thet are generated. Also these cells might be a powerful tool for ATCT.

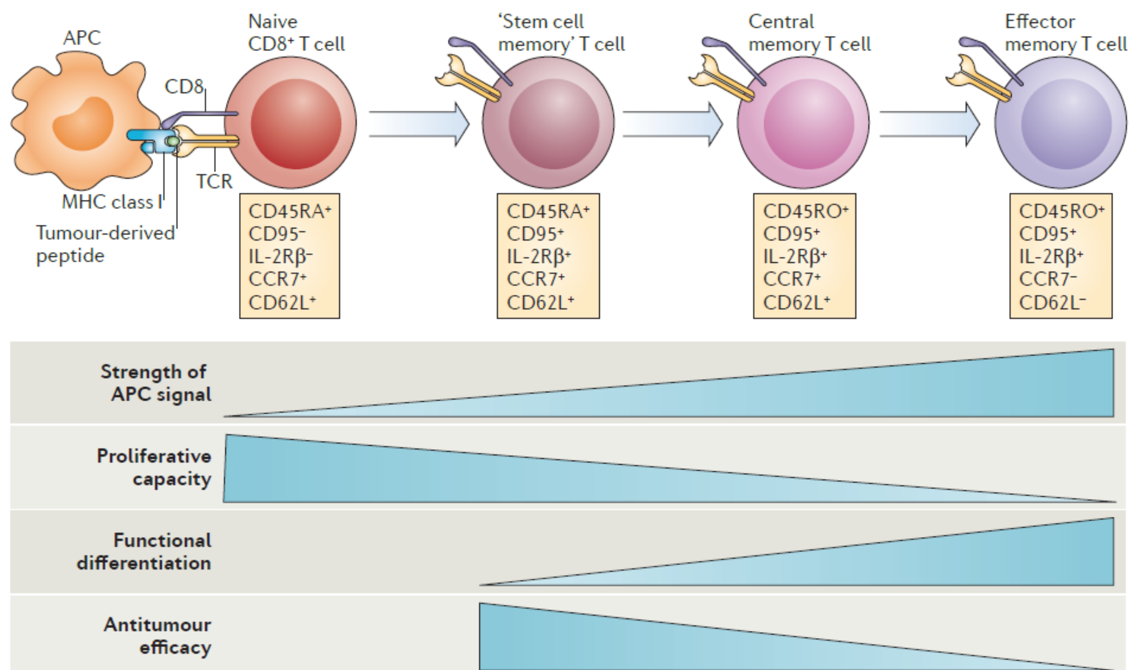

Figure 1.10. Phenotypic and functional characteristics of the early differentiation stages of $CD8^+$ T cells. T_{SCM} represent the first differentiation stage after engagement with the cognate antigen, and seem to have the highest anti-tumor effect (Restifo et al 2012)

1.2.2.2.3. Role of interleukins in T lymphocyte expansion

The cytokine that plays a major role both *in vitro* and *in vivo* in driving proliferation and differentiation of T lymphocytes is IL-2 (Murphy et al., 2011). Instead, IL-7 supports the

homeostatic expansion of T_N outside the thymus. $CD8^+$ T_N lymphocytes activated in the presence of IL-7 expand retaining their naïve phenotype (Vieira et al., 1998; Tan et al., 2001). Moreover, in $CD8^+$ T_N lymphocytes, IL-7 without other co-stimuli prevents apoptosis and, like IL-15, induces expansion of the cells without loss of their phenotype and reduction in telomere length (Wallace et al., 2006).

1.2.2.2.4. T lymphocyte exhaustion

T-cell exhaustion was initially described in mice during chronic viral infections (Gallimore et al., 1998), but the phenomenon was later found to occur also in humans, in association with chronic infection with CMV, HIV or hepatitis B or C virus, or with cancer, and in elderly people (Fletcher et al., 2005; Wherry, 2011; Baitsch et al., 2011; Trzonkowski et al., 2010). Initially, exhaustion can be reverted by removing the activating stimulus, but with time it becomes irreversible (Blattman et al., 2009). The different functions are lost following a precise sequence. $CD8^+$ T lymphocytes first lose the ability to secrete IL-2, to proliferate, and to exert cytotoxicity; afterward the ability to secrete tumor necrosis factor (TNF) is lost; lastly the cells become unable to produce interferon (IFN) γ and to degranulate (Wherry, 2011). Also $CD4^+$ T lymphocytes lose their functions sequentially, but the order the functions are lost is less clear. Eventually, exhausted T lymphocytes undergo apoptosis.

Membrane receptors, like PD-1, LAG-3, CTLA-4, and TIM-3 are known to be involved in T lymphocyte exhaustion and the transcriptional and epigenetic mechanisms involved are being discovered (Pereira et al., 2017). All these molecules are promising targets for checkpoint inhibitor therapy.

1.2.3. ANTIGEN PROCESSING AND PRESENTATION

Protein antigens are not presented to T cells in their native form but are first digested into short peptides, and the presentation pathway follows different routes for the antigens presented via HLA Class I and those presented via HLA Class II.

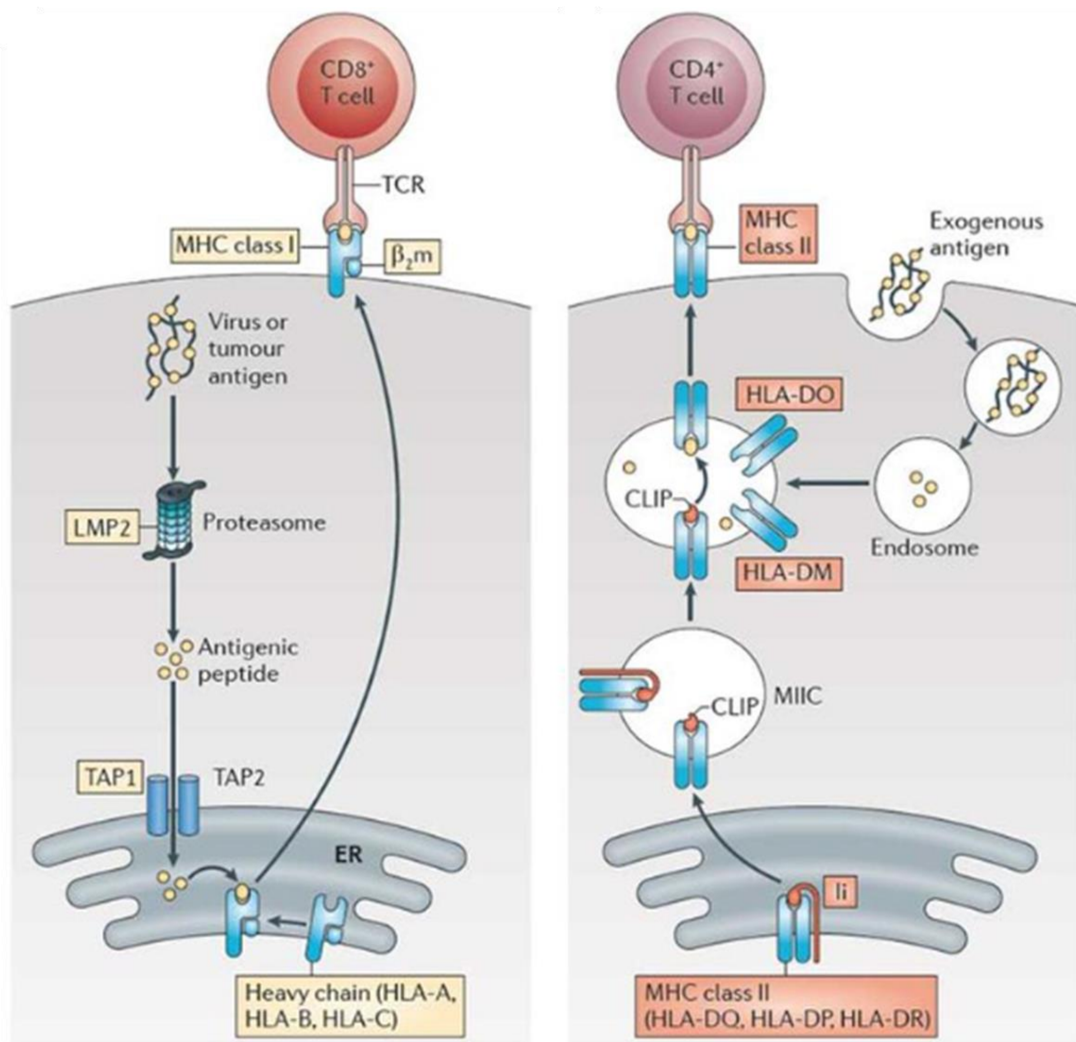

Figure 1.11. The mechanisms of antigen presentation via Class I and Class II. Endogenous peptides are generated in the cytosol by proteolytic clivage, transferred into the ER where they may be bound by the newly synthesized Class I molecules. Exogenous peptides are generated in the phagolysosomes from digestion of endocytosed particles and may be bound Class II molecules (Kobayashi and van den Elsen, 2012).

1.2.3.1. ANTIGEN PROCESSING AND PRESENTATION VIA CLASS I

HLA Class I molecules are expressed by all the nucleated cells of the body, and in general they participate in monitoring the inner antigens, presenting peptides derived from degradation of endogenously synthesized proteins (Figure 1.11). The misfolded or denatured proteins, together with part of the newly synthesized ones, are ubiquitinated and transported to the proteasome where they undergo enzymatic digestion generating both linear and spliced peptides (Vigneron et al., 2017). The resulting peptides ranging in length from 8 to 16 residues are then transferred into the endoplasmic reticulum (ER) via the coordinated activity of transporter associated with antigen processing (TAP) 1 and 2 (Neefjes et al, 1993). Associated with these molecules are calreticulin, ERp57 and tapasin (Sadasivan et al., 1997; Dong et al., 2009), which mediate the interaction between the newly assembled HLA Class I molecules and the translocated peptides that display the required characteristics, i.e. generally 9 residues in length and the appropriate common motif. Once the peptide is stably bound to the Class I binding groove the peptide-loading complex breaks down and the Class I-peptide comples is transported to the cell surface.

DCs can take up exogenous antigens and present them in association with Class I (Bevan, 1976), although it is not clear which DC subset exerts cross presentation in humans (Joffre et al., 2012).

The complex Class I-peptide is, with rare exceptions (Bendelac et al., 1994), recognized by $CD8^+$ T lymphocytes

1.2.3.2. ANTIGEN PROCESSING AND PRESENTATION VIA CLASS II

HLA Class II molecules are expressed by APCs, i.e. DCs, B lymphocytes, macrophages and by activated T cells, and they contribute to predominantly monitor the extracellular milieu (Figure 1.11). In the ER the newly synthesized Class II molecules are associated with the invariant chain (CD74) which partakes to the correct assembling of the complex and largely prevents the bindig of peptides by blocking with its distal portion (CLIP) the Class II peptide binding site. CD74 also acts as a chaperon, transferring the complex to the endocytic pathway, where are also targeted the particles and molecules internalised via endocytosis, pinocytosis or autophagy. In the phagolysosome the CD74 molecule is degraded by the lysosomal proteases, particularly by cathepsin S (Riese et al., 1996), the CLIP peptide and other peptides eventually present in the peptide binding groove are uptaken by the non-classical HLA complex DM (Denzin and Cresswell, 1995), and the binding site is now free to bind the peptides that are generated by the proteolytic machinery, in particular by cathepsins F, L and S (Shi et al., 2000; Hsieh et al., 2002). In this case the bound peptides are longer that those bound by Class I, generally ranging from 12 to 15 residues in length, although longer peptides can be bound and then trimmed afterwards, and the motif requirements are less stringent.

The complex Class II-peptide is generally recognized by $CD4^+$ T lymphocytes, although in rare instances it may engage the TCR of $CD8^+$ T cells (Kirberg et al., 1994).

1.2.3.3. MECHANISMS OF PEPTIDE EDITING AND BINDING TO HLA MOLECULES

The number of peptides that can be generated by digestion of all the potential non-self proteins is huge, therefore mechanisms are in place to restrict this number to a size that could be managed by the immune system.

Some mechanisms are operative during processing. It was known for a long time that the requirements for clivage by the proteasome enzymes, and those for transport by TAP molecules reduce the number of the peptides available in the ER, but more recently it has been demonstrated that also tapasin contributes to the peptide editing for Class I. In fact, only peptides with high affinity can close the Class I binding groove, nullifying the effort of tapasin to keep it open (Fisette et al., 2016). For Class II the number of peptides available for binding is mainly determined by the cleavage properties of the lysosome enzyme, but it was suggested that also the complexes DM and DO might contribute to modulate the repertoire of peptides available for binding (Sloan et al., 1995; van Ham et al., 2000).

Whether a peptide will be bound or not by an HLA molecule depends on the respective amino acid sequences. Since in HLA molecules the polymorphic sites are located mainly in the peptide binding groove, different HLA molecules have sterically different grooves and will bind peptides with different amino acid sequence. It has also to be considered that only some amino acid residues in the peptides interact with the HLA molecule, while others point to the outside, to be eventually recognized by a TCR. Figure 1.12 shematically shows the interactions of the peptide with the TCR and the HLA molecules.

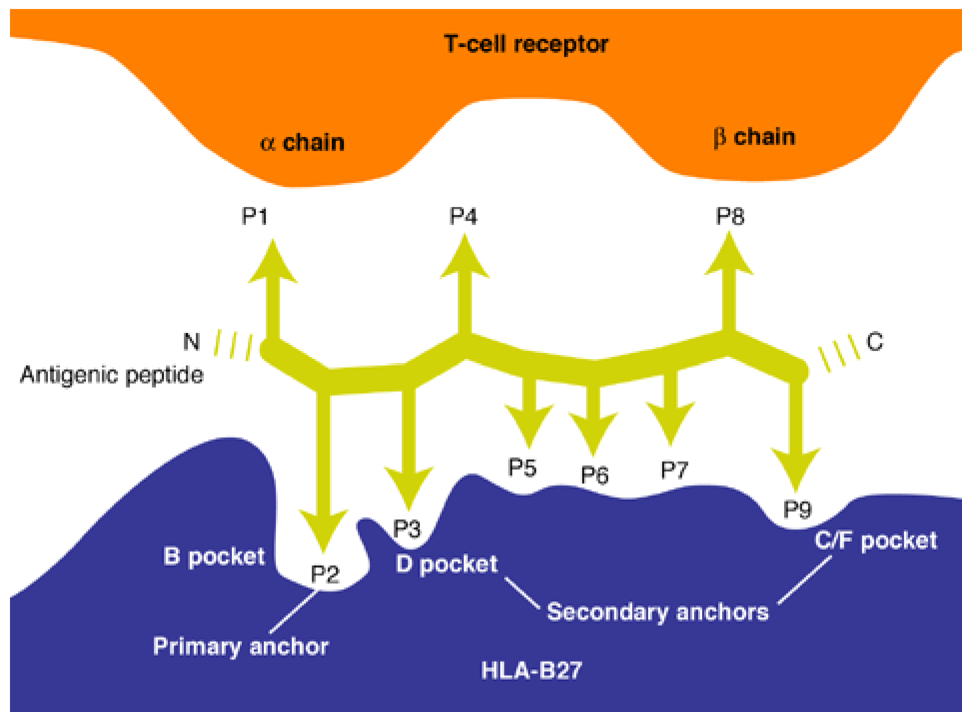

Figure 1.12. Scheme of the interactions of the peptide with the TCR and the HLA molecules. Some peptides residues anchor the peptide to specific pockets in the HLA binding groove, while others interact withn the TCR. (Bowness et al., 1994)

Generally, a peptide binds to Class I with the residues in position 2 and 9, which represent the primary anchor positions and interact with specific pockets, i.e. pocket B and F, in the peptide binding groove. Since these pockets differ in the different Class I alleles, the peptides bound to a given Class I allele share the same characteristics at the anchor positions, that is, display a common motif. For instance, the common motif for HLA-A*02:01 is leucine or methionine at position 2 and valine or leucine at position 9. The requirements for peptide binding to Class II molecules are less stringent, for instance the anchor positions for HLA-DRB1*0101 are 1, 4, 6 and 9, each displaying preference for at least six different amino acids. Not surprisingly, Class II molecules may display degenerate binding (Southwood et al., 1998).

Using experimental data and computational analysis, some alorithms have been created which predict the chances of a given peptide to be bound by a given HLA molecule. The fist was SYFPEITHI (Rammensee et al., 1999; http://www.syfpeithi.de/) and several others have followed, till the two just published For Class II (Jensen et al., 2018; http://www.cbs.dtu.dk/services/NetMHCIIpan-3.2/ and http://www.cbs.dtu.dk/services/NetMHCII-2.3/). Useful in HSCT and SOT is PIRCHE (https://www.pirche.org/pirche/#/), which calculates the permissibility of HLA mismatches between recipient and donor by evaluating the relevance of indirect recognition of the peptides resulting from the mismatched alleles. Others algorithms predict the proteasome clivage sites of a protein (http://www.paproc.de/), or compare the common motives in different MHC alleles (http://www.cbs.dtu.dk/biotools/MHCMotifViewer/Home.html).

1.2.4. INTERACTIONS BETWEEN CD4$^+$ AND CD8$^+$ T LYMPHOCYTES AND PERIPHERAL TOLERANCE

CD4$^+$ T lymphocytes may occasionally exert cytolytic activity, but in general they role is to regulate the innate and the adaptive immune response. The help from CD4$^+$ T cells is fundamental in B cell antibody class switching, in enhancing the bactericidal activity of phagocytes and, last but not least, in supporting CD8$^+$ T cell function. The latter effect is achieved via different mechanisms. By releasing IL-2 CD4$^+$ T cells sustain the antigen-driven clonal expansion of CTLs, and this help is provided both for primary and secondary response. Moreover, CD4$^+$ T cells are also fundamental in maintaining the

memory $CD8^+$ T cells, since the latter cells require continuous exposure to $CD4^+$ T cells during the generation of the memory pool after acute infection (Sun et al., 2004). The help is provided also via indirect mechanisms. $CD4^+$ T cells via CD40-CD40L prime APCs (Schoenberger et al., 1998), which in turn prime the $CD8^+$ T cells, thus enabling their expansion and differentiation. Also indirect is the mechanism through which $CD4^+$ T helper cells promote migration of CTLs into inflamed tissues, i.e. by secreting IFN-γ which induces chemokine secretion in the infected tissues (Nakanishi et al., 2009).

$CD4^+$ and, to some extent, $CD8^+$ T cells can also play a suppressive role on immune response. T_{regs} inhibit T cell function in different ways (Figure 1.13). They can kill T cells by direct cytolysis or by inducing apoptosis, also by IL-2 starvation (Ren et al., 2007; Pandyian et al., 2007), and they can release inhibitory cytokines, namely IL-10, IL-35 and TGFβ. Moreover, T_{regs} can induce metabolic disruption by releasing adenosine or by transferring cyclic AMP into effector T cells (T_{Eff}) (Deaglio et al., 2007; Bopp et al., 2007). In addition, T_{regs} express CTLA-4, which outcompetes the molecule CD28 for the binding to CD80 and CD86, therefore depriving activated T cells of an important co-stimulus. CTLA-4 engagement of CD80 and CD86 also induces in APCs the activation of the potent immune suppressive enzyme indoleamine 2,3-dioxygenase (Arce-Silas et al., 2016).

Although T_{regs} generally express the CD4 molecule there is also evidence of $CD4^-/CD8^+$ T cells with regulatory activity (Rifa'i et al., 2004; Xystrakis et al., 2004; Boor et al., 2011).

T_{regs} are essential for preventing autoimmune responses, limiting chronic inflammatory diseases and for maintaining peripheral tolerance.

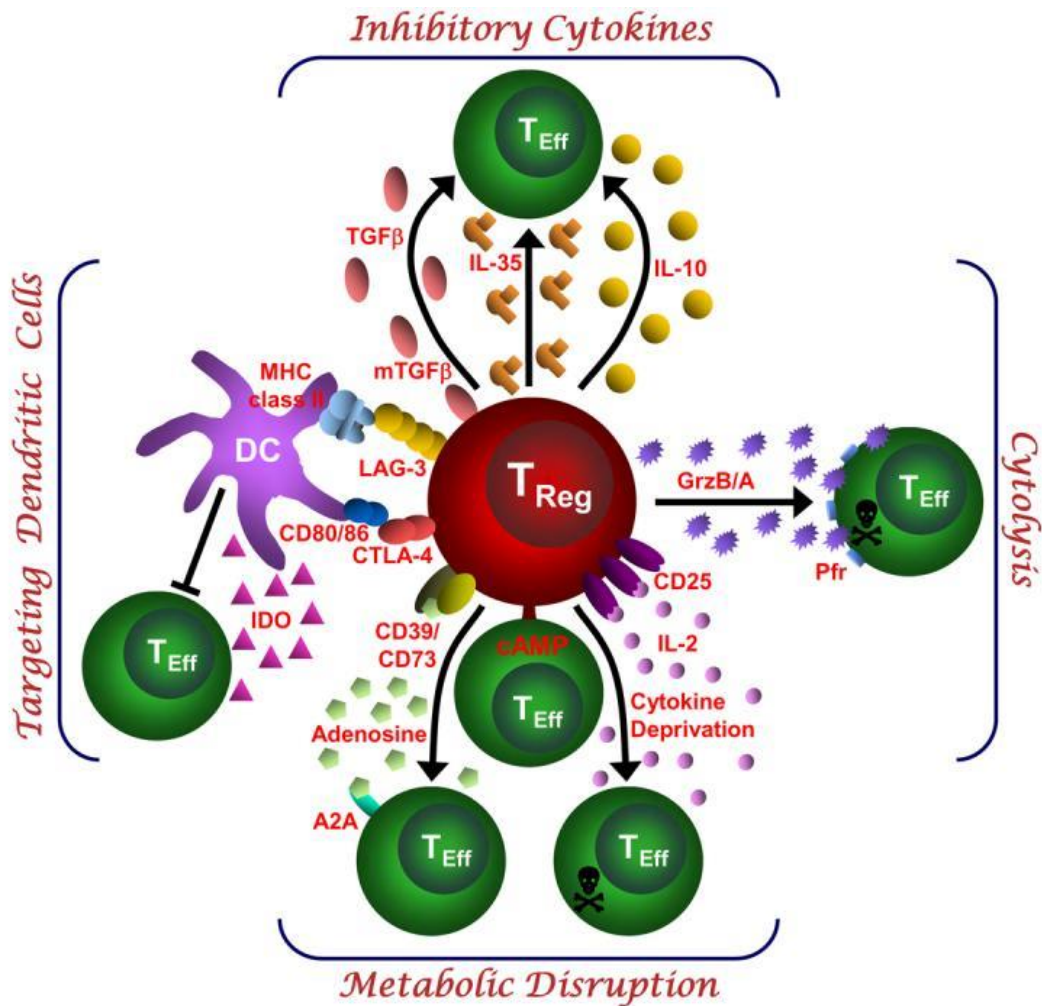

Figure 1.13. The mechanisms of T_{reg}-mediated suppression of T_{Eff} function. T_{reg} cells suppress T cell function by direct killing, by releasing immune suppressive cytokines, by interfering with T_{Eff} metabolism and by inducing DCs to switch to a suppressive phenotype. (Vignali et al., 2008)

Despite the fact that most of the self-reactve T cells are negatively selected in the thymus, some escape this selection, due to inadequate presentation in the thymus of tissue-restricted antigens, or to cross reactivity of TCRs having passed the thymic selection. These cells, which pose the threat of autoimmune diseases, will undergo deletion or suppression in periphery. DCs control the balance between T_{Eff} and T_{reg} cells and are therefore the major actors in maintaining peripheral tolerance. This effect is achieved through different mechanisms. Immature DCs constitutively cross present

tissue-specific antigens via Class I and this results in deletion or inactivation of the responding $CD8^+$ T cells (Luckashenak et al., 2008). The same activity is also displayed by lymph node stromal cells (Lee et al., 2007). Moreover, DCs can both expand the T_{regs} derived from the thymus and induce the conversion into T_{regs} of recently activated autoreactive T_N cells (Yogev et al., 2012). The circulating T_{regs}, either of thymic origin or generated in periphery, also contribute to keep at bay autoreactive T cells.

1.3. IMMUNE RESPONSE TO EBV

1.3.1. IMMUNE RESPONSE TO EBV IN PRIMARY INFECTION

In healthy individuals primary EBV infection is controlled mainly via T and B lymphocytes. During the acute phase, a cytolytic response mediated by $CD8^+$ T lymphocytes is mounted predominantly against the lytic antigens BZLF1, BMLF1, BMRF1, and BHRF1 (Rickinson and Moss, 1997; Oumade et al., 2011). Moreover, antibodies directed against capsid proteins and some early antigens appear early during infection (Linde, 1996; Oumade et al., 2011). This response leads to the clearance of the EBV-infected B cells in peripheral blood, which drop from 10 % during the acute phase to 1 out of 10^6 cells during convalescence.

Latent EBV infection is controlled mainly via cytolytic T-cell response: the EBV specific $CD8^+$ T lymphocytes directed against EBNA-3A, EBNA-3B and EBNA-3C epitopes are the most frequent (Tan et al., 1999), although more than 50 epitopes, either restricted to HLA Class I or Class II molecules, have been identified so far (Hislop et al., 2007).

A role in controlling EBV infection has been suggested also for $CD4^+$ T lymphocytes, since one out of three cells recognizing EBV-positive lymphoblastoid cell lines (LCLs) belongs to this cell subset (Bhaduri-McIntosh et al., 2008). It has been demonstrated that EBV-infected cells can effectively be lysed by cytolytic $CD4^+$ T lymphocytes; these cells, as compared to their $CD8^+$ counterpart, show a broader recognition of epitopes derived from lytic antigens (Long et al., 2011). Most importantly, $CD4^+$ T lymphocytes

can also provide help, therefore improving the CD8-mediated CTL activity, as demonstrated *in vitro* by analyzing the immune response to CMV (Hammoud et al., 2013). Finally, $CD4^+$ T lymphocytes can enhance the anti-viral response through mechanisms that are independent of helper or cytotoxic activity: they activate the APCs and release effector cytokines, namely IFNγ and TNF (Swain et al., 2012).

1.3.2. EBV-DEPENDENT IMMUNE ESCAPE

The immune response to EBV is effective in controlling the primary EBV infection and in general in clearing the virus in the lytic phase, but is less effective in destroying the virus in the latent phase, being hampered by the several immune escape mechanisms that EBV puts in action at this stage.

The main escape mechanism is based on the fact that the restricted gene expression profile during latency reduces the number of antigens potentially detectable by the immune system. Moreover, EBV deeply affects the mechanisms of antigen presentation via HLA Class I and Class II molecules. EBNA-1 contains a Gly/Ala repeat that affects proteasome function, reducing the number of EBNA-1-derived epitopes that can be presented via HLA Class I molecules (God and Haque, 2010). The mechanisms of EBV evasion of presentation via HLA Class II molecules are still unclear, but they seem to be related more to functional impairment of antigen presentation than to reduced levels of Class II expression. In this regard the EBV protein BZLF2 seems to interfere with the TCR binding at the cell surface level (Ressing et al., 2005). Furthermore, it was found that EBV-positive tumor cell lines express much more Class II associated invariant-chain peptide (CLIP) at the cell surface than EBV-negative cell lines of the same histotype, possibly affecting TCR binding (God and Haque, 2010).

In addition, the viral late gene product BCRF1, the analogous of the human IL-10, exerts a strong immune suppressive activity. It inhibits the antigen presenting activity of monocytes and macrophages (Salek-Ardakani et al., 2002), and inhibits the expression of TAP-1 (Rowe and Zou, 2010). TAP activity is also inhibited by BNFL2 that binds to the TAP complex and prevents peptide translocation (Rowe and Zou, 2010).

In EBV-positive BL an effective immune response is also hampered by the fact that tumor cells express low levels of the costimulatory molecules CD80 and CD86 (God and Haque, 2010). In HL, instead, EBV infection increases through LMP1 the expression of PDL-1, the ligand of the immune checkpoint PD-1 (Green et al., 2012). PDL-1 is also expressed by more than70 % of PTLD.

1.3.3. T-CELL RESPONSE IN EBV-ASSOCIATED TUMORS

The EBV-associated malignancies can be divided on the basis of the patient's immune response potential into two groups: those arising in immune competent patients, such as HL or NPC, and those arising in immune depressed patients, such as PTLD or the EBV-associated malignancies occurring in HIV-positive individuals.

There is convincing evidence that, despite the numerous mechanisms put in action by the EBV to escape or suppress the immune response, recognition of EBV antigens takes place in EBV-associated malignancies occurring in immunocompetent patients. The level of cell membrane HLA Class I expression and the number of activated $CD8^{+}$ T lymphocytes in peripheral blood is higher in EBV-positive cases of HL than in EBV-negative ones (Murray et al., 1998; Oudejans et al., 1997). Moreover, circulating EBV-specific cytotoxic T lymphocytes (CTLs) can be found in HL patients, and HL cells can

be lysed by EBV-specific CTLs *in vitro* (Tsang and Muntz, 2011; Lee et al., 1998). Accumulating evidence indicates that also $CD4^+$ T lymphocytes might play a role in the immune response to EBV-associated malignancies. Indeed it has been demonstrated that $CD4^+$ T lymphocytes specific for EBNA-1 can recognize BL cells *in vitro* and prevent EBV-associated tumor growth in a mouse model (Voo et al., 2002; Fu et al., 2004). Despite this evidence the EBV-specific immune response never reaches a level sufficient to completely destroy the tumor.

Instead, in the EBV-associated malignancies arising in immune suppressed patients the immune system is constitutively unable to mount an efficient response, although these tumors are potentially more immunogenic that those arising in immune competent patients. Each of the different EBV-associated human tumor has a peculiar pattern of latency gene expression (Table 1.4), and the tumors arising in immune depressed patients are those that have the largest spectrum of expressed EBV antigen.

Table 1.4. Latency patterns in the most common EBV-associated malignancies.
(Modified from Thompson and Kurzrock, 2004)

Latency I	Burkitt lymphoma
	Gastric carcinoma
Latency II	Hodgkin lymphoma
	Nasopharyngeal carcinoma
	Extranodal T/NK lymphoma
Latency III	PTLD
	HIV-associated lymphomas

One might speculate that these tumours do not occur in immune competent individuals because an intact immune system is able to erase this type of neoplastic cells. In this regard a study on 150 EBV-positive patients receiving either unmanipulated or T-cell depleted HSCT showed that only the latter developed PTLD (van Esser et al., 2001). On this basis, patients with PTLD or HIV-associated EBV-positive lymphomas might get advantage of ATCT more than those with EBV-associated tumours arising in immune competent individuals. Moreover, the former tumours have the broadest pattern of EBV antigen expression, therefore providing a better target for CTL activity. However, some degree of immune alteration can also be observed in the latter tumors, either being intrinsic to the tumor, as in the case of lymphomas, or iatrogenic.

1.4. T-CELL IMMUNOTHERAPY IN EBV ASSOCIATED MALIGNANCIES

Several attempts to treat EBV-associated malignancies with ATCT have been performed. Beside a pilot study in 2001 (Chua et al., 2001), five trials have been carried out in NPC (Straathof et al., 2005; Comoli et al., 2005; Louis et al., 2010; Chia et al., 2014; Smith et al., 2016) and all showed that the treatment had no significant short or long term toxicity while inducing partial or complete remission roughly in half of the patients. In particular the most recent trials showed that ATCT resulted on average in 18 months progression-free survival in patients with no or minimal residual disease, and in a prolonged survival in patients with refractory locally advanced or metastatic disease. Encouraging results have also been obtained in trials carried out in HL, where 4 complete remissions were obtained out of 20 cases refractory to conventional therapy (Roskrow et al., 1998; Straathof et al., 2003; Bollard et al., 2004; Lucas et al., 2004). However, the most encouraging trials so far have been performed on PTLD.

1.4.1. EFFICACY OF ATCT IN PTLD

PTLD can arise in patients having received HSCT or SOT, and EBV-specific T-cell immunotherapy has been conducted in both cases, with slightly different, but nevertheless encouraging results.

1.4.1.1. PTLD AFTER HSCT

The first observation that PTLD arising after HSCT can be treated by donor lymphocyte infusion (DLI) dates back to more than 20 years ago (Papadopulos et al., 1994), but the use of DLI in this setting has been hampered by the risk of inducing severe GVHD. Indeed in a cohort of 27 HSCT patients, mainly pediatric ones, that in most cases received lymphocyte infusion for PTLD from HLA-matched donors, 5 developed acute GVHD (Dubrovina et al., 2012). To circumvent this risk, starting from 1995 (Rooney et al., 1995), EBV-specific CTLs have been used, generally with good results, albeit most of the trials were carried out on a limited set of patients in non-randomised single arm studies. Focusing on the ones with the largest cohorts, in a study on 13 patients with EBV-positive PTLD, 11 achieved complete remission (Shaffer et al., 2010). In another trial complete remission was achieved by 7 out of 10 patients (Icheva et al., 2013). In a third study 3 out of 6 patients achieved complete remission (Moosman et al., 2010). Finally, in a cohort of 17 patients, 12 achieved complete remission, including 5 patients that failed to respond to conventional therapy; noteworthy, those that did not respond showed an impaired recognition of tumor antigens *in vitro* (Doubrovina et al., 2012).

EBV-specific ATCT has also been used as a pre-emptive approach to prevent PTLD. In this case a retrospective analysis showed that none of the 101 HSCT recipients receiving EBV-specific CTLs developed PTLD, whilst in the group of 47 patients that received no prophylaxis 5 developed PTLD (Heslop et al., 2010).

In most of the above mentioned cases the sources of T lymphocytes were the patients themselves.

1.4.1.2. PTLD AFTER SOT

The largest trial assessing the effect of adoptive T-cell therapy on PTLD after SOT involved 32 patients refractory to conventional treatment that received *in vitro* expanded EBV-specific CTLs from their donors; complete remission was achieved in 14 cases (Haque et al., 2007). Better response seemed to correlate with higher percentages of $CD4^{+}$ T lymphocytes in the infused cells and with their longer *in vivo* persistence. It has been suggested that the reduced effectiveness of EBV-specific ATCT in PTLD arising after SOT might be related to a shorter *in vivo* persistence of the infused CTLs, possibly related to a better alloreactive response of SOT patients, compared to HSCT ones (Shaffer et al., 2010). In this regard a trial was conducted, infusing autologous, *in vitro* expanded CTLs for either prophylaxis or treatment, and it was found that the survival in the blood of the infused cells ranged from 2 to 6 months (Savoldo et al., 2006).

1.4.2. EBV ANTIGENS TO BE TARGETED FOR T-CELL ATCT IN PTLD

So far, most of the peptides used to induce a CTL response for ATCT were derived from latent proteins, since PTLD expresses the latency III antigens, i.e. EBERs, LMP1 and 2, and all the EBNA proteins (Rickinson and Moss, 1997; Michelow et al., 2012), In a study assessing the specificity of immune response in one case of PTLD treated with CTLs stimulated with autologous LCLs, the main response was directed against EBNA3 (Gottschalk et al., 2001); interestingly, when the lymphoma relapsed, the EBNA3 gene was found to have mutated.

It is widely assumed that, as in other EBV-associated tumors, lytic antigens are not expressed in PTLD, despite several evidences indicating that these antigens might be expressed as well. Expression of lytic antigens, predominantly BZLF1, was found by immunohystochemistry in PTLD specimens (Montone et al., 1996; Oertel et al., 2002). In other studies transcripts of lytic antigens were found in high percentages of PTLD cases (Hopwood et al., 2002; Porcu et al., 2002). Most importantly, CTL recognition of lytic antigens seems to correlate with response to conventional therapy (Porcu et al., 2002). On these bases, lytic antigens should be included as targets for EBV-specific ATCT in PTLD. Evidence is also emerging that, besides $CD8^+$, $CD4^+$ T lymphocytes are also important for effective ATCT. Indeed, cytolytic $CD4^+$ T cell lines specific for lytic epitopes effectively lyse LCL cells *in vitro* (Long et al., 2011). The Class II-restricted antigenic repertoire for these epitopes is broader than the Class I-restricted repertoire. It was also found that EBNA-1-specific $CD4^+$ T lymphocytes expanded *in vivo* after infusion of poly-specific EBV CTL into PTLD patients (Icheva et al., 2013). Moreover, a better response to T-cell immunotherapy in PTLD seems to be related to a higher number of EBV-specific $CD4^+$ T lymphocytes in the infused sample (Haque et al., 2007). These data support the using of $CD4^+$ T lymphocytes to target Class II epitopes for EBV-specific T-cell immunotherapy.

1.4.3. THE DIFFERENT STRATEGIES FOR OBTAINING EBV-SPECIFIC T LYMPHOCYTES

In order to obtain antigen-specific T lymphocytes one may follow different strategies and employ different techniques, each of them having pros and cons. Early studies used

in vitro expansion methods, but technological advances have led to using Ag-specific T lymphocytes isolated ex vivo, or T lymphocytes transduced with specific TCR genes.

1.4.3.1. IN VITRO EXPANSION OF ANTIGEN-SPECIFIC T LYMPHOCYTES

EBV-specific T cell lines are usually generated by repeatedly stimulating T lymphocytes with autologous, EBV-transformed B LCL cells in the presence of IL-2. In one study, 39 HSCT patients considered at risk of PTLD received prophylaxis with *in vitro* expanded EBV-specific T cells and none developed lymphoma (Rooney et al., 1998). *In vivo* monitoring post-cell infusion showed a sharp drop in EBV DNA levels in circulating peripheral blood mononuclear cells (PBMC). In the same study 2 additional patients that developed overt PTLD were also treated with *in vitro* expanded EBV-specific T cells and both experienced a completed and sustained remission.

The advantage of this approach is that a large number of EBV-specific T lymphocytes can be generated, and eventually administered in repeated infusions. On the other hand a severe drawback of this approach is the long time it takes to reach a number of antigen-specific T lymphocytes sufficient for infusion: up to 4 months (Shaffer et al., 2010; Bollard, 2013). This length of time might be too much when the disease is in a phase of rapid progression, and even when it is not, the delay in treating the patient might affect the outcome. Nucleofection of DCs with immunodominant viral antigens has been proposed as an effective strategy for reducing the production time of viral-specific CTL lines (Gerdemann et al., 2009). Alternatively, the establishment of a bank of allogeneic, EBV-specific CTL lines to be used “off the shelf” might overcome the time limitation (Shaffer et al., 2010). A small bank with these characteristics has been established by the Scottish Blood and Transplant Service (Vickers et al., 2014).

However, it is worth mentioning that, when LCL cells are used as stimulators, the coverage of latent antigens is incomplete, the response to lytic antigens is missing, and there is the risk of the presence of free virus in the preparation (Eiz-Vesper et al., 2012). Another problem is related to the fact that, by using LCL cells as stimulators, most of the resulting CTLs are directed against EBNA3 and to a lesser extent to LMP2 but only minimally against the other latent antigens (Bollard, 2013). The use of DCs loaded with peptides derived from the different EBV antigens as APCs might overcome this problem.

Last but not least, the repeated stimulations that lymphocytes undergo during *in vitro* expansion induce the cells to express a late differentiation phenotype, and become easily prone to cell exhaustion (Klebanoff et al., 2005; Berger et al., 2008; Baitsch et al., 2011).

1.4.3.2. ISOLATION OF ANTIGEN-SPECIFIC T LYMPHOCYTES

Two methods are available for the *ex vivo* isolation of antigen-specific T lymphocytes from PBMC at clinical grade level: both were set up for selecting CTLs recognizing CMV, and both are based on immunomagnetic selection, but one uses HLA-multimers for selection (Cobbold et al., 2005), while the other selects for IFNγ-secreting cells (Rauser et al., 2004) using cytokine capture antibodies. Both these techniques have been used to isolate EBV-specific T lymphocytes (Weisner et al., 2005; Mackinnon et al., 2008). HLA-multimer selection was used in one case of PTLD, resulting in complete remission (Uhlin et al., 2010). IFNγ capture was used in two small trials on PTLD, achieving in both trials a 50 % of complete remissions (Moosman et al., 2010; Icheva et al., 2012). PBMC from the HSCT donor were incubated with a pool of EBV-derived

peptides, the activated, IFNγ-secreting cells isolated, and then infused into the recipient (Figure 1.11).

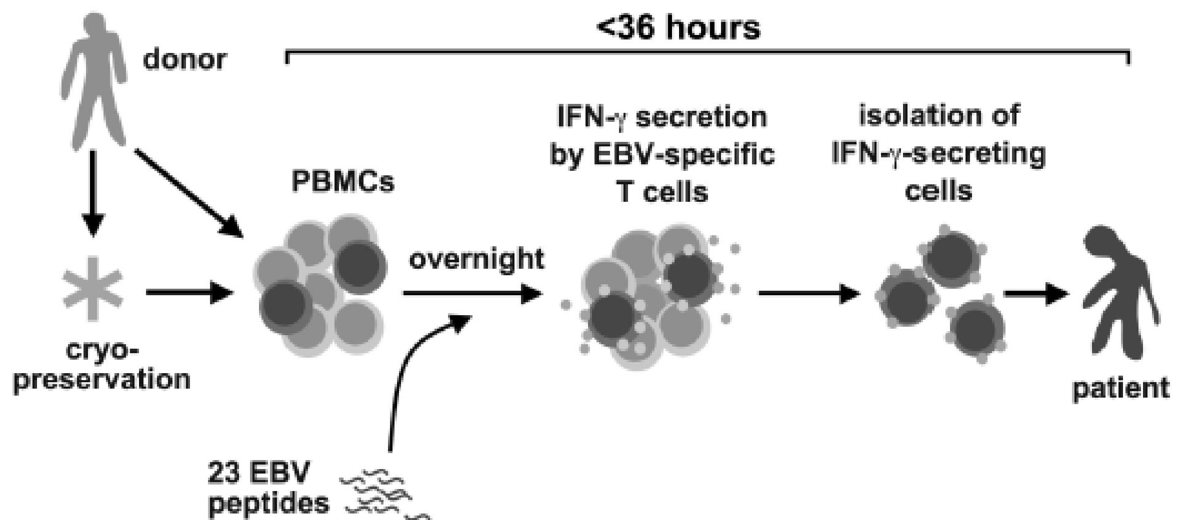

Figure 1.14. Schematic representation of the method for rapid isolation of EBV-specific T lymphocytes for ATCT. PBMC are stimulated overnight with EBV-derived peptides, then the EBV-specific T lymphocytes are immunomagnetically selected using the IFNγ capture technique, and immediately infused into the patient. (Moosman et al., 2010)

The 3 patients with advanced disease did not respond, but the others experienced a complete and sustained remission, accompanied by a significant increase in the number of circulating EBV-specific $CD8^+$ T lymphocytes. The great advantage of these approaches resides in two facts: the infused cells are minimally manipulated, and the whole procedure takes less than two days to perform.

The purity of antigen-specific T lymphocytes seems to be reduced following IFNγ capture as compared to multimer-based selection (Eiz-Vesper et al., 2013), but it has to be said that in the trials using the former technique no increase in GVHD was

detected (Feuchtinger et al., 2006; Moosman et al., 2010; Peggs et al, 2011; Icheva et al., 2012). On the other hand, only a limited, although expanding, set of Class II multimers is currently available, restricting the effectiveness of this technique for the selection of $CD4^+$ T lymphocytes. Instead, a number of clinical grade EBV peptides is currently available, and pools from LMP-2A, EBNA-1, and BZLF-1 have been used for the selection by IFNγ capture of both $CD4^+$ and $CD8^+$ T lymphocytes (Eiz-Vesper et al., 2012).

To improve the efficacy of this approach the creation of a registry of suitable T lymphocytes donors, HLA typed, and with high numbers of EBV-specific T cells could be useful for those cases where a third party donor is the only option.

1.4.3.3. Gene transduction of T lymphocytes

T lymphocytes can be effectively transduced with genes coding for chimeric antigen receptors (CARs) or for antigen-specific TCRs (Pule et al., 2008; Hinrichs et al., 2011). The major advantage of this technique relies on the fact that autologous cells can be used. In case of CAR transduction there is the possibility of off-target toxicity.

In transduction of exogenous TCR gene, mispairing of α and β chains, leading to creation of new TCR specificity can occur, albeit no evidence of alloreactive response have so far been detected in humans. A more relevant risk is related to the ability of the viral vectors, usually adenovirus and more recently lentivirus, to integrate into the cell genome. Indeed, insertional mutagenesis resulted in oncogene activation in 5 cases out of 19 in a trial of gene therapy for X-linked severe combined immune deficiency, and induced genome instability in 2 cases of chronic granulomatous disease treated with gene therapy (Hacein-Bey-Abina et al., 2008; Stein et al., 2010). However, this

complication has only been observed in gene therapy of haemopoietic stem cells and not in mature lymphocytes. Nevertheless, this has led gene transduction-based ATCT to undergo a severe scrutiny. A recent phase I trial on fourteen patients with metastatic cancer however showed no side effects for this treatment and suggested its efficacy in some cases (Yong-Chen et al., 2016).

1.4.4. AUTOLOGOUS VS. ALLOGENEIC SOURCE OF T LYMPHOCYTES FOR ATCT

In order to minimize the risk of alloreactive response, the ideal source of T lymphocytes for immunotherapy is the patient himself, and this is the case for the gene transduction approach. However, in some instances this option is not feasible. This is the case in EBV-negative patients, a frequent occurrence in children; but it is also the case of elderly people, that have a reduced T-cell repertoire and mount a less effective immune response. Moreover, most of the patients at the time of ATCT have received a myeloablative treatment, as is the case of HSCT patients, are under immune suppressive therapy, as is the case of SOT patients, or have received massive chemo- and/or radiotherapy, as is the case of tumor patients, all conditions that severely reduce the number and impair the function of their T lymphocytes.

In the case of HSCT or SOT from living donor, the donor is the source of EBV-specific T lymphocytes. Indeed most of the trials conducted so far have used HLA matched allogeneic HSCT donors as source of T lymphocytes. However, there is evidence that granulocyte colony-stimulating factor (GCSF) might affect functional activity of T lymphocytes by polarizing them to a Th2 profile, suggesting that GCSF-treated HSCT

donors might not be the best source of T lymphocytes for immunotherapy early after HSCT; in these cases a third party donor should be considered (Franzke et al., 2003; Toh et al., 2009).

A third party is required when the donor is EBV-negative, or is not available, as in the case of SOT from cadaveric donors or HSCT from umbilical cord blood (CB), or in patients with EBV-associated solid tumors. In a trial with 10 patients with severe viral infection, ATCT with T lymphocytes selected with multimers from third party donors was performed with encouraging results (Uhlin et al., 2012). In another study, two patients with PTLD after HSCT receiving *in vitro* expanded EBV-specific CTLs from third party donors showed complete remission (Barker et al., 2010). Another trial, including third party donors, confirmed the efficacy of this approach (Doubrovina et al., 2012).

1.4.4.1. THE POSSIBILITY OF GVHD AFTER INFUSION OF ALLOGENEIC T LYMPHOCYTES

A life-threatening complication of infusing CTLs from HLA-mismatched donors might be represented by severe GVHD, however evidence from literature suggests that this risk is possibly overestimated. Indeed, in a study on 101 HSCT recipients receiving EBV-specific CTLs as prophylaxis for PTLD no increase in GVHD was found, nor *de novo* GVHD episodes were detected after CTL infusion (Heslop et al., 2010). Also in a retrospective analysis on 73 HSCT patients receiving T-cell ATCT for EBV alone or in combination with CMV and/or adenovirus from HLA mismatched donors, no increase in acute or chronic GVHD was detected, as compared to patients receiving CTLs from HLA-matched donors (Melenhorst et al., 2010). In a cohort of 44 patients, 17 treated

with EBV-specific T cells and 27 with DLI, GVHD was observed only in the latter group (Doubrovina et al., 20120).

As it regards SOT, in a study involving 32 patients, neither GVHD nor any other adverse affect were observed (Haque et al., 2007).

1.4.5. THE IMPORTANCE OF T-CELL SUBSETS IN ATCT

The ability of transfused antigen-specific T lymphocytes to survive and expand into the host is crucial for the outcome of ATCT: only a significant clonal expansion can induce an effective immune response *in vivo*. The fact that clinical response to ATCT is related to the long term *in vivo* persistence of transfused cells was first observed in trials on melanoma patients (Yee et al., 2002). As discussed above, the ability to expand is progressively lost during differentiation, from T_N to T_{EMRA}. It was demonstrated in primates that in contrast to T_{CM}, T_{EM} lymphocytes albeit endowed with proliferative potential *in vitro* fail to replicate *in vivo* (Berger et al., 2008). Moreover, it has been recently found that antigen experienced T cells promote via Fas-mediated cell-to-cell contact the differentiation of T_N and the rapid loss of the less differentiated T-cell subsets (Klebanoff et al., 2016) In a mouse model of adoptive T cell therapy, this phenomenon resulted in an increased *in vivo* persistence of the transfused cells and an improved survival of the mice receiving purified T_N cells, compared with mice infused with unfractionated T cells (Klebanoff et al., 2016). Notwithstanding this, in most of the cases unfractionated T lymphocytes are used for ATCT.

Another cause of failure of ATCT is related to the rapid exhaustion of transfused cells. It was demonstrated in melanoma patients that infused T lymphocytes infiltrating

metastases showed signs of exhaustion (Baitsch et al., 2011). In addition, it was found that clinical responses and *in vivo* persistence of infused T lymphocytes were related to longer telomeres (Zhou et al., 2005), a marker of less differentiated memory T-cell subsets.

These findings indicate that T_{EM} lymphocytes may be poor candidates for T-cell ATCT: although they are endowed with better effector functions, they have less proliferative potential than T_{CM} lymphocytes and are more prone to cell exhaustion. Indeed, T_{CM} lymphocytes are considered by some the best cells for infusion (Klebanoff et al., 2005; Hinrichs et al., 2011). This has to be taken into consideration in the choice of strategy for ATCT. Whereas the *in vitro* expansion results, due to repeated and prolonged stimulation, in generation of highly differentiated T lymphocytes, gene transduction or *ex vivo* antigen-specific cell selection result in minimal changes to T lymphocyte differentiation.

1.4.5.1. T LYMPOCYTES FROM UMBILICAL CORD BLOOD IN ATCT

CB is widely used for HSCT, but its use as source of T lymphocytes for ATCT has been scarcely investigated, despite evidence suggests that T lymphocytes from CB display a normal response to tumor antigens but a reduced one to alloantigens (Merindol et al., 2010). Functionally active CTL lines that react to CMV, EBV and adenovirus have been produced by *in vitro* expansion from CB (Micklethwaite et al., 2010). Moreover, a pilot trial using CB as source of T lymphocytes on two case of PTLD refractory to conventional therapy resulted in both the patients achieving a sustained complete remission without adverse side effects (Baker et al., 2010).

1.5. AIMS OF THE STUDY

Based on the considerations put forward in paragraph 1.4, it is evident that ATCT, albeit promising, is still in its infancy, and needs to be ameliorated in order to become a first or a second line treatment for human diseases. In particular there is the necessity to improve the performance of ATCT for those cases where a third party donor of EBV-specific lymphocytes is the only available possibility. Therefore, focus has been put on three areas that in our opinion deserved to be further investigated.

1.5.1. THE SET UP OF A REGISTRY OF THIRD PARTY DONORS

So far, the frequency of EBV-specific T lymphocytes in the blood of third party donors used for ATCT trials has not been checked. However, the *in vitro* expansion of EBV-specific T cells is a time-consuming procedure, requiring 3-4 weeks for the cells to reach an adequate number, and 1 or 2 for the quality controls. This length of time does not fit well with the treatment of rapidly progressing diseases, such as PTLD. So we have aimed at creating a panel of HLA-typed healthy volunteers with high levels of circulating EBV-specific T lymphocytes. In this way, it would be possible to provide at short notice fresh T lymphocytes for effective *ex vivo* selection or *in vitro* expansion of EBV-specific T cells. Moreover, the registry could benefit pediatric transplant patients at high risk of donor-derived EBV reactivation; in this case the EBV-negative donors could provide safe blood products (Eiz-Vesper et al., 2012).

1.5.2. THE STUDY OF PHENOTYPICALLY NAÏVE MEMORY T CELLS.

The two T-cell types with naïve-like phenotype and memory traits, T_{SCM} and T_{MNP}, are currently ranked among the best candidates for ATCT (Restifo et al., 2012; Pulko et al., 2016), however al lot still needs to be done in order to fully understand their origin and function.

Despite sharing several phenotypic and functional characteristics, T_{SCM} and T_{MNP} differ in their extended phenotype, particularly in the expression of CD95 (Pulko et al., 2016), and it is not clear whether they represent different stable T-cell subsets or are the same cells transiently exhibiting different membrane markers, as expression of their plasticity. Moreover, the mechanisms leading to the generation of these cell subsets still need to be fully elucidated. Although $CD8^+$ T_{SCM} can be produced *in vitro* by activating T_N cells with IL-7, IL-21, and the glycogen synthase-3β inhibitor TWS119 (Sabatino et al., 2016), the physiological mechanisms generating these cells are unknown. As for T_{MNP}, nothing is known about their origin.

Therefore, we have aimed at elucidating the relationship between these two T-cell subsets, and to investigate how they are generated.

1.5.3. THE STUDY OF CB-DERIVED $CD8^+$ T LYMPHOCYTES AS SOURCE OF CELLS FOR TCR GENE TRANSDUCTION

An important prerequisite to move T-cell immunotherapy from promising pilot trials to established therapy consists in improving the *in vivo* performance of the infused T cells.

As shown in paragraph 1.4.5, the less differentiated are the infused cells and the longer is the *in vivo* survival, a correlate of therapeutic efficacy.

This makes T lymphocytes from adult donors, being predominantly memory cells, not the best candidates for T-cell immunotherapy. After the activation that is required to perform gene transduction, and the several rounds of activation necessary for *in vitro* expansion, these cells become more differentiated and senescent. The further activation they undergo *in vivo* after infusion likely induces a shift toward terminally differentiated subsets, and eventually results in exhaustion and cell death. Ideally, the best candidate should be T_N cells, which have the greatest proliferative and differentiative potential. However, gene transduction for ATCT has been carried out so far on blood lymphocytes from adults, which are predominantly memory cells. Since T lymphocytes from CB are mostly T_N (Beck and Lam-Po-Tang, 1994), our aim was to determine whether CB provides a better source of T cells for TCR engineering, in comparison to peripheral blood from adult donors

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2. ESTABLISHING AND CHARACTERISING A REGISTRY OF THIRD PARTY DONORS OF EBV-SPECIFIC T LYMPHOCYTES FOR ADOPTIVE IMMUNOTHERAPY

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Manuscript in preparation

2.1. ABSTRACT

Early phase clinical trials have demonstrated that ATCT with autologous cells is effective and safe in Epstein-Barr virus (EBV)-associated malignancies refractory to conventional therapy. Similar outcome and safety was also achieved using EBV-specific CTL generated from third party donors for treatment of both HSCT- and SOT-associated PTLD. However, to move from early proof-of-principle trials to established practice, it is important to establish a registry of HLA-typed, third party T-cell donors with high levels of circulating EBV-specific T lymphocytes so that donors can be recruited at short notice to donate PBMC for CTL generation. We have selected 17 HLA Class I restricted and 12 Class II-restricted EBV peptides derived from both lytic and latent antigens, and used them to activate T lymphocytes from 108 HLA-typed platelet donors expressing the Class I and Class II alleles that are most common in the UK, covering greater than 90% of the population. Screening was performed using interferon (IFN)γ Elispot, as preliminary experiments demonstrated it to be more sensitive and suitable than intracellular IFNγ staining or HLA-multimer staining. We found a remarkable inter- and intra-donor variability in the magnitude of the response to the different peptides, but strong responses were found for peptides derived from both latent and lytic antigens, and for both Class I- and Class II-restricted peptides. For the latter peptides, there was evidence of promiscuous response. For each donor we were able to define the pattern of response to the peptides restricted to the donor's haplotypes, identifying for each allele at least one high-responding donor. Our results support the feasibility of setting up a registry of third party donors of T lymphocytes for adoptive immunotherapy of EBV-associated diseases.

2.2. INTRODUCTION

About 1% of human tumours are associated with the expression of Epstein-Barr virus (EBV) antigens: almost all the endemic forms of Burkitt lymphoma (BL), from 50 to 90 % of Hodgkin lymphoma (HL), more than 90 % of nasopharyngeal carcinoma (NPC), and about 80 % of post transplant lymphoproliferative disorders (PTLD) express various patterns of EBV antigens (1-4). This makes EBV-positive tumours suitable targets for EBV-specific cytotoxic T lymphocytes (CTL). Indeed, several trials have been conducted to assess safety and efficacy of ATCT in EBV-associated malignancies. Although limited results have been obtained in HL (5-8), the results obtained in other tumor types are encouraging. The three trials for NPC (9-11) showed that the treatment had no significant toxicity whilst inducing complete remission in more than half of the patients. The same conclusions can be drawn for PTLD, the EBV disease expressing the broadest set of viral antigens. The largest trial for PTLD arising after solid organ transplant (SOT) involved 32 patients refractory to conventional treatment; complete remission was achieved in 14 cases (12). Regarding PTLD after hematopoietic stem cell transplant (HSCT), complete remission was achieved in 11 out of 13 patients (13), 7 out of 10 patients (14), 3 out of 6 patients (15), and 12 out of 17 patients (16). EBV-specific ATCT was also used as pre-emptive therapy on reactivation of EBV before onset of disease. A retrospective analysis showed that none of the 101 HSCT recipients receiving EBV-specific CTLs developed PTLD, compared to 5 out of the 42 that received no prophylaxis (17).

Adoptive T cell immunotherapy for EBV-associated tumours seems therefore to be a safe and effective treatment for patients not responding to conventional therapy.

For PTLD arising after HSCT, EBV-specific CTL were mostly generated from PBMC derived from the original HLA-matched stem cell donors. However, in settings where the donor is EBV-negative or is not available, such as after cadaveric SOT, umbilical cord blood transplant, or in patients with EBV-associated solid tumours, one solution is to resort to third party donors. Several trials using third party cells have proved effective. ATCT with T lymphocytes from third party donors was effective in 5 cases out of 6 with severe infection (18). In another study, two patients with PTLD after HSCT receiving *in vitro* expanded EBV-specific CTLs from third party donors showed complete remission (19). A further trial, including third party donors, confirmed the efficacy of this approach (16). No graft versus host disease (GVHD) attributable to the ATCT was reported.

Since infusion of cells from third party donors puts the patient at risk of GVHD, it is critical to infuse T lymphocytes that have been selected for their antigen specificity with minimal contamination by non-specfic and potentially alloreactive T cells. So far two techniques with clinical grade reagents are available for isolation of antigen-specific T lymphocytes: IFNγ capture antibodies and HLA-multimers for immunomagnetic selection. Both methods proved to be effective without increasing alloreactivity (14,15,18).

On these basis the creation of a registry of third party donors of known HLA type, and with high levels of circulating EBV-specific T lymphocytes, should ensure the rapid availability of cells for ATCT.

A crucial consideration in EBV-specific T-cell immunotherapy is the choice of antigens to be targeted by the infused T lymphocytes. It is widely assumed that, as in other EBV-associated tumours, lytic antigens are not expressed in PTLD. However several studies indicate that these antigens are expressed. Lytic antigens, predominantly BZLF1, were

detected by immunohystochemistry in PTLD specimens (20,21). In other studies transcripts of lytic antigens were found in high percentages of PTLD cases (22,23). Moreover, CTL recognition of lytic antigens seems to correlate with resolution of the tumour with conventional therapy (23). Therefore, lytic antigens should not be dismissed as suitable targets of EBV-specific T-cell ATCT in PTLD.

Recent emerging evidence suggests that, besides $CD8^+$ T lymphocytes, $CD4^+$ T cells are also important for effective ATCT. In fact, the Class II-restricted antigenic repertoire for the EBV epitopes is broader than the Class I-restricted repertoire. Cytolytic $CD4^+$ T lymphocytes specific for lytic epitopes effectively lyse LCL cells *in vitro* (24), and a better response to T-cell immunotherapy in PTLD seems to be related to a higher number of EBV-specific $CD4^+$ lymphocytes in the infused sample (12). These data support the targeting of Class II epitopes for EBV-specific T-cell immunotherapy.

So far expanded polyspecific CTL, which include lots of redundant specificities, have been used. A more targeted, epitope-specific approach is still innovative and to be tested. This is cheaper than engineering T cells, and it is easier to get approval, because the cells are minimally manipulated and with low risk profile.

On the basis of the considerations addressed above, we set up a panel of HLA-typed donors with high levels of circulating $CD4^+$ and $CD8^+$ T lymphocytes recognising both latent and lytic EBV epitopes. In addition to the magnitude of EBV-specific responses we have characterised the selectabilty of EBV T cells using different selection methods, and the effector functionality of ex vivo selected, minimally manipulated T cells. Our data demonstrate the feasibility of establishing a registry of HLA-typed third party donors for T-cell ATCT in EBV-associated diseases.

2.3. MATERIALS AND METHODS

2.3.1. PEPTIDES

The peptides were selected on the basis of: i) being able to induce a significant T-cell response, and ii) being restricted to the HLA Class I and Class II alleles that are the most common in the UK population. HLA A02, A11, B07, B08, B44, DR03, DR04, DR07, DR13, DQ05 and DP04 were identified as the HLA alleles most frequently expressed (25). Care was taken to select peptides derived from lytic together with peptides derived from latent EBV antigens, and restricted to either HLA Class I or Class II. The chosen peptides are shown in Table 2.1.

Table 2.1. List of the EBV-derived peptides used in this study. (From 29)**.**

CLASS I RESTRICTED			CLASS II RESTRICTED		
Peptide	**Allele**	**Protein**	**Peptide**	**Allele**	**Protein**
SVRDRLARL	A2	EBNA3A	VKLTMEYDDKVSKSH	DR0301	BMRF1
FLYALALLL	A2	LMP2	PYYVVDLSVRGM	DR4	BHRF1
LLWTLVVLL	A0201	LMP2	PGNDSTVQTAAAVVF	DR13	BZLF1
CLGGLLTMV	A0201	LMP2	VYGGSKTSLYNLRRGTALAI	DR7	EBNA1
YVLDHLIVV	A0201	BRLF1	PRSPTVFYNIPPMPLPPSQL	DR7 + 52b	EBNA2
GLCTLVAML	A0201	BMLF1	MVFLQTHIFAEVLKD	DR15	EBNA1
TLDYKPLSV	A0201	BMRF1	LDLDFGQLTPHTKAVYQPRG	DR15	BLLF1
RLRAEAQVK	A0301	EBNA3A	LEKQLFYYIGTMLPNTRPHS*	DR51	BXLF2
IVTDFSVIK	A11	EBNA3B	LTAYHVSTAPTGSWF*	DR52b	BZLF1
SSCSSCPLSK	A11	LMP2	ILRQLLTGGVKKGRPSLKLQ*	DR53	EBNA3B
RPPIFIRRL	B0702	EBNA3A	SDDELPYIDPNMEPV*	DQ5	EBNA3C
QPRAPIRPI	B0702	EBNA3C	GSFSVEDLFGANLNRYAWHR*	DP4	BKRF2
FLRGRAYGL	B8	EBNA3A			
QAKWRLQTL	B0801	EBNA3A			
RAKFKQLL	B0801	BZLF1			
KEHVIQNAF	B4402	EBNA3C			
EENLLDFVRF	B4402	EBNA3C			

Blue: latent antigens; red: lytic antigens.
*These peptides were tested in all the donors.

The peptides (Biosynthesis, Lewisville, TX, USA) were all dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml stock solution.

2.3.2. DONORS

Platelet donors undergoing apheresis at the NHS Blood Donor Centre, Birmingham, UK were screened on the basis of their HLA typing. The typing of the HLA A, B, C and DRβ1 loci was available for all the donors, while the typing of the remaining loci was largely incomplete. 108 donors expressing at least one of the alleles representing the restricting element for the above mentioned peptides were selected for our study. Informed consent was sought from each donor prior to sample collection. Before platelets apheresis, each donor was bled, and 5 ml of coagulated blood was colected for serum separation. After apheresis, the leukocyte-containing cone, a by-product of platelet apheresis, was collected and peripheral blood mononuclear cells (PBMC) isolated.

The project was approved by the NRES committee West Midlands, REC ref 11/WM/0315.

2.3.3. SERUM AND CELL COLLECTION

A tube with coagulated blood was centrifuged at 500 g for 10 min, and serum was collected.

Leukocyte-enriched blood was collected from apheresis cones with a syringe, diluted 1:2 with sterile phosphate buffer solution (PBS), and a Ficoll gradient separation was performed. PBMC were collected, washed and resuspended in RPMI1640 plus 10 % autologous serum for further tests.

2.3.4. ELISPOT

A standard IFNγ Elispot was performed using the Elispot Assay for Human Interferon-γ (Mabtech, Nacka Strand, Sweden), according to manufacturer's instructions. Briefly, MAIPN45 Elispot plates (Millipore, Billerica, MA, USA) were first treated for 1 min with 30 % ethanol. The primary antibody was then added and incubated overnight at 4 C. After washing, 10^5 PBMC for Class I-restricted peptides and $5x10^5$ PBMC for Class II-restricted peptides were seeded in each well. Peptides were added at a final concentration of 40 and 80 μg/ml for Class I- and Class II-restricted peptides, respectively; as positive control phytohemagglutinin (PHA) was used, whilst DMSO was used to establish background level. After 24 hrs in a CO_2 incubator, plates were washed and incubated for 2 hrs at RT with the secondary antibody. Afterward, plates were washed and incubated for 1 hr at RT with the streptavidin-alkaline phosphatase conjugate. Staining was performed using the AP Conjugate Substrate Kit (Bio-Rad, Hercules, CA, USA), exactly following manufacturer's instructions. Dots were counted on an AID Elispot reader (Autoimmun Diagnostika, Strassberg, Germany). Each test was performed in duplicate, and repeated in case of inconsistency between the readings. Since donors were not HLA typed for DQ and DP genes, nor for DR genes other than DRβ1, PBMC from all the selected donors were tested for reactivity against all the peptides restricted to DR51, DR52, DR53, DQ05 and DP04.

4.3.5. FLOW CYTOMETRY

For intracellular IFNγ staining, cells were first stimulated with peptide at the concentrations used for Elispot, and 1 h later 1 μl/ml Golgi Stop (BD, Franklin Lakes, NJ, USA) was added. After overnight incubation cells were fixed and permeabilised using Fix & Perm Kit (An Der Grub, Kaumberg, Austria), and stained with anti CD3

PE, anti CD4 PE-Cy7, anti IFNγ APC (all from BD), and anti CD8 APC-eFluor 780 (eBioscience, San Diego, CA, USA). For staining with GLC Dextramers (Immudex, Copenhagen, Denmark), manufacturer's instructions were followed. Briefly, 10^6 PBMC were incubated with 10 μl GLC Dextramer at RT in the dark for 10 min, then anti CD3 PE, 7AAD (BD) and anti CD8 APC-eFluor 780 were added. After 20 min at RT in the dark, cells were washed and examined. Samples were read by a FacsCanto II (BD) flow cytometer.

2.4. RESULTS

2.4.1. IFNγ ELISPOT IS A SUITABLE TECHNIQUE FOR SCREENING T-CELL RESPONSE TO EBV

Preliminary experiments were carried out in order to identify the technique best suited for screening T-cell response to EBV-derived peptides in PBMC from healthy people. Three techniques were taken into account: Dextramer staining, IFNγ Elispot, and intracellular IFNγ staining.

PBMC from two donors were activated with a pool of peptides, and Elispot and intracellular IFNγ staining were performed in parallel (Figure 2.1A). While a specific response for both Class I and Class II could be detected with Elispot, no difference between unstimulated and stimulated cells was found using intracellular IFNγ staining.

Dextramer staining and Elispot were then compared (Figure 2.1B). A specific response to GLC peptide was found with Elispot, and a specific staining was detected with the GLC Dextramer.

It is of note that the percentage of GLC-specific PBMC detected with the two techniques is very close, indicating that they have roughly the same sensitivity.

Since Elispot is less expensive than multimer staining, and allows for the detection of all the Class II-restricted peptides in our panel, we used this technique to screen our cohort of platelet donors.

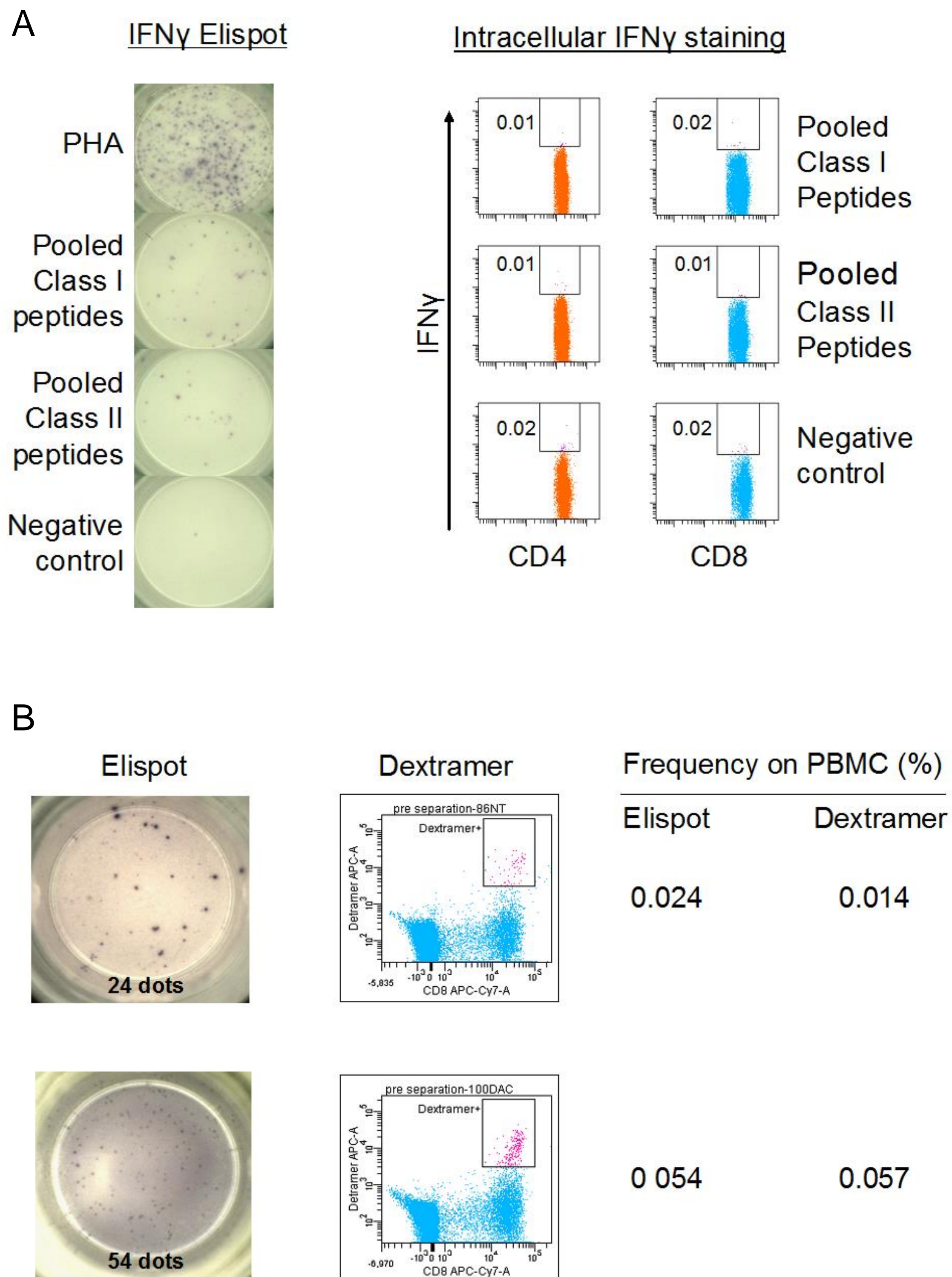

Figure 2.1. Comparison of different techniques for the enumeration of EBV-specific T lymphocytes. (A) PBMC were incubated with a pool of either Class I-restricted or Class II-restricted peptides, afterward IFNγ Elispot and intracellular IFNγ staining were performed. For the latter technique the percentage of $CD8^+$ T lymphocytes expressing high levels of IFNγ is indicated. Single representative experiment out of two. (B) PBMC from two donors were incubated with GLC peptide and either Elispot was performed, or cells were stained with GLC Dextramer.
(From 29).

2.4.2. THERE IS AN INTRA- AND INTER-DONOR VARIABILITY IN THE RESPONSE TO EBV PEPTIDES

Elispot was performed on the 108 healthy donors enrolled in our study, using 17 HLA Class I-restricted peptides, 13 derived from latent and 4 from lytic proteins, and 12 HLA Class II-restricted peptides, 5 from latent and 7 from lytic proteins. Since most of the donors were not typed for the HLA Class II loci, with the exception of DRB1, the peptides not restricted to DRB1 gene products were tested on all the donors.

Table 2.2 summarizes the results obtained. We found a large difference in the amplitude of response to the different peptides within the same donor, but there was also a quite relevant difference in the responses elicited by the same peptide in different donors. Despite this remarkable inter-individual variability, we were able to detect at least one high-responding donor for all the peptides in our panel.

However, 14 donors did not react to any of the peptides they were tested against. Since our donors were not checked for the positivity to EBV, it likely that these non-responders were individuals that had not been infected by the virus.

Table 2.2. Evaluation of T-cell response to EBV epitopes in a panel of healthy donors. (From 29).

Code	A*02	A*02	A*0201	A*0201	A*0201	A*0201	A*0201	A*0301	A*11	A*11	B*0702	B*0702	B*08	B*0801	B*0801	B*4402	B*4402	DR0301	DR4	DR7	DR7+52b	DR13	DR15	DR15	DR51	DR52b	DR53	DQ5	DPB1*04
	SVR	FLY	LLW	CLG	YVL	GLC	TLD	RLR	IVT	SSC	RPP	QPR	FLR	QAK	RAK	KEH	EEN	VKL	PYY	VYG	PRS	PGN	MVF	LDL	LEK	LTA	ILR	SDD	GSF
1AB																													
2AP																													
4PS																													
5PM																													
6RC																													
7AP																													
8GT																													
9MJS																													
10PJM																													
11SM																													
12AJ																													
13AW																													
14JF																													
15AJ																													
16JG																													
17RS																													
18SB																													
19GS																													
20AM																													
21TC																													
23TMF																													
24SC																													
25ND																													
26RE																													
27SSW																													
28MH																													
29JW																													
31GG																													
32SF																													
33PT																													
34GH																													
35MP																													
36SRT																													
37JH																													
38CW																													
39SP																													
40DS																													
41DH																													
42SJT																													
43CMM																													
44TJ																													
46RY																													
47RM																													
48AW																													
49PR																													
50DB																													
51KSP																													
52KC																													
53PW																													
54PS																													
55KP																													
56CW																													
58 CB																													
59JH																													
60SW																													
62RJ																													
63KW																													
64AD																													
65RMA																													
66REM																													
67KPH																													
68MR																													
69PH																													
70PW																													
71SH																													
72KK																													
73JAG																													
74DKM																													
75AJP																													
76MN																													
77SP																													
78PR																													
79GJB																													
80PG																													
81 AC																													
82MC																													
85NJH																													
86NT																													
87AW																													
88DM																													
89GD																													
90AA																													
91MO																													
92BH																													
93AC																													
94CAM																													
95AD																													
96JW																													
97DC																													
98MB																													
100DAC																													
101NV																													
102RW																													
103PS																													
104TR																													
105FWW																													
106RWW																													
107 JSC																													
108MB																													

White cell: not tested (the donor did not express the restricting HLA allele); green cell: no response (below 2 times the background); yellow cell: weak response (between 2 and 5 times background); amber cell: strong response (above 5 times background); red cell: presence of promiscuous response.

2.4.3. THERE ARE DOMINANT AND SUBDOMINANT PEPTIDES

Although the reactivity to a given peptide in each donor was absolutely unpredictable, we found that there is a hierarchy among both Class I- and Class II-restricted peptides (Table 2.3). For the former set of peptides the frequency of donors responding to RAK was 12 times the frequency of donors responding to SVR. This phenomenon also occurs for Class II-restricted peptides, but is less evident.

Table 2.3. Frequency of positive response to the different peptides. (From 29).

CLASS I RESTRICTED				CLASS II RESTRICTED			
Peptide	**Allele**	**Frequency**	**%**	**Peptide**	**Allele**	**Frequency**	**%**
RAK	B0801	22/28	78.6	GSF	DP4	58/108[a]	53.7
GLC	A0201	30/44	68.2	PYY	DR4	15/28	53,6
RPP	B0702	16/26	61.5	PRS	DR7+52b	8/16	50.0
FLR	B8	17/28	60.7	LEK	DR51	51/108	47.2
IVT	A11	5/10	50	VYG	DR7	7/16	43.8
EEN	B4402	12/37	32.4	LDL	DR15	7/16	43.8
YVL	A0201	14/44	31.8	SDD	DQ5	46/108	43.8
QAK	B0801	8/28	28.6	LTA	DR52	47/108	43.5
CLG	A0201	10/44	22.7	MVF	DR15	6/17	35.3
RLR	A0301	7/32	21.9	VKL	DR3	6/20	30.0
QPR	B0702	5/26	19.2	ILR	DR53	26/108	24.1
KEH	B4402	7/37	18.9	PGN	DR13	2/9	22.2
LLW	A0201	6/44	13.6				
FLY	A2	6/44	13.6				
TLD	A0201	5/44	11.4				
SSC	A11	1/10	10				
SVR	A2	3/44	6.8				

[a]The promiscuous responses were included

It has to be noted that for some peptides the restricting allele was defined at molecular level, while for others the restricting antigen was identified by serology. An attempt to predict the restricting allele for the latter peptides using three algorithms available on line, i.e. SYFPETHI, DTU (http://www.cbs.dtu.dk/services/NetMHCpan-3.0/) and IEDB (http://tools.immuneepitope.org/mhci/) gave disappointing results, since all the algorithms allowed for the analysis to be carried out only on a very limited set of alleles. As an example, regarding the many alleles encompassed by the antigen HLA-A2 SYFPETHI and DTU provide prediction for the sole allele A*02:01 and IEDB for A*02:01 and A*02:06.

Instead, good correspondence was observed when the predictions were compared with the nominal restricting element resulting from laboratory data. For instance, the peptides LLWTLVVLL and GLCTLVAML, nominally restriced to the allele A*02:01 (25,26), got predictions of high affinity binding to this allele but not to HLA-A*03:01 (Table 2.4).

Table 2.4. Comparison of three predictive algorithms

Peptide	HLA allele	SYFPEITHI	IEDB	DTU
LLWTLVVLL	A*02:01	5 [a]	0.3 [b]	0.4 [b]
LLWTLVVLL	A*03:01	NA	11.05	11
GLCTLVAML	A*02:01	3	2.3	1.1
GLCTLVAML	A*03:01	NA	19	19

[a]arbitrary score, the highest the value, the highest the affinity
[b]percentile rank, the lowest the value, the highest the affinity

The degeneration of the common motives did not allow for such an analysis to be also performed with satisfactory results on Class II alleles.

2.4.4. THE RESPONSE TO SOME CLASS II-RESTRICTED PEPTIDES SEEMS TO BE PROMISCUOUS

In 8 cases we recorded a concomitant strong response to LEK, LTA and ILR (Table 2.2). Since an individual can express only up to two HLA molecules among DR51, DR52 and DR53, this finding suggests the occurrence for these peptides of a promiscuous response. The same conclusion can be drawn from the observation of a strong response to peptide SDD in a donor not expressing DQ05.

2.5. DISCUSSION

With a series of preliminary experiments we found that intracellular IFNγ staining is less sensitive than Elispot and Dextramers for screening T-cell response to EBV-derived peptides, whilst the sensitivity of the two latter techniques is similar. Considerations on costs and logistics of bulk testing prompted us to choose Elispot over HLA multimers, and with this technique we have assayed a panel of 108 healthy donors for the reactivity of their T lymphocytes to EBV-derived peptides restricted to the HLA alleles that are the most common in the UK population.

14 donors did not respond to any of the peptides tested. As platelet donors are not tested for EBV, we cannot say whether these donors were EBV-negative or reacted to peptides other than those we tested. In order to establish a registry of third party donor, the EBV serostatus should be tested.

The remaining 94 donors responded to one or more peptides. We could find at least one donor with a strong response for each of the peptides tested. In general we detected a remarkable intra- and inter-donor variability, i.e. a single donor reacting differently to different peptides, and different donors mounting different response to the same peptide. It has also to be noted that we had strong responses to peptides derived from both lytic and latent EBV antigens.

Although the individual response to a given peptide is unpredictable, a hierarchy is identifiable with some peptides being able to induce response in most cases, and others in only a few. This holds true particularly for Class I-restricted peptides, where the responses span from 81.5 to 6.8 %. With Class II-restricted peptides we found that the magnitude of responses was comparable to Class I. However, in some instances we had evidence that the response to Class II-restricted peptides was promiscuous, involving

alleles other than the cognate ones. This might be related to the fact that the long Class II-restricted peptides may harbour in their sequence epitopes for more than one allele, or that they may undergo proteolytic cleavage during incubation, generating new epitopes, including Class I restricted ones. Promiscuity in the response to SDD and GSF peptides might in part explain the high percentage of responders: since the percentage of DQ05 and DP04 in UK population is about 50 % (27), either all the individuals expressing these antigens responded to by chance EBV, or other HLA alleles were also involved. More studies are required to elucidate this point.

The algorithms we examined proved effective in predicting the peptide affinity for the HLA Class I alleles that are in their data base but these data bases are largely incomplete and do not allow to unmistakably identify for most peptides the restricting HLA allele.

In general our data demonstrate the feasibility of establishing a registry of HLA-typed third party donors for T-cell ATCT in EBV-associated diseases. Indeed, the establishment of an allogenic T-cell donor registry will greatly facilitate T-cell ATCT in a number of virus-associated diseases (28), providing at short notice fresh EBV-specific T cells for immediate administration to the patient, or for further *in vitro* expansion.

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3. THE EARLY T-CELL MEMORY POOL IS REPLENISHED THROUGH REVERSION OF RECENTLY DIFFERENTIATED CD8$^+$ T CELLS TO MEMORY STEM CELL VARIANTS

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Acknowledgment of collaborative work

GF designed and performed the experiments, interpreted the data and wrote the manuscript; AW performed part of flow cytometry experiments; ZN analyzed the microarray data; SK performed the gene expression analysis; SPL provided the TCR construct and advised on experiments; GA advised on experiments and edited the manuscript; PM advised and edited the manuscript; FEC advised on designing the experiments and interpreting the data, and edited the manuscript.

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3.1. ABSTRACT

The memory T-cell pool is essential for long-term health. Antigen-experienced $CD8^+$ T cells with a naïve (T_N) phenotype and stem cell features, T-memory stem cells (T_{SCM}) and memory T cells with naïve phenotype (T_{MNP}), have been described as contributing to the maintenance of the pool, but the mechanisms underlying their generation remain unknown. Primary antigen stimulation of T cells is believed to trigger a one-way differentiation from T_N to memory T-cell subsets. However, we demonstrated that recently differentiated central memory and effector memory T cells can de-differentiate into cells with a revertant T_N-like phenotype (T_{Nrev}) in the presence of homeostatic cytokines, of which IL-7 was the most potent. Despite the naïve phenotype, molecular analysis showed that T_{Nrev} had an activated T-memory profile. Functionally T_{Nrev} displayed the same characteristics as T_{SCM} and $T_{MNP,}$ including high proliferative and differentiative capacity, polyfunctionality and cytolytic activity upon re-stimulation. They could undergo multiple cycles of differentiation and reversion. Depending on cytokine milieu, T_{Nrev} acquired the phenotype of T_{SCM} or T_{MNP}, which we suggest are the same cells exposed to different environmental conditions. We propose that cytokine-dependent reversion of recently differentiated $CD8^+$ T cells represents a novel mechanism for replenishing the human memory T-cell pool, contributing to the preservation of immunity over an individual's lifetime.

3.2. INTRODUCTION

The development of the memory T-cell pool is essential for long-term health and survival, but how this is maintained remains unresolved. Thymic output is age-limited and considered insufficient for maintaining the TCR repertoire. Current $CD8^+$ T-cell differentiation models presume a one-way pathway (1) where initial antigen stimulation triggers differentiation from naive (T_N) to central memory (T_{CM}), effector memory (T_{EM}), and effector (T_{Eff}) T cells. Along this pathway, $CD8^+$ T cells acquire increasing effector function but progressively loose survival and proliferative capacity.

However, two subsets of phenotypically naïve $CD8^+$ T cells with stem cell features and memory traits that have enhanced differentiative and proliferative potential have been recently described.

T-memory stem cells (T_{SCM}) have been proposed to constitute the precursor memory T-cell pool, which lies between T_N and T_{CM} in the differentiation pathway (2). Despite displaying some phenotypic features in common with T_N cells and showing ability of self-renewal, T_{SCM} exhibit functional characteristics of antigen-experienced cells and rapidly differentiate into memory and T_{Eff} subsets upon antigenic re-challenge.

The recently described memory T cells with naïve phenotype (T_{MNP}) also represent a subset of antigen-experienced $CD8^+$ T cells with naïve phenotype, high proliferative potential and polyfunctional capability (3). Half of these cells express granzyme B (GrB), a molecule thought to be expressed only by T_{EM} and T_{EFF} (4). Despite sharing several phenotypic and functional characteristics, T_{SCM} and T_{MNP} differ in their extended phenotype, particularly in the expression of CD95, and it is not clear whether they represent different T-cell subsets (3) or are the same cells transiently exhibiting different membrane markers for contingent reasons. Moreover, the mechanisms leading to the

generation of these cell subsets still need to be fully elucidated. Although $CD8^+$ T_{SCM} can be produced *in vitro* by activating T_N cells with interleukin (IL)-7, IL-21, and the glycogen synthase-3β inhibitor TWS119 (5), the physiological mechanisms generating these cells are unknown. As for T_{MNP}, nothing is known about their origin.

Given the importance of cytokines as key regulators of T-cell mediated immunity, we analyzed the effect of different cytokines on the pattern of T-cell differentiation after initial stimulation. We used lymphocytes from human cord blood (CB) which are less likely to have encountered antigens and have a very low frequency of T_{SCM} (2). We demonstrated, for the first time, that recently differentiated $CD8^+$ memory T cells can revert to a naïve-like phenotype in a process that is controlled by a hierarchy of g-chain (γc) cytokines, the most potent of which is IL-7. We show that these revertant $CD8^+$ T_N cells (T_{Nrev}) transiently share phenotypic and functional characteristics with T_{SCM} and T_{MNP}, before displaying a trend to re-acquire a phenotype similar to primary T_N cells. Collectively, our study unravels the mechanism generating T_{SCM} and T_{MNP} and provides a unifying theory for these subsets, and identifies a novel model for human $CD8^+$ T-cell differentiation/de-differentiation with important biological implication for understanding the maintenance of immunity over human lifetime.

3.3. RESULTS

3.3.1. IL-7 INDUCES RECENTLY DIFFERENTIATED CD8$^+$ MEMORY T CELLS TO REVERT TO A NAÏVE-LIKE PHENOTYPE.

Cord Blood Mononuclear Cells (CBMCs) were activated with anti-CD3 plus IL-2 and the differentiation of CD8$^+$ T cells was monitored following CD45RA and CCR7 expression. These markers are conventionally used to categorize T cells into T_N (CD45RA$^+$/CCR7$^+$), T_{CM} (CD45RA$^-$/CCR7$^+$), T_{EM} (CD45RA$^-$/CCR7$^-$) and T_{Eff} (CD45RA$^+$/CCR7$^-$) subsets (1). As expected, activation induced an expansion of T_{CM} and T_{EM} subsets with a concurrent reduction in T_N (Fig. 3.1A,B). T_{Eff} were not generated in significant number following one single stimulation and were not considered further.

In order to investigate the role of cytokines in modulating the differentiation of T cells after activation, IL-7 was added to the culture medium when the proportion of CD8$^+$ T_N dropped below 20%, usually a week after activation (6.7 ± 1.91 days, mean ± 1 SD, n=50). In the first three days following addition of IL-7, the percentage of CD8$^+$ T_N continued to diminish, reaching a nadir of 8.39% ± 6.40 (Fig. 3.1*B*). However, after further incubation with IL-7 the great majority of CD8$^+$ T cells started to re-express CD45RA and reverted back to a phenotype resembling T_N, characterized by co-expression of CD45RA, CCR7, CD62L and CD27 and lack of expression of CD45RO (Fig. 3.1A and Supplemental Information Appendix, Fig. S1).

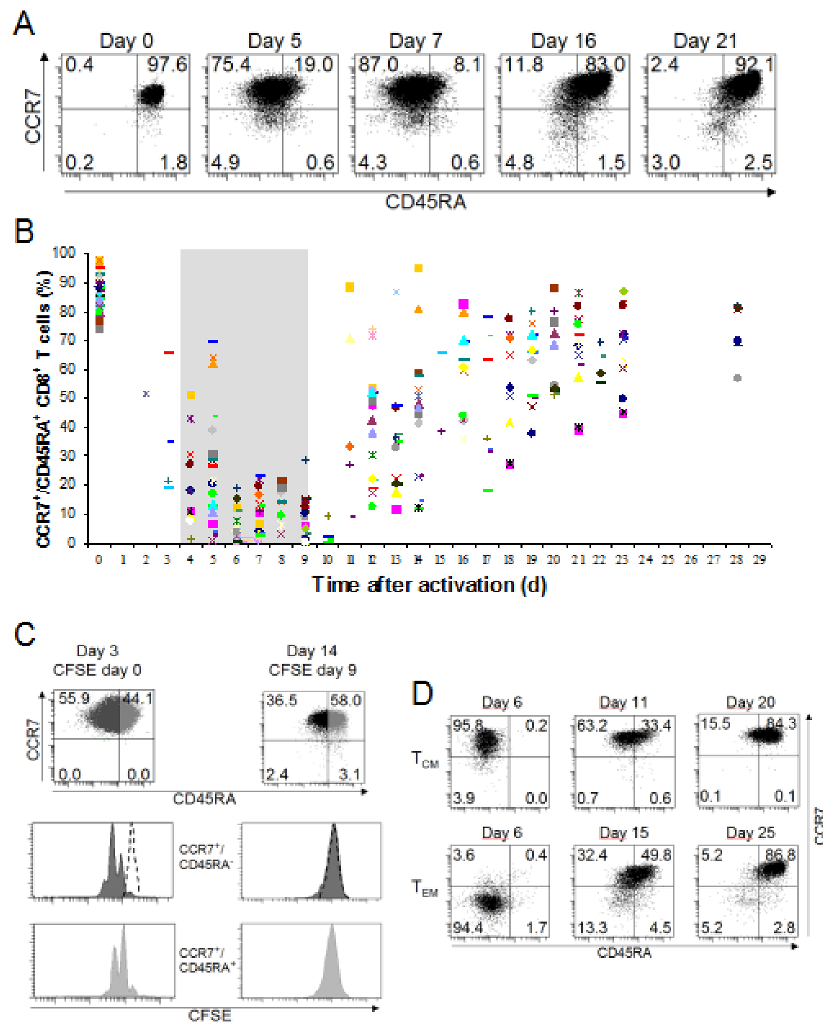

Fig. 3.1. IL-7 induces reversion of recently differentiated memory CD8⁺ T cells to a naïve-like phenotype. (A) Flow cytometric analysis of phenotypic changes in CD8⁺ T cells after activation and successive incubation with IL-7. CBMCs were activated with anti CD3 and the phenotype of CD8⁺ T-cell was monitored over time. At day 5, when the percentage of T_N dropped below 20%, IL7 was added to the cultures. Numbers indicate the percentage of cells in each quadrant. Single representative experiment out of 50. (B) Kinetics of phenotype reversion of CD8⁺ T cells from 50 different CB samples. The shaded area represents the times when IL-7 was added for the first time. (C) CD8⁺ T-cell proliferation after activation and after IL-7 administration. CBMCs were stained with CFSE either before activation (left panels) or at day 14, during phenotype reversion (right panels). At the indicated time points, cell phenotype and CFSE content were assessed for T_N (light gray dots) and T_{CM} (dark gray dots). Dashed lines indicate basal content in CFSE. Single representative experiment out of three. (D) Flow cytometry evaluation of IL-7-dependent phenotype reversion in recently differentiated T_{CM} and T_{EM}. Enriched CD8⁺ T_N were stimulated and, when T_N percentage dropped below 20%, untouched T_{CM} and T_{EM} were isolated. The two cell subpopulations were then incubated with IL-7 and monitored for phenotype changes over time. The T_{CM} and T_{EM} shown here are from two different CB samples. Single representative experiment out of three, for each subset.
(From 37).

The re-acquisition of a naive phenotype by $CD8^+$ memory T cells reached a plateau by 13-28 days after initial activation (19.8 ±4.66 days), and typically represented over 70% of the $CD8^+$ T-cell population (mean 71.1% ±11.68, range 44.7%- 95.3%). As such, this value was only slightly below the mean of 86.6 % of $CD8^+$ T_N at day 0 (±5.84, range 74.3-97.7). We called T_{Nrev} the cells that had reverted to a naïve-like phenotype. Although all samples followed a similar pattern, there was significant variation in the time taken to reach nadir in T_N and peak in T_{Nrev} levels, and in the magnitude of the T_{Nrev} population at plateau (Fig. 3.1B).

To demonstrate that the phenotypic reversion of differentiated T cells was not due to enhanced proliferation or selective death of individual T-cell subsets, we enumerated the cells within each cell subset and monitored their proliferation. The total number of all cell subsets before and after reversion remained largely unchanged (Supplemental Information Appendix, Table S1), and no cell proliferation was detected after the addition of IL-7 and during phenotype reversion (Fig. 3.1C).

To confirm that the phenomenon was due to modulation of cellular phenotype, recently differentiated $CD8^+$ T_{CM} and T_{EM} were purified and incubated with IL-7. Phenotype reversion was again demonstrated for over 80% of the purified T_{CM} and T_{EM}, with the latter cells showing slightly delayed kinetics (Fig. 3.1D).

3.3.2. PHENOTYPIC REVERSION IS SUPPORTED BY CYTOKINES THAT MAINTAIN CELL SURVIVAL AND T_{NREV} CAN UNDERGO SEVERAL ROUNDS OF REVERSION.

We next assessed whether this property was unique to IL-7 or shared by other cytokines. Recently differentiated CBMCs were incubated with single and multiple combinations of the γc cytokines IL-2, IL-7, IL-15, IL-4 and IL-21. IL-6, an inflammatory cytokine that is known to deliver pro-apoptotic signals, was also incorporated.

Phenotypic reversion was indeed observed for several of these cultures but it was less marked than with IL-7 (Table 3.1).

Table 3.1. The effect of different cytokines on phenotypic reversion. (From 37).

		IL-7	IL-2	IL-4	IL-15	IL-21	IL-6	Medium	IL-7 + IL-2	IL-7 + IL-15	IL-7 + IL-4
T_N nadir	T_N	3.5 ± 3.6[A]	0.7 ± 0.8	5.0 ± 2.4	1.2 ± 1.8	5.3 ± 5.1	1.0 ± 0.9	7.9 ± 5.9	5.1 ± 5.2	9.1 ± 3.6	9.3 ± 3.1
	T_{CM}	60.5 ± 8.5	42.5 ± 8.8	60.4 ± 10.5	37.0 ± 12.5	52.2 ± 12.3	72.6 ± 16.4	54.6 ± 19.2	66.1 ± 15.0	71.2 ± 8.9	64.4 ± 7.6
	T_{EM}	34.5 ± 8.5	55.7 ± 12.1	31.7 ±8.8	58.9 ± 14.6	38.6 ± 6.6	26.3 ± 13.5	34.8 ± 14.0	24.7 ± 7.7	18.8 ± 8.8	21.3 ± 5.7
	T_{Eff}	1.6 ± 0.7	1.1 ± 0.8	1.9 ± 0.8	2.9 ± 1.7	3.9 ± 4.5	0.2 ± 0.3	2.7 ± 2.4	4.2 ± 6.4	0.9 ± 0.7	5.0 ± 3.8
T_{Nrev} plateau	T_N	75.8 ± 9.2	41.3 ± 8.4	30.1 ± 4.5	32.1 ± 5.6	6.4 ± 6.4	4.2 ± 3.2	10.1 ± 4.5	68.3 ± 11.2	73.1 ± 3.9	74,7 ± 3.6
	T_{CM}	15.0 ± 5.5	22.9 ± 7.9	47.4 ± 7.0	16.6 ± 7.7	22.1 ± 12.8	13.2 ± 6.5	54.9 ± 14.9	21.7 ± 12.7	21.2 ± 4.5	17.6 ± 4.7
	T_{EM}	3.1 ± 2.9	23.7 ± 6.2	11.3 ± 5.2	45.1 ± 9.1	66.9 ± 5.9	72.7 ± 8.8	27.9 ± 13.3	6.7 ± 7.9	2.4 ± 2.1	2.3 ± 1.7
	T_{Eff}	6.0 ± 4.0	12.1 ± 4.7	11.2 ± 5.0	26.3 ± 7.8	14.6 ± 8.9	9.9 ± 9.0	7.1 ± 6.3	3.4 ± 6.4	4.3 ± 4.4	5.1 ± 1.8

[A]The percentage of CD8+ T cells within each subset at T_N nadir and T_{Nrev} plateau is shown.

Interestingly, $CD8^+$ T cells cultured with IL-6 or IL-21 were driven toward a more differentiated phenotype with a substantial increase in T_{Eff} cells. No synergistic effect was observed when IL-7 was administered together with IL-2, IL-4 or IL-15. Of further interest was the observation that the ability of individual cytokines to promote de-differentiation to T_{Nrev} cells correlated with their ability to support $CD8^+$ T-cell survival

in vitro (Fig. 3.2A). Particularly the use of IL-6, IL-21, or medium alone led to massive cell death. This suggests that reversion may be a default physiological program of recently differentiated $CD8^+$ T cells when a survival stimulus is provided.

Phenotypic reversion also takes place following differentiation induced by mitogenic stimuli other than soluble anti-CD3 (Fig. 3.2B).

The percentages of differentiated $CD8^+$ T cells undergoing IL-7-dependent phenotype reversion after activation with PHA and SEB were similar to those found in cells activated with anti-CD3. Activation with CD3/CD28 beads led to a smaller proportion of differentiated cells reverting to a naïve phenotype. However, in these experiments we were unable to fully remove the beads, some of which were still attached to the cells when IL-7 was added. It is possible that the resulting continuous stimulation may explain the lower degree of reversion achieved. Indeed, the $CD8^+$ T cells activated with anti-CD3 alone and those activated with anti-CD3 plus cross-linked anti-CD28 showed similar kinetics of reversion, demonstrating that co-stimulation did not prevent the cells from reverting their phenotype.

Further, we assessed whether cells could undergo more than one cycle of phenotypic reversion. Since serial rounds of anti-CD3 stimulation led to a high rate of cell death, PHA was used for two further rounds of activation; each was followed by IL-7 incubation (Fig. 3.2C). After each cycle of activation and IL-7 treatment, a phenotype reversion was observed, indicating $CD8^+$ T_N can undergo repeated cycles of differentiation and de-differentiation.

In order to demonstrate that phenotypic reversion is possible after activation with cognate antigen, CBMCs were transduced with a gene encoding a TCR specific for the SSC peptide (6). Following activation and retroviral transduction, cells acquired a predominantly T_{CM}/T_{EM} phenotype, but reverted to T_{Nrev} when incubated with IL-7.

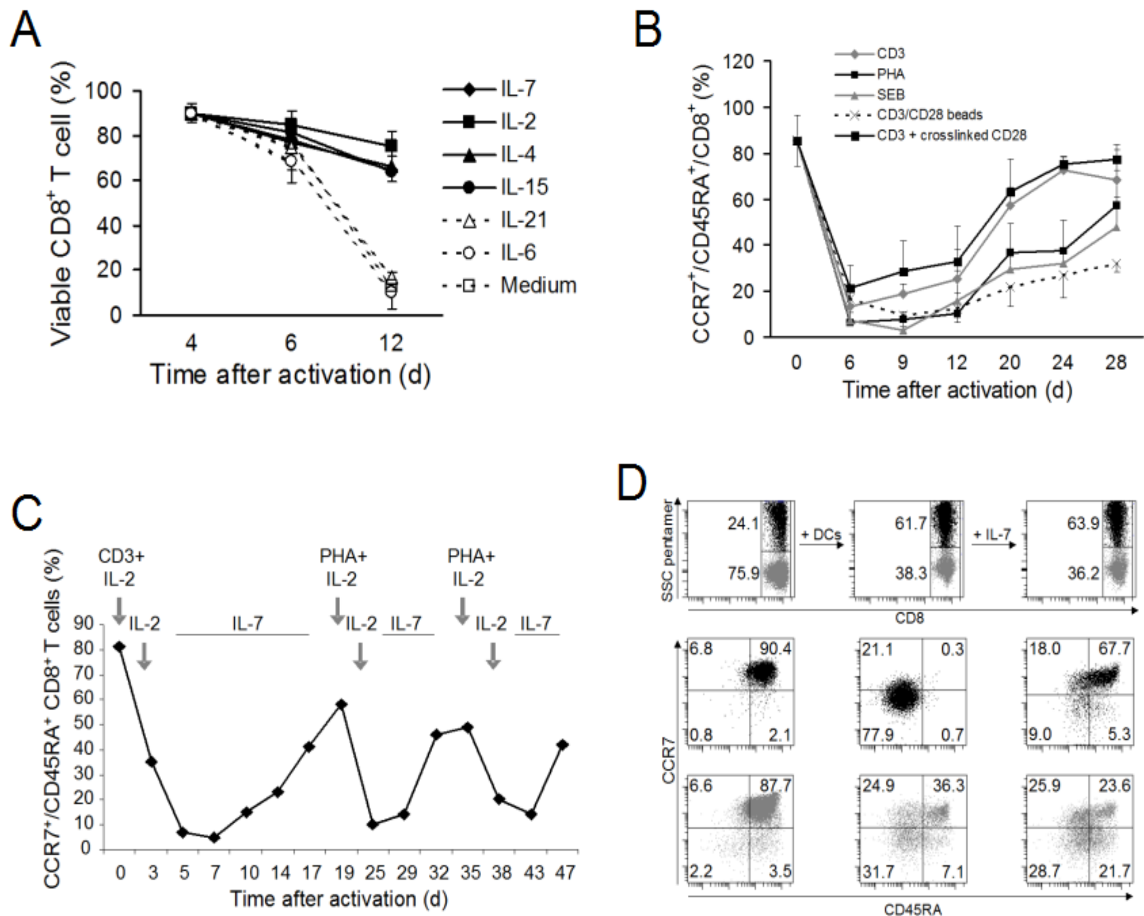

Fig. 3.2. Phenotype reversion can be induced by cytokines other than IL-7 and following activation with different stimuli. (A) Viability of cells incubated with different cytokines. Activated CBMCs were incubated from day 4 with each cytokine or medium, and CD8$^+$ T-cell viability was evaluated by flow cytometry using 7-AAD uptake. Data are represented as means ± 1SD of three samples. (B) The kinetics of phenotype reversion of CD8$^+$ T cells activated with different artificial stimuli. IL-7 was added on day 6, and CD8$^+$ T-cell phenotype was monitored throughout. Data are represented as means ± 1SD of three samples. (C) The kinetics of phenotype reversion of CD8$^+$ T cells undergoing successive cycles of actvation/IL-7 incubation. Newly generated CD8$^+$ T_{Nrev} cells were twice re-stimulated with PHA and induced to revert twice with IL-7 when the percentage of T_N dropped below 20%. (D) Flow cytometry analysis of phenotype changes of CD8$^+$ T_{Nrev} upon activation with the cognate antigen. CB T-lymphocytes were activated, retrovirally-transduced with the SSC-TCR and induced to revert their phenotype with IL-7 (left panels). Afterward, cells were incubated with peptide-pulsed DCs to induce specific differentiation of transduced cells (central panels). IL-7 was then added again, driving the transduced cells to revert their phenotype (right panels). Plots were gated on CD8$^+$ T cells. The upper panels show the percentage of transduced and non-transduced CD8$^+$ T cells.
(From 37).

Cells were then re-challenged with SSC-pulsed autologous DCs which differentiated them again to $CD8^+$ T_{EM} within 5 days (Fig.3. 2D). At this point IL-7 was re-added, and after a further 9 days a second reversion to T_{Nrev} was attained, demonstrating that phenotype reversion was also possible after stimulation with cognate antigen presented by professional antigen-presenting cells.

3.3.3. $CD8^+$ T_{NREV} PROLIFERATE AND DIFFERENTIATE RAPIDLY INTO FUNCTIONAL EFFECTOR CELLS FOLLOWING SECONDARY STIMULATION.

Since T cells progressing along the differentiation pathway increasingly lose their ability to proliferate (Klebanoff et al., 2006), and since T_{Nrev} are antigen-experienced cells that have undergo differentiation and expansion, it might be expected that they would have diminished proliferative potential when compared to primary T_N.

The ability to differentiate and proliferate over time upon re-stimulation was therefore checked in T_{Nrev} and T_N. T_{Nrev} differentiated into memory subsets more rapidly than T_N, acquiring at the same time point a more differentiated phenotype (Fig. 3.3A). They also exhibited a higher proliferation rate (Fig. 3.3B).

The capacity to achieve a more differentiated stage suggested that T_{Nrev} might also acquire effector function following re-stimulation. To check this hypothesis CB T-lymphocytes were activated, retrovirally-transduced with the SSC-TCR and induced to revert their phenotype with IL-7. These SSC-specific T_{Nrev} were then re-stimulated with peptide-pulsed DCs. Once reaching the T_{EM} phenotype, the cells expressed intracellular perforin and GrB (Fig. 3.3C). Moreover, in a standard ^{51}Cr release assay, they exerted

cytolytic activity against SSC-loaded, HLA A*1101-transduced T2 target cells (Fig. 3.3D).

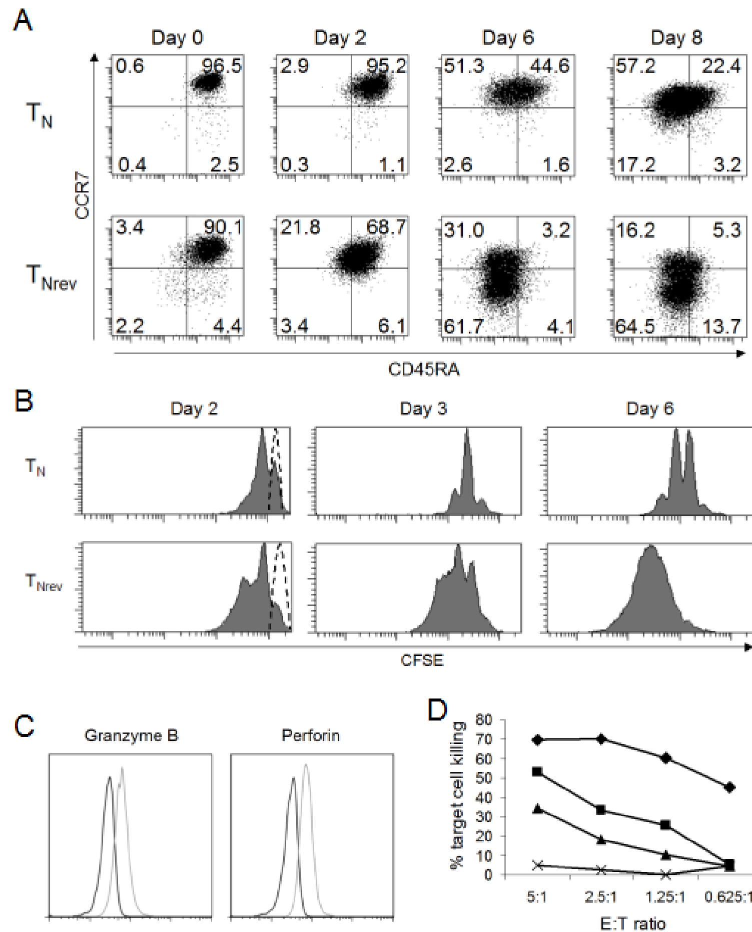

Fig. 3.3. CD8⁺ T_{Nrev} cells have excellent differentiative and proliferative potential, and can acquire effector function upon secondary activation. (A) Flow cytometry analysis comparing the kinetics of phenotype change in $CD8^+$ T_{Nrev} and T_N cells from the same CB sample following activation with PHA. Single representative experiment out of three. (B) Flow cytometry analysis of the proliferation of $CD8^+$ T_{Nrev} and T_N cells. Cells from the samples shown in the panel above were stained with CFSE at day 0 and activated with PHA. The CFSE content in the two cell subsets is shown at the indicated time points. Dashed lines represent basal content of CFSE. Single representative experiment out of three. (C) Flow cytometry analysis of perforin and GrB expression by the re-stimulated TCR-transduced $CD8^+$ T_{Nrev}. The intracellular expression of perforin and GrB by T_{Nrev} cells transduced with the SSC-TCR were assessed after re-stimulating the cells twice with cognate peptide-pulsed DCs. Black histograms indicate resting $CD8^+$ T_{Nrev} cells, gray indicate re-stimulated cells. (D) Cytotoxic assay of re-stimulated, SSC-specific TCR-transduced T_{Nrev}. The T cells were incubated in a standard ^{51}Cr cytolytic assay with target cells consisting of HLA A*1101-transduced T2 cells loaded with either 1 µg/ml (diamonds), 10 ng/ml (squares) or 1 ng/ml (triangles) of SSC peptide. The peptide solvent, i.e. DMSO, was used as control (crosses). The percentage of target cell killing at different E:T ratios is indicated. (From 37).

3.3.4. REVERSION OCCURS IN CD8⁺ T CELLS DERIVED FROM BOTH CBMCS AND PBMCS.

To check whether reversion also occurs in T cells from adult donors, $CD8^+$ T_N from PB and from CB were compared for their ability to revert. After activation, PB T cells were also found capable of reversion to T_{Nrev}, but the magnitude of reversion appeared to be smaller than from CB T cells (Fig. 3.4).

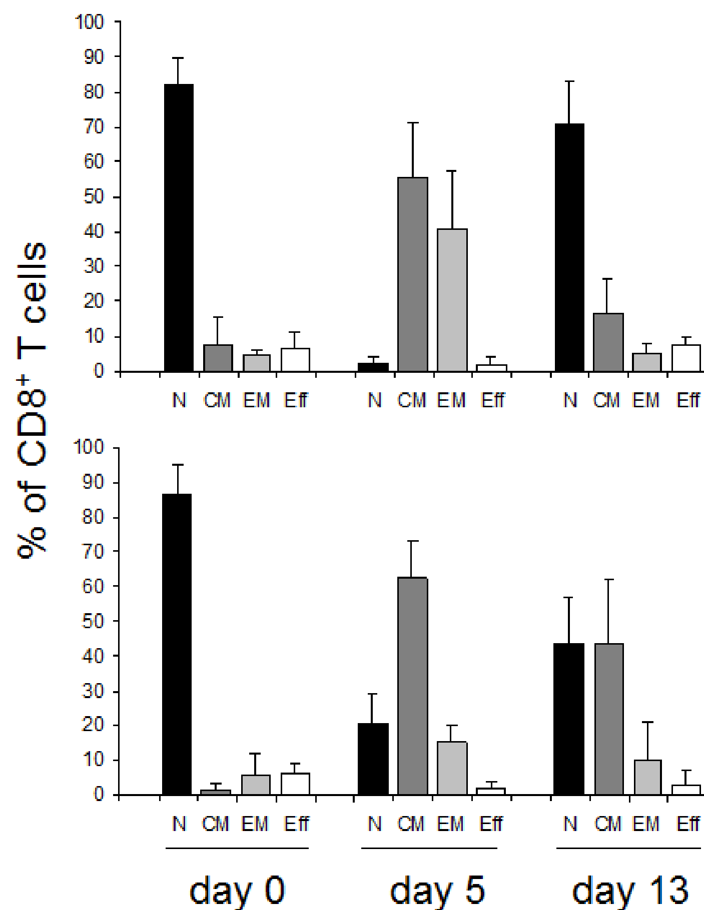

Fig. 3.4. PB-derived $CD8^+$ T_N cells revert less than CB-derived ones. $CD8^+$ T_N were isolated from CB (upper panel) and adult PB (lower panel), activated with anti CD3 and incubated with IL-7 from day 5. The percentage of differentiation subsets was calculated for each cell source before and after reversion. Data are represented as mean ± 1SD of five CB samples and eight PB samples.
(From 37).

3.3.5. CD8$^+$ T_{NREV} AND T_N DIFFER AT THE A TRANSCRIPTOME LEVEL.

To investigate the properties of CD8$^+$ T_{Nrev} at the molecular level, and to identify distinct markers for these cells, the gene expression profiles of CD8$^+$ T_{Nrev} were compared with those of T_N.

The analysis of the transcriptomes identified 1938 genes having statistically significant changes in expression levels (Supplemental Information Appendix, Table S2). 58.9% of these genes were over-expressed in T_{Nrev}, the rest in T_N.

At the cell level, the most significantly enriched pathways within T_{Nrev} are related to cell proliferation, cell cycle control and cell death (Fig. 3.5). These results are likely related to the fact that we compared activated with resting lymphocytes.

Similarly, at the systemic level, the functions affected by the differential gene expression mainly reflect the activation states they represent (Fig. 3.5B), and T_{Nrev} showed an overall increase in the expression of genes involved in T-cell pathways affecting proliferation, migration and function of T-lymphocytes (Fig. 3.5C).

These findings indicate that recently reverted T_{Nrev} are functionally quite distinct from primary T_N, and indeed they show a transcriptome profile of activated T cells, typical of memory cells.

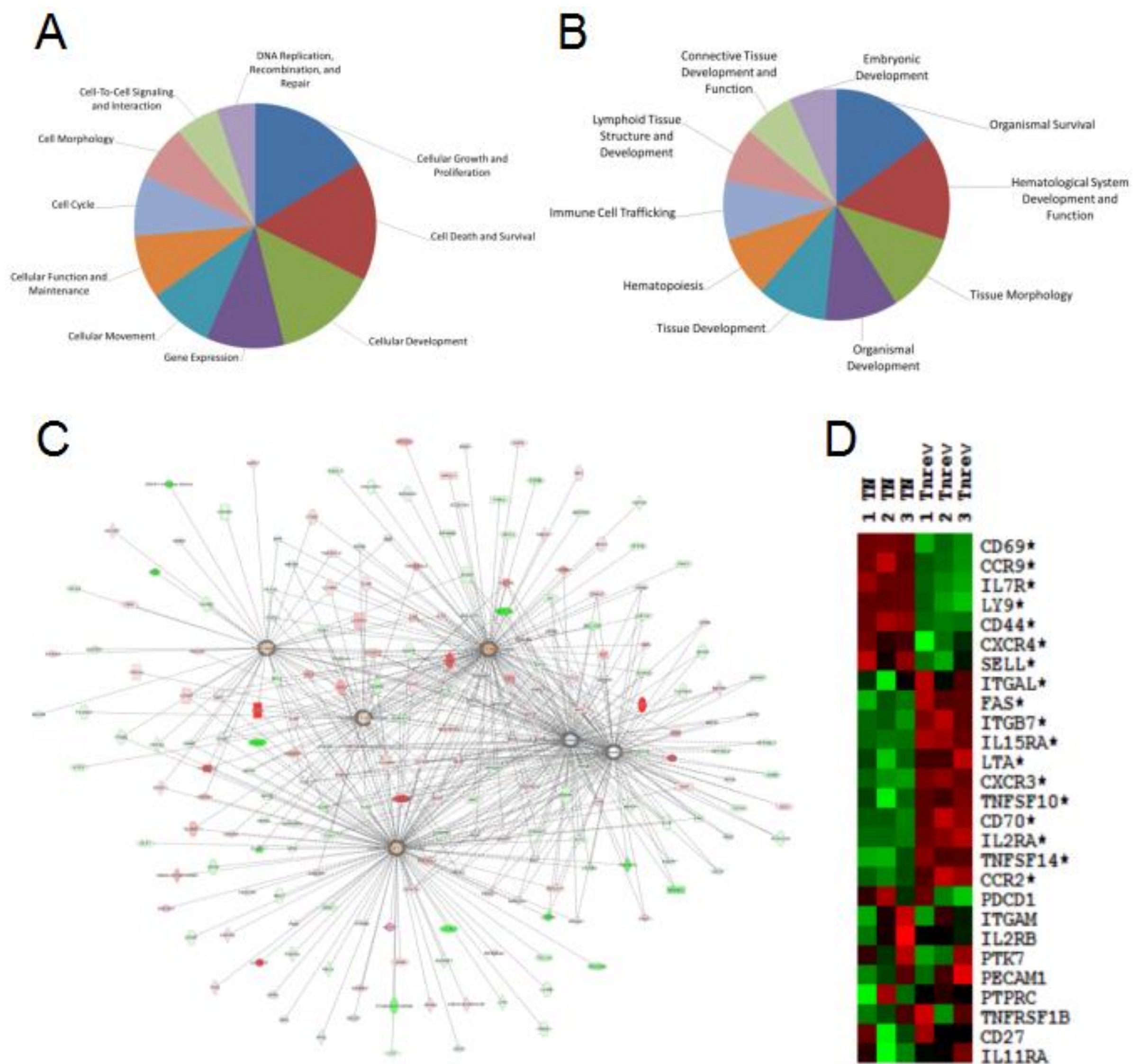

Fig. 3.5. Functions and systems affected by differentially expressed genes in CD8^{+} $T_{Nrev}$and T_N cells. Ingenuity Pathway Analysis was used to identify the pathways involved. (A) The pie-chart shows the molecular and cellular functions affected by the differentially expressed genes. (B) The pie-chart shows the physiological systems (development and function) affected by the differentially expressed genes. (C) Molecular network of genes involved in T-cell development. Red and green colors indicate increased and decreased expression, respectively. (D) Expression heat map of the genes encoding for putative markers for distinguishing T_N, from T_{Nrev}. * indicates the genes with statistically significant difference in the expression in the two cell subsets. (From 37).

3.3.6. T_{NREV} HAVE MEMBRANE MARKERS DISTINCT FROM T_N.

Using the data from the microarray analysis and from literature (7-14), 28 membrane-bound proteins were selected in order to identify distinct markers that could be used for discriminating T_{Nrev} from T_N subsets.

Fig. 3.5D shows the heat map of the RNA expression data from the microarray analysis for these markers.

Afterward, the protein expression for these markers was measured by flow cytometry in the two subsets mentioned above, and in T_{CM}. Most of these markers showed similar expression levels in both T_{Nrev} and T_N (Supplemental Information Appendix, Fig. S2), proving useless for discriminating between these two cell subsets. Instead, integrin β7, CD95, CD25, CXCR3, and CD49d were expressed by T_{Nrev} only (Fig. 3.6A), which for these proteins showed expression levels similar to those found in T_{CM}.

Interestingly, CD95, CD25 and CXCR3 are also distinct markers of T_{SCM}, and CD49d is a distinct marker of T_{MNP} (2,3).

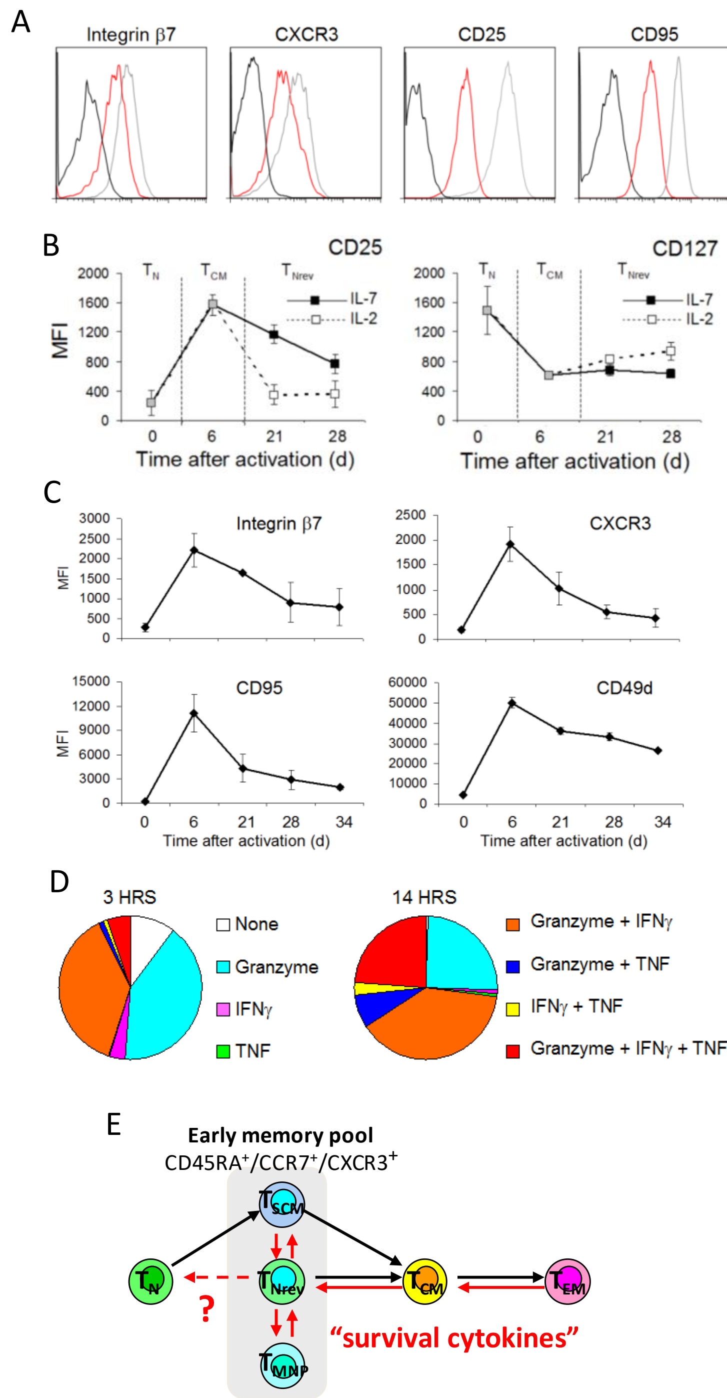

Fig. 3.6. Comparative phenotype of CB CD8$^+$ T_{Nrev}, T_{SCM} and T_N cells, and dependence on culture conditions. (A) The expression of the markers that discriminate early T_{Nrev} from T_N was measured by flow cytometry. T_N (black histograms), recently differentiated T_{CM} (gray histograms) and early reverted T_{Nrev} (red histograms). Single representative experiment out of three. (B) Kinetics of CD25 and CD127 expression by reverted CD8 T_{Nrev} cells in the presence of either IL-2 or IL-7. After phenotype reversion had occurred following activation, cells were either maintained in 25 ng/ml IL-7 or switched to culture in 30 U/ml IL-2. The absence of one cytokine led to increased expression of its cognate receptor. The mean fluorescence intensity for CD25 and CD127 is shown. Data are represented as means ± 1SD of three samples. (C) Kinetics of the expression of discriminatory markers for early T_{Nrev} cells. The mean fluorescence intensity of integrin β7, CXCR3, CD95 and CD49d was monitored at different time points during activation and reversion as in (B). Data are represented as means ± 1SD of three samples. (D) Polyfunctionality of re-activated T_{Nrev}. T_{Nrev} were re-stimulated with PMA plus ionomycin, and the percentages of cells expressing GrB, IFNγ, TNF-α and combinations thereof were measured after 3 and 14 hours. Data are represented as mean of three samples. (E) Proposed model of CD8$^+$ differentiation/ reversion.
(From 37).

3.3.7. CULTURE CONDITIONS INDUCE PHENOTYPIC AND FUNCTIONAL CHANGES IN T_{Nrev}.

We next investigated if culture conditions could alter T_{Nrev} phenotype and function, and how T_{Nrev} relate to T_{SCM} and T_{MNP}. Here we will refer to the recently reverted T_{Nrev} that we have described so far as early T_{Nrev}, and to the revertants that have been incubated in long-term cultures, as late T_{Nrev}. We found that early $CD8^+$ T_{Nrev} cells had a phenotype that was very similar to the phenotype of T_{SCM}, both being $CD45RA^+/CCR7^+/CD62L^+/CD27^+/CXCR3^+/CD31^+/CD95^+/CD122^{lo}/CXCR4^{lo}/CD45RO^-$ $/CD69^-$ (2), and to the phenotype of T_{MNP}, both expressing $CD45RA^+/CCR7^+/CD62L^+/$ $CD27^+/CXCR3^+/CD31^+/CD49d^+/CD31^+/CD122^{lo}/CD45RO^-$ (3).

The only difference between early T_{Nrev} and the other two subsets was that T_{MNP} are $CD127^+$ and T_{SCM} are $CD127^+/CD25^-$, whilst T_{Nrev} are $CD127^{low}/CD25^{high}$. However, the expression of the receptors for IL-7 and IL-2 by T cells can be down-regulated by their respective cytokines (16,17). When early T_{Nrev} were deprived of IL-7 and maintained in IL-2, the cells shifted to a phenotype almost indistinguishable from T_{SCM} and T_{MNP}, with rapid decrease in CD25 expression and progressive increase in CD127 expression (Fig. 3.6B). After a further two weeks culture with IL-2, we observed a progressive loss of CD49d, CD95, CXCR3 and integrin β7 (Fig. 3.6C), so that late T_{Nrev} acquired a phenotype approaching that of primary T_N, displaying $CD49d^-/CD95^-/CXCR3^-$/integrin $\beta7^-$. We were unable to monitor the cells further, due to increased cell death.

T_{MNP} cells are functionally characterized by their ability to express GrB and secrete IFNγ and TNF-α after re-stimulation for 3 hrs with PMA plus ionomycin (3). We used the same stimuli for re-stimulating early T_{Nrev},. Although the cells were mainly GrB^+ or $IFN\gamma^+$ or both, only a minority expressed TNF-α (Fig. 3.6D). However, when re-stimulation was

continued up to 14 hrs, a sharp increase in the percentage of cells expressing all the three molecules was obtained.

We suggest that T_{Nrev}, T_{SCM} and T_{MNP} belong to the same subset of early lineage T cells, where the minor differences in phenotype are related to the stage of activation and cytokine milieu. Given the considerable plasticity in early T-cell differentiation, we propose that the T-cell differentiation model should take into account the reversion of newly differentiated $CD8^+$ T cells, from T_{EM} to T_{CM} and to $T_{Nrev/SCM/MNP}$, and possibly to cells with a T_N phenotype (Fig. 3.6E).

3.4. DISCUSSION

Here we described a previously unknown phenomenon of cytokine-driven reversion of newly generated memory $CD8^+$ T cells into cells with T_N-like phenotype, but with enhanced ability to proliferate, differentiate and acquire effector function upon re-stimulation. We also identified the mechanisms by which these T_{Nrev} acquire function and phenotype of T_{SCM} or T_{MNP}.

Self-renewal is a characteristic feature of T_{SCM}, and this may be a correlate of the property of T_{Nrev} to revert repeatedly back to the T_{Nrev} stage after multiple rounds of stimulation. Like T_{MNP}, T_{Nrev} became functionally lytic, expressed GrB, and secreted IFNγ and TNF-α upon re-stimulation.

Our experiments demonstrated that T_{Nrev} are endowed with remarkable plasticity, where phenotypic changes are dependant on the cytokine milieu and on the time in culture from antigenic stimulation. The difference in CD25 and CD127 expression between early T_{Nrev}, and T_{MNP} and T_{SCM} were related to exposure of the former cells to IL-7, which led to down-regulation of the cognate receptor (15-17). Replacing IL-7 with IL-2 led T_{Nrev} to express a phenotype that for these markers was indistinguishable from that of the other two cell subsets.

The difference in the expression of CD95 between T_{MNP} and T_{SCM} is instead time-dependant, and the T_{MNP} phenotype corresponds to a later time point in the reversion process, where CD95 down-regulates.

In contrast to $CD95^{lo}/CD49d^+$ $T_{MNP,}$ T_{SCM} are $CD95^+$. Although CD49d expression by T_{SCM} has not been previously described, we found that upon prolonged incubation CD95 down-regulated more rapidly than CD49d, so that cells becoming $CD95^{lo}$ still retained high levels of CD49d. It is likely that T_{SCM} represent an earlier stage than T_{MNP} in the de-

differentiation pathway. As such, we propose that early T_{Nrev}, T_{MNP} and T_{SCM}, despite the former cells being generated *in vitro* and the latter ones identified *in vivo*, belong to the same subset of recently differentiated T cells, but modulated by different cytokine milieu, and time from activation.

Early T_{Nrev} retained phenotypic features typical of activated memory cells, including CD95, CD25, CXCR3, integrin β7 and CD49d expression (18-21), that correlate with their rapid functional response to stimulation. A further feature of $CD8^+$ T_{Nrev} was the re-expression of CD45RA, a critical regulator of the signaling threshold in T-lymphocytes (12, 22), which render T_{Nrev} more responsive to antigenic stimulation than T_{CM} or T_{EM}. We hypothesize that early T_{Nrev} cells are generated from recently differentiated T_{CM} and T_{EM} during the resolution of a viral infection. In this way an antigen-specific memory pool with heightened responsiveness to early infective re-challenge can be generated. Phenotype reversion would replenish the phenotypically naïve $CD8^+$ T-cell pool, which would otherwise be depleted of the cells responding to the stimulating antigens if differentiation were a one-way process, and allow for the retention in this pool of antigen-experienced cells. Indeed, besides the discovery of T_{MNP} and T_{SCM}, there is further evidence to support the concept that antigen-experienced T cells are contained within the naïve $CD8^+$ T-cell pool: expanded populations of $CD8^+$ T_N specific for Cytomegalovirus, Epstein Barr virus or Influenza virus have been found in individuals positive for these viruses (23). Phenotype reversion may also explain how the T_N pool remains preserved into old age, whilst the thymus involutes. Both *in vivo* analyses and mathematical modeling have shown that thymic output is insufficient to maintain the naïve T-cell pool in adults (24-26). Interestingly, the ability to revert was greater in $CD8^+$ T cells derived from CB than from adults. This suggests that reversible $CD8^+$ T cells may represent a subpopulation of T_N that diminishes with age. This seems contrary to the finding that the

frequency of T_{MNP} increases with age, but it can be explained by the fact that the latter cells respond to chronic viral infections.

With time, early T_{Nrev} reverted further to a late T_{Nrev} stage, characterized by a $CD95^{lo}$/integrin $\beta7^{lo}$/$CXCR3^{lo}$ phenotype. Despite this trend, we cannot conclude that late T_{Nrev} cells can de-differentiate further, and the possibility for these cells to achieve a phenotype indistinguishable from primary T_N remains a mere speculation.

Our findings indicate that the generation of the human T-cell memory pool is a more complex phenomenon than hitherto thought, challenging the model of unidirectional T-cell differentiation. Although the mechanism leading to the generation of T_{MNP} is unknown, T_{SCM} is thought to differentiate straight from T_N (27), but we suggest that the current T-cell differentiation model should be modified to also take into account the reversion of memory T cells to $T_{SCM/MNP/Nrev}$. Indeed, phenotypic reversion from T_{EM} to T_{CM} has been observed previously in murine models and in patients following hematopoietic stem cell transplant (HSCT) (28-30). Our model does not rule out the possibility that memory $CD8^+$ T-cell precursors can also exist within different phenotypic subsets, such as T_{CM} (31).

The capacity to undergo reversion is likely an innate property of recently differentiated memory T cells, that becomes effective if cells are kept surviving long enough by ‘survival’ cytokines, of which IL-7 was the most potent. Both IL-7 and IL-15 are known to promote the survival of memory $CD8^+$ T cells (32), but IL-7 is unique in having a key role in providing survival signals and supporting homeostatic expansion of T cells during thymic differentiation (33, 34). IL-7 was also found to have a dominant role in the peripheral expansion of T_{SCM} directly from T_N in patients following HSCT (30, 35). IL-7

has therefore the ability to drive the generation of T_{SCM} in both directions: from T_N or through reversion from T_{CM} or T_{EM}.

We propose that reversion is a critical physiological mechanism by which the pool of early antigen-specific memory T cells is replenished in order to maintain a competent immune system over the lifetime of an individual. Understanding this mechanism also permits the development of new methods for generating early lineage memory T cells for ATCT, which we have demonstrated with the transduction of an EBV-specific TCR, and where such cells used for immune receptor modification retain properties associated with superior clinical efficacy (6).

3.5. MATERIALS AND METHODS

3.5.1 CELL SEPARATION AND CULTURE.

The study was approved by the National Research Ethics Committee, UK REC no. 11/WM/0315, and by the Non-Clinical Issue committee of the NHS Blood and Transplant. Human CB from anonymized collections unsuitable for banking, was provided by the NHS Cord Blood Bank, UK, as Non-Clinical Issue. Peripheral blood (PB) was collected from consenting adult healthy blood donors from the NHS Blood and Transplant Donor Centre, Birmingham, UK.

PB mononuclear cells (PBMCs) and CB mononuclear cells (CBMCs) were obtained by Ficoll separation. $CD8^+$ T_N and T_{Nrev} were enriched using the Naïve $CD8^+$ T-cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany). $CD8^+$ T_{EM} cells were negatively isolated after activation of enriched $CD8^+$ T_N by removal of $CCR7^+$ and $CD45RA^+$ cells with anti-CCR7-APC, anti-CD45RA-APC and anti-APC MicroBeads (Miltenyi). $CD8^+$ T_{CM} cells were isolated from less differentiated samples by depletion of $CD45RA^+$ cells with anti CD45RA-APC and anti-APC MicroBeads. Cells were cultured in RPMI 1640 plus 10% FCS.

3.5.2. TCR GENE TRANSDUCTION.

CBMCs were retrovirally transduced with a TCR, specific for the SSCSSCPLSK (SSC) peptide of the LMP2 protein of Epstein Barr virus, as previously described.[6] Also previously described are the generation of dendritic cells (DCs), their loading with the peptide, and the re-stimulation of transduced T cells with peptide-pulsed DCs.

3.5.3. CELL ACTIVATION AND TREATMENT.

Cells were activated, or re-activated, with either of the following stimuli.

Anti-CD3: cells were incubated with 66 ng/ml anti-CD3 antibody (OKT3), plus 300 U/ml IL-2 (Miltenyi); cells were activated in this way throughout the study, unless otherwise indicated.

Anti-CD3 and crosslinked anti-CD28: cells were incubated with 66 ng/ml OKT3, 66 ng/ml LEAF anti-CD28 (BioLegend, San Diego, CA), and 66 ng/ml rat anti-mouse IgG1 (BioLegend), plus 50 U/ml IL-2. CD3/CD28 beads: Dynabeads T Activator CD3/CD28 (Life Technologies, Grand Island, NY) were incubated with CBMCs at 1:1 ratio in the presence of 30 U/ml IL-2. Phytohemagglutinin (PHA): cells were incubated with 1% PHA M (Life Technologies), plus 50 U/ml IL-2. Staphylococcus enterotoxin B (SEB): cells were incubated with 1 μg/ml SEB (Sigma-Aldrich), plus 50 U/ml IL-2. From day 2, IL-2 100 U/ml, for soluble anti-CD3, or 30 U/ml, for the other cases, was added thrice a week. Phenotype was checked alternate daily. When the percentage of $CCR7^+/CD45RA^+$ $CD8^+$ T cells dropped below 20%, half of the medium was replaced with fresh medium containing either IL-2, IL-4, IL-6, IL-7, IL-15 or IL-21 (all from Miltenyi), or combinations thereof, at final concentrations of 30 U/ml, 25 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml, and 50 ng/ml, respectively. Thrice a week, half of the culture medium was replaced with new medium plus cytokine(s). Re-activation by phorbol myristate acetate (PMA) plus ionomycin was performed as previously described.[3]

3.5.4. FLOW CYTOMETRY.

The antibodies used for cell staining are listed in Supplemental Information Appendix, Table S3. Gating strategy involved selection of single cells and use of a “dump channel” including either 7-aminoactinomycin D (BD) and PerCP-conjugated CD14, CD16 and

CD19, or Live/Dead Fixable Violet (Thermo Fisher Scientific, Wilminghton, DE) and Pacific Blue-conjugated CD14, CD16 and CD19. Proliferation was evaluated by staining cells with 1 μM CFSE. For intracellular staining, cells were fixed and permeabilized using the FIX&PERM kit (ADG, Kaumberg, Austria). In some experiments cells were enumerated using Trucount Beads (BD). Transduced lymphocytes were identified using HLA A*1101:SSC peptide-specific pentamers and Tag/PE (Proimmune, Oxford, UK). Flow cytometry was performed on either a FACSCanto II, or a Fortressa (BD).

3.5.5. CYTOTOXICITY ASSAY.

Cytotoxicity of transduced T cells was assessed in a standard ^{51}Cr assay.[6] Briefly, HLA A*1101-transduced T2 cells were loaded with different concentrations of SSC peptide, then used as targets at 2500 cells/well in a 5 hrs test.

3.5.6. MICROARRAY ANALYSIS.

Gene expression analysis was performed on 3 $CD8^+$ T_N and 3 $CD8^+$ T_{Nrev} samples. RNA extraction was performed using RNeasy columns (Qiagen, Hilden, Germany). Source RNA was confirmed as high quality by use of a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). RNA Integrity Numbers of 6.0 were confirmed for all samples using a RNA 6000 Pico Chip kit (Agilent). 25ng of each source sample RNA was labeled with Cy3 dye using the Low Input Quick Amp Labelling Kit (Agilent). A specific activity of greater than 6.0 was confirmed by measurement with a spectrophotometer. 600 ng of labeled RNA was hybridized to SurePrint G3 Human 8x60K microarray slides (Agilent). After hybridization, slides were scanned with a High Resolution C Scanner (Agilent), using a scan resolution of 3 mm. Feature extraction was performed using Feature Extraction Software (Agilent), with no background subtraction. Extracted data were

normalized using the R 3.0.1 software environment with the limma 3.16.8 analysis package (36). Log transformed expression values were analyzed using two class paired analysis, SAM v4.01.

3.5.7. STATISTICS

Differential gene expression was accepted as statistically significant if the False Discovery Rate was below 5% and the fold change in gene expression was >2. Functional and network analysis was performed on the pool of significant genes using Ingenuity Pathway Analysis (Qiagen).

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3.7. SUPPLEMENTAL INFORMATION APPENDIX

Table S1. Cell numbers are not altered by phenotypic reversion. (From 37).

	CB 1		CB 2		CB 3	
	Day6	**Day 16**	**Day8**	**Day 18**	**Day6**	**Day 20**
$CCR7^+/CD45RA^+$	32,1[A]	872,1	9	345,5	21,3	1769,3
$CCR7^+/CD45RA^-$	982,2	256,6	782,2	303,2	1560,9	487,9
$CCR7^-/CD45RA^-$	308	79,2	183,7	101,4	213,2	33,1
$CCR7-/CD45RA^+$	5,4	72	1,4	32	2,9	43
Total	1327,7	1279,9	976,3	782,1	1798,3	2333,3

[A] Absolute number of CD8+ T cells within different subsets at T_N nadir (days 6 and 8) and T_{Nrev} plateau (days 16, 18 and 20). Cell counts (x10^3) from 3 different CB samples, enumerated using Trucount beads.

Table S2. Genes showing statistically significant difference in the levels of expression between T_N and T_{Nrev}. (From 37).

Gene ID	Fold change	Gene ID	Fold change	Gene ID	Fold change	Gene ID	Fold change	Gene ID	Fold change	Gene ID	Fold change	Gene ID	Fold change	Gene ID	Fold chan
AAK1	-2.114	CD300A	-6.060	FAM72C/F	5.043	KCMF1	2.495	MOSPD2	2.141	PPM1F	-2.553	SLCO3A1	2.356	TUBA1C	3.383
AAR2	2.020	CD300E	-3.242	FAM92A1	2.646	KCNJ14	-2.247	MPEG1	-12.313	PPM1N	-2.396	SLCO4A1	8.824	TUBA3C/T	-2.134
AARS	3.539	CD3EAP	3.084	FANCF	2.060	KCNJ18	-3.890	MPHOSPH	3.253	PPP1R2	-3.102	SLED1	-3.501	TUBA4A	-2.697
ABCA11P	3.007	CD8B	2.035	FAS	2.430	KCNK6	2.258	MPRIP	-3.613	PPP1R8	2.410	SLIRP	3.267	TUBB2A	-25.733
ABCB1	-7.766	CD99P1	2.011	FASTKD2	2.471	KCNQ1	-2.099	MR1	2.155	PPP1R10	2.518	SMAD7	-6.617	TUBD1	2.247
ABCD2	4.358	CDA	-2.308	FASTKD3	3.450	KCTD5	2.544	MRFAP1L	3.926	PPP1R15A	-23.385	SMAP1	-2.026	TUBG1	2.016
ABCD3	-2.379	CDC42	-10.844	FASTKD5	-2.450	KDELC1	3.217	MRPL3	2.181	PPP1R15B	-2.756	SMAP2	-3.019	TVP23B	2.926
ABI2	-2.043	CDC123	2.401	FBLN2	-2.539	KDM4A	2.118	MRPL13	4.015	PPP3CC	-2.007	SMARCAD	2.137	TVP23C	2.832
ABTB2	-2.273	CDC25C	4.242	FBP1	-2.918	KDM4C	-4.148	MRPL15	3.581	PRAM1	-3.049	SMARCD2	2.119	TXN	2.955
ACAA2	2.041	CDCA2	15.375	FBXO5	6.757	KEAP1	3.339	MRPL17	4.804	PRDM1	-4.086	SMARCD3	-4.289	TXNDC5	2.241
ACAD9	2.085	CDCA3	7.019	FBXO8	3.122	KIAA0125	-4.646	MRPL18	2.448	PRDX1	2.463	SMC2	4.198	TXNDC9	3.769
ACAT1	2.293	CDCA8	10.114	FBXO11	-2.290	KIAA1551	2.738	MRPL19	-2.993	PRDX3	6.894	SMC3	2.277	TYMSOS	2.920
ACOT13	2.056	CDHR3	-3.172	FBXO22	2.302	KIAA1683	-3.040	MRPL20	2.558	PRDX4	3.482	SMEK1	-2.048	TYW3	2.132
ACRBP	-3.955	CDK1	19.376	FBXO30	3.190	KIF11	11.539	MRPL27	6.156	PRH2	-2.348	SMG8	3.156	UBA3	2.460
ACTL10	4.224	CDK4	2.789	FBXO33	-2.836	KIF14	5.747	MRPL32	2.067	PRKX	2.290	SMIM3	-2.787	UBA6-AS1	2.076
ACTL6A	3.901	CDK12	-2.579	FBXO43	5.309	KIF15	13.452	MRPL35	2.651	PRL	-7.115	SMIM5	-5.445	UBE2A	-2.642
ACTN1	-3.337	CDK16	-2.473	FBXW4	-2.085	KIF18A	4.659	MRPL39	2.977	PRMT6	3.720	SMIM7	2.170	UBE2C	18.446
ACTR3	2.029	CDK2AP2	3.618	FBXW9	2.074	KIF1BP	3.962	MRPL42	3.278	PRNP	-2.706	SMIM13	2.444	UBE2D3	-4.097
ADAM19	5.340	CDK5R1	-6.644	FCAR	-6.806	KIF20A	5.800	MRPL46	3.756	PRO2852	-3.872	SMIM19	2.079	UBE2H	-2.491
ADGRA3	-4.663	CDKN3	6.307	FCGR1B	-4.503	KIF20B	3.140	MRPL47	2.308	PRPS1	-2.119	SMIM10L2	2.670	UBE2Q2P	2.042
ADGRE2	-2.350	CDKN1C	-4.001	FCGR2A	-7.629	KIT	4.298	MRPL50	2.733	PRR7	-3.324	SMIM10L2	3.244	UBE2R2	-2.540
ADGRG1	-42.638	CDKN2A	15.909	FCGR2C	-10.162	KLF4	-6.204	MRPL51	3.421	PSAT1	3.723	SMPD1	-2.295	UBE2T	5.662
ADM	-11.780	CDKN2B-A	2.482	FCGR3A/F	-54.901	KLF6	-5.700	MRPL55	-2.081	PSD	-2.334	SMPD3	10.796	UBL4A	2.180
ADPRHL2	2.204	CDKN2D	-3.116	FCGRT	-2.598	KLF9	-5.195	MRPS12	2.245	PSEN1	2.001	SNAI1	-5.413	UFSP1	2.470
ADRA1A	-2.507	CEBPB	-2.890	FCN1	-68.446	KLF10	-5.842	MRPS14	6.161	PSMA2	2.124	SNAI3	3.262	UHRF1BP	-2.584
ADRBK2	2.333	CELA1	-5.410	FDPSP2	2.086	KLF16	-2.228	MRPS17	3.219	PSMA3	3.155	SNAPC5	2.254	ULK2	-4.244
AGAP3	-2.116	CENPA	9.274	FDXR	4.312	KLHDC9	2.798	MRPS21	2.139	PSMA4	3.160	SNAR-B2	7.857	UMPS	10.044
AGFG2	3.950	CENPE	8.928	FEM1B	-2.901	KLHL5	2.256	MRPS22	2.461	PSMA5	3.519	SNAR-D	6.772	UQCRQ	2.280
AGO2	-2.482	CENPF	3.870	FEM1C	-2.777	KLHL6	-3.933	MRPS23	4.165	PSMB2	-4.014	SNAR-F	6.995	USP1	2.072
AGPAT4	-2.386	CENPN	3.706	FGGY	-2.382	KLHL9	4.396	MRPS28	4.807	PSMB5	2.363	SNAR-G2	12.039	USP3	-3.827
AHNAK	2.263	CENPQ	2.604	FGR	-38.315	KLRF1	-18.680	MRPS33	2.259	PSMC6	2.547	SNAR-H	6.757	USP31	-3.940
APLP2	-2.732	CLUAP1	3.083	GGT7	-2.189	LINC0056	-2.263	NAA38	3.222	RAPGEF2	-2.285	SNORD32	-2.421	WHAMMP	-2.189
APOBEC3	4.046	CMAHP	2.853	GIMAP4	2.930	LINC0066	2.333	NABP1	3.654	RAPGEF6	-5.713	SNORD35	-5.246	WHAMMP	-4.361
APOE	-4.045	CMPK2	3.171	GIMAP7	3.515	LINC0067	-2.549	NAGK	2.581	RARG	2.996	SNORD36	-3.465	WIPF1	-6.124
APOLD1	2.852	CNPY2	2.029	GINS1	4.763	LINC0084	2.036	NAMPT	-17.075	RARS	3.391	SNORD36	-2.638	WNT10B	-2.268
APP	-2.331	CNST	-5.882	GLB1	2.556	LINC0090	2.014	NANOS1	2.175	RASA2	-2.900	SNORD36	-3.152	WRB	2.345
APTX	2.565	CNTRL	-3.455	GLRX2	3.525	LINC0092	-5.666	NAT1	5.784	RASD1	-2.621	SNORD50	-2.451	WSB2	3.483
AQP3	2.148	COA1	-2.581	GLT1D1	-3.244	LINC0093	-3.514	NBAT1	-2.161	RBBP6	-4.331	SNORD59	-2.017	WTAP	-4.415
AREG	-12.067	COA3	6.743	GLUL	2.688	LINC0100	2.416	NBEA	-3.072	RBBP8	3.309	SNRK	-2.388	XLOC_00	2.575
ARF6	2.202	COA7	2.482	GMFB	2.920	LINC0113	2.025	NBN	2.585	RBM12	2.480	SNRPD1	2.315	XPO5	2.754
ARGLU1	-2.253	COL18A1	-3.608	GMPPB	2.767	LINC0116	3.255	NCAPG	2.597	RBM19	-2.157	SNRPG	2.776	XYLT1	2.060
ARHGAP1	2.128	COL5A1	-2.612	GNAS	-4.027	LINC0135	2.039	NCF1	-5.878	RBM38	-13.054	SNTG2	-2.812	YAE1D1	3.565
ARHGEF7	-9.350	COL9A2	2.463	GNAZ	-2.846	LINC0148	-2.881	NCF4	3.555	RBM39	-3.129	SNX29	-3.197	YME1L1	2.721
ARHGEF4	-5.594	COMMD3	2.039	GNG7	-2.538	LINC0150	-2.211	NCK1-AS	2.387	RBM12B	2.909	SOCS3	-6.237	YOD1	-2.178
ARHGEF1	-4.706	COMMD4	2.733	GNG10	2.853	LINC0155	-2.227	NCR3	4.523	RBP7	-4.212	SON	-3.632	YPEL5	-5.359
ARID5A	-6.520	COMMD5	3.362	GOLGA4	2.259	LINC0156	2.053	NDEL1	-2.708	RBX1	3.542	SORD	-2.335	YRDC	-2.201
ARL4A	-2.614	COMMD8	2.514	GON4L	-2.264	LINC-PINT	-6.042	NDN	2.280	RDM1	2.584	SOS1	3.549	YTHDC1	-2.238
ARL6IP1	2.554	COMMD10	2.272	GPAM	3.407	LINC0100	-3.452	NDNL2	-4.550	RDX	2.340	SOX4	-9.396	ZAK	2.662
ARL6IP5	2.725	COPB2	4.390	GPANK1	2.587	LINGO3	2.097	NDUFA13	2.556	REC8	-2.169	SPC25	13.405	ZBED3	-2.784
ARMC8	-2.398	COPG2IT1	-3.140	GPATCH3	2.595	LITAF	-2.387	NDUFAB1	2.317	RECQL	2.081	SPDL1	2.105	ZBTB1	-2.109
ARMCX1	-4.744	COPS4	3.224	GPBAR1	-2.714	LMF1	-11.937	NDUFB1	2.056	RELT	-2.332	SPIN4	2.859	ZBTB3	6.647
ARMCX4	-2.587	COQ3	2.010	GPCPD1	-3.164	LMNB1	2.891	NDUFB3	3.543	REM2	-3.885	SPINK2	-7.733	ZBTB6	2.751
ARMT1	3.002	COQ5	2.042	GPKOW	2.205	LMNTD2	-2.004	NDUFB7	2.121	RFESD	2.774	SPOCK2	-2.433	ZBTB10	-6.053
ARRDC2	-4.995	COX16	3.317	GPN1	2.029	LMO2	-9.535	NDUFC2	3.471	RFX3	-2.371	SPON2	-59.369	ZBTB20	-2.580
ARRDC4	-2.142	COX17	3.495	GPN3	2.896	LNX2	2.431	NDUFS3	2.197	RGCC	-2.884	SPRY1	-8.981	ZBTB21	-19.078
ASB7	-2.135	CPXM1	-2.123	GPR19	3.552	LOC28391	-2.019	NECAB3	4.832	RGS1	-158.617	SPSB2	2.088	ZBTB24	-13.326
ASGR1	-3.464	CR2	-14.188	GPR35	-2.195	LOC28437	-2.011	NECAP2	2.092	RGS2	-18.028	SPTBN1	-12.385	ZBTB25	-2.409
ASH2L	2.095	CREBRF	-4.578	GPR171	5.233	LOC33998	2.461	NEIL1	-4.665	RGS16	2.536	SPTLC2	3.849	ZBTB45	2.739
ASPM	10.185	CREG1	-2.141	GPR180	2.117	LOC37489	-3.346	NEURL1B	7.156	RGS9BP	2.086	SPTSSA	2.614	ZC3H12A	-6.460
ASXL1	-2.330	CRHR1-IT	-2.423	GPR183	-9.472	LOC39990	-2.113	NFAM1	-4.618	RHNO1	4.362	SQLE	3.463	ZC3HAV1	-2.557
ATF3	-6.498	CRNDE	4.030	GPRASP1	-5.892	LOC4013	-2.267	NFE2	-7.562	RHOB	-4.862	SRFBP1	2.649	ZC3HC1	2.442
ATG2A	-2.383	CRYZ	2.451	GPSM2	3.240	LOC44029	3.081	NFE2L3	4.769	RHOH	-3.338	SRGAP2B	-2.237	ZC4H2	2.041
ATL1	-2.245	CSF1R	-17.470	GRAP2	2.232	LOC49375	-2.231	NFIL3	-5.465	RHPN1	-3.390	SRRD	2.192	ZCCHC2	-2.051
ATP11C	-4.547	CSF3R	-11.272	GSAP	-4.263	LOC64223	2.546	NFKBIA	-12.866	RIMKLB	-3.833	SRRM2	-2.285	ZCCHC10	2.423
ATP1A1-A	2.008	CSNK1E	-6.615	GSKIP	3.967	LOC64663	-3.090	NFKBIB	-2.546	RIOK2	2.404	SRSF1	-4.162	ZDHHC11	-4.899
ATP5F1	2.527	CSRNP1	-35.426	GSTM2	-3.071	LOC64676	-2.616	NFKBIE	-3.970	RIPK1	2.084	SS18L2	2.627	ZDHHC12	2.154
ATP5J2	2.062	CSTF1	3.002	GTF2E1	3.019	LOC72848	2.574	NFS1	2.269	RIPK2	-7.603	SSH2	-4.424	ZDHHC23	3.544
ATP6V0C	-2.129	CSTF2	4.334	GTF2H5	3.105	LOC10012	2.585	NGFRAP1	-3.099	RIPK3	2.539	ST13	-2.322	ZDHHC11	-4.899
ATP6V0E1	2.168	CTDSP2	-2.092	GTPBP1	-2.627	LOC10013	-3.880	NIF3L1	3.037	RIPK4	-3.479	ST6GALN	-2.645	ZEB2	-6.929
ATP6V1C1	3.013	CTH	-4.511	GYPB	-2.225	LOC10014	2.052	NIFK	4.478	RLF	-2.301	STAC3	-2.188	ZFAND5	-9.653
ATP6V1E2	-2.099	CTNNBIP1	-2.055	GZMH	-78.525	LOC10028	-3.666	NIPSNAP3	2.838	RMI1	2.518	STAG2	-2.230	ZFAND2A	-2.111
ATXN1	2.223	CTR9	3.153	H3F3A/H3	-9.357	LOC10028	3.490	NLN	2.202	RMND5A	-3.328	STAMBPL	3.548	ZFC3H1	-2.410
ATXN7L1	-2.794	CTSF	-2.689	HAMP	-2.090	LOC10029	-9.606	NLRP3	-17.296	RMRP	-2.376	STAT4	2.032	ZFP14	2.016
AZIN2	2.423	CTSH	7.357	HAPLN3	2.929	LOC10050	-4.780	NME7	2.188	RNASE6	-2.954	STIP1	2.251	ZFP30	4.449
B2M	2.073	CUX1	-2.548	HARS2	2.969	LOC10050	-2.702	NOD2	-2.637	RNF6	2.200	STRBP	-2.293	ZFP36	-14.329
B3GALT2	7.943	CWC25	2.271	HAS1	-5.788	LOC10050	2.629	NOL11	2.474	RNF11	-2.752	STXBP1	2.258	ZFP82	2.565
B3GALT4	2.878	CXCL2	-9.162	HAT1	4.213	LOC10050	-2.121	NOL4L	-3.160	RNF14	2.072	SUCLA2	3.144	ZFP36L1	-4.634
B3GNT2	-2.666	CXCL8	-4.481	HAUS1	2.285	LOC10050	-3.526	NOP16	4.259	RNF34	4.151	SUCO	-2.065	ZFP36L2	-8.159
B4GALT1	-3.221	CXCR3	3.042	HAUS2	2.165	LOC10192	4.021	NOP56	2.145	RNF38	-2.270	SULF2	-7.289	ZFYVE21	2.998
BAG2	4.678	CXCR5	-4.956	HBA1/HBA	-758.669	LOC10192	-2.887	NOXA1	-2.106	RNF43	-2.992	SUV39H1	2.382	ZG16B	4.862
BAG3	-3.922	CXCR6	5.280	HBB	-2954.175	LOC10192	6.286	NPAS2	-3.231	RNF103	-19.725	SYF2	-3.161	ZKSCAN2	3.050
BAMBI	-8.561	CYB561D	2.051	HBD	-374.713	LOC10192	-3.341	NPIPA8 (i	-2.038	RNF122	-5.747	SYK	-9.639	ZKSCAN4	2.370
BAX	2.240	CYBB	-14.941	HBG1	-2019.402	LOC10192	13.895	NPRL2	2.195	RNF125	-3.102	SYNE2	2.348	ZMIZ1	-2.002
BBS12	4.559	CYCS	-2.317	HBM	-44.123	LOC10192	2.027	NR1D1	-6.029	RNF130	-5.424	SYNE4	-2.376	ZMYM2	-2.187
BBX	-2.273	CYP2F1	-2.944	HBQ1	-4.543	LOC10192	-3.950	NR1D2	-2.615	RNF138	-2.878	SYNRG	-2.073	ZMYM5	-2.706
BCAS2	-3.871	DARS2	3.220	HBS1L	-2.574	LOC10272	-2.544	NR3C1	2.063	RNF139	-3.967	SYS1	-2.438	ZMYND8	-2.039
BCAT1	4.953	DBH-AS1	-12.320	HCAR3	-8.839	LOC10536	-2.498	NR4A2	-52.420	RNF146	2.161	SYTL2	-2.518	ZNF2	2.009
BCAT2	2.690	DBNL	-2.114	HCFC2	4.329	LOC10537	-2.415	NRDE2	2.222	RNF149	2.463	TACO1	2.459	ZNF10	-2.629
BCDIN3D	5.912	DCAF7	4.274	HCP5	3.488	LOC10065	-2.192	NSD1	-2.642	RNF181	2.468	TAF2	2.003	ZNF17	2.996
BCL11A	-27.478	DCAF13	3.135	HDAC5	-2.425	LOC10099	2.338	NUAK2	-3.296	RNF185	2.094	TAF1A	2.277	ZNF20	2.101
BCL2L11	-4.715	DCAF13P	2.458	HDAC7	-2.090	LOC10272	-17.048	NUCKS1	2.213	RNF212	-2.122	TAF9B	2.539	ZNF35	2.561
BCL7A	-2.034	DCHS1	-3.665	HDAC10	2.194	LOC38983	2.203	NUDT6	3.272	RNF219	4.012	TAGAP	-3.582	ZNF45	2.604
BCL9L	-3.382	DCK	2.808	HDGFRP3	8.720	LPAR2	-2.140	NUDT21	2.147	RNF144A	3.269	TANC2	-2.920	ZNF57	3.917
BDH1	3.677	DCLRE1C	2.112	HEATR1	2.349	LPAR5	3.851	NUF2	7.869	RNF144A-	6.205	TAPT1	-2.164	ZNF79	3.613
BEX2	-2.239	DCTPP1	3.065	HEATR6	2.416	LPAR6	2.641	NUP35	2.654	ROBO3	-2.223	TARS	2.942	ZNF91	2.617

BFSP2-AS	-3.011	DDB2	3.800	HEATR5A	2.215	LRFN2	3.693	NUP37	3.254	RPA1	2.001	TATDN3	2.263	ZNF107	2.376
BHLHE40	3.873	DDIT4	-7.783	HECA	-2.814	LRP6	-2.255	NUP85	2.036	RPA3	2.440	TBC1D1	-2.004	ZNF132	2.212
BIVM	2.043	DDX1	2.638	HEG1	3.083	LRR1	5.717	NUP133	2.398	RPL17	-2.140	TBL2	4.614	ZNF146	2.627
BLM	3.717	DDX3X	-2.205	HEMGN	-2.571	LRRC2	2.097	NUSAP1	13.601	RPL28	-3.796	TBXAS1	-2.552	ZNF155	2.020
BLOC1S2	2.192	DEDD2	-2.027	HENMT1	2.359	LRRC24	-2.827	NUTM2A-A	-2.404	RPL26L1	2.427	TCAP	-3.548	ZNF165	-2.888
BLOC1S3	2.509	DEFA1 (in	-83.483	HERPUD2	-3.526	LRRC25	-3.865	NUTM2B-A	-2.720	RPL27A	-2.144	TCEAL4	-2.096	ZNF180	5.035
BLZF1	3.600	DEGS1	2.201	HES4	-50.406	LRRC34	6.005	NUTM2F/N	-5.742	RPS6	-2.321	TCF7	-2.022	ZNF181	2.596
BNIP3L	-2.135	DEK	2.179	HEXIM1	-2.155	LRRC70	-3.081	NXT1	-3.644	RPS27	-4.287	TCF12	-2.444	ZNF189	2.935
BOLA1	2.672	DENND6A	-2.242	HIAT1	-2.934	LRRC37BP	-2.643	OBSCN	-3.661	RPS16P5	-2.405	TCL1A	-21.051	ZNF200	2.967
BOLA2/BC	2.007	DEPDC1	4.508	HIATL1	2.888	LRRC75A-	-3.694	ODF2	-2.779	RPS27L	6.534	TCP11L2	-3.057	ZNF219	-2.578
BORA	3.730	DEPDC1B	3.145	HIF1A	-3.655	LRRN3	11.139	OIP5	4.770	RPS6KA5	4.061	TEFM	3.119	ZNF226	4.519
BRCA1	2.440	DERL2	2.040	HIGD1A	2.160	LSM1	2.495	OIP5-AS1	2.706	RPUSD2	2.758	TEPP	2.030	ZNF227	4.078
BRCA2	2.085	DESI2	2.351	HIPK1	-2.636	LSMEM1	-2.743	OLFM4	-4.584	RRAS2	4.186	TET2	-4.644	ZNF230	2.093
BRD4	-2.577	DHFR	2.127	HIRIP3	2.103	LTA	3.501	OLIG1	-6.078	RRM2	6.212	TET3	-2.013	ZNF232	2.310
BRIP1	2.343	DHFRL1	2.580	HJURP	16.577	LTB	5.518	OPLAH	-2.319	RRM2B	2.352	TEX30	3.080	ZNF235	2.163
BRIX1	4.094	DHX32	2.034	HLA-A	-4.294	LTBP3	-2.375	OPTN	2.119	RRN3P1	-2.173	TFIP11	2.231	ZNF250	3.173
BST1	-2.917	DIAPH3	5.870	HLA-DOA	-2.248	LTF	-11.379	OR6C4	2.325	RRP8	2.552	TGFBI	-19.162	ZNF253	2.186
BTBD10	2.637	DICER1-A	-2.738	HMGB2	7.437	LUZP1	-2.775	ORMDL2	4.278	RRS1	2.561	TGFBR1	2.268	ZNF254	2.705
BTG2	-4.129	DIDO1	2.351	HMGB3	3.059	LY9	-8.157	OSCAR	-2.627	RSAD2	3.343	TGFBR2	-2.856	ZNF268	3.793
BTG3	-3.619	DIP2A	-2.204	HMGB3P1	2.778	LY86	-4.618	OSER1	-6.944	RSRP1	-2.802	TGOLN2	-2.457	ZNF283	3.311
BTK	-2.894	DISC1	-3.003	HMGCR	6.472	LYPD3	-8.471	OSM	3.925	RTKN2	2.998	TGS1	2.242	ZNF292	-2.135
BUB1	8.603	DLGAP5	17.181	HMMR	11.704	LYPLAL1	2.736	OSTC	2.568	RTN4R	2.369	THAP7	2.421	ZNF302	2.054
BUB1B	7.793	DMPK	-2.198	HNRNPA1	-4.512	LYRM4	-2.036	OSTCP2	2.760	RTP4	5.527	THBD	-12.388	ZNF304	2.033
BUD13	2.156	DNAI2	3.728	HNRNPDL	-3.206	LZTS1	13.104	OTUD1	-3.041	RUNX1	-20.275	THOC3	2.704	ZNF320	3.197
BYSL	8.432	DNAJA4	-2.549	HNRNPLL	2.353	MAD2L1	4.999	OTULIN	-8.089	RUNX2	-2.272	THRA	-5.741	ZNF322	3.264
C10orf76	-4.257	DNAJB1	-3.873	HOMER3	-6.096	MAD2L1BP	3.356	OXNAD1	-3.966	S100A4	3.148	TIMM21	2.250	ZNF331	-22.246
C11orf96	-4.747	DNAJB6	-3.585	HOTAIRM1	-3.559	MAFB	-12.699	OXR1	-3.106	S100A6	2.495	TIMM8A	2.639	ZNF347	2.449
C12orf42	-2.976	DNAJC17	2.232	HP	-7.350	MAFF	-22.040	P2RX4	2.572	S100A8	-454.981	TIMM8B	5.071	ZNF350	2.992
C12orf73	3.100	DNTTIP1	2.082	HPDL	6.606	MAFTRR	-2.611	PA2G4	2.354	S100A9	-62.113	TIMP2	-2.543	ZNF394	-4.845
C14orf142	2.592	DOCK2	-2.282	HPR	-2.937	MAGOHB	3.303	PABPC1L	-2.353	S100A11	7.503	TIPARP	-2.785	ZNF416	2.857
C14orf169	2.475	DOHH	2.172	HPS5	2.581	MAK16	3.405	PAFAH1B	3.194	S100A12	-99.420	TIPIN	2.997	ZNF419	2.020
C15orf52	-3.035	DOK3	-5.508	HPS6	4.119	MAL	4.108	PAFAH1B	-3.056	SAC3D1	5.289	TLE2	-3.241	ZNF420	3.514
C17orf75	2.542	DOLK	3.106	HSBP1	4.527	MALAT1	-5.534	PAICS	2.339	SACM1L	2.223	TLE3	-2.265	ZNF436	3.433
C17orf80	2.747	DOT1L	-2.411	HSPA6	-4.877	MAP1LC3	-2.583	PALB2	2.137	SACS	4.041	TLE4	-4.684	ZNF441	2.003
C18orf21	2.078	DPP4	5.199	HSPA8	2.014	MAP1LC3	-2.371	PANK2	2.546	SAMD9L	3.780	TLK2	-2.253	ZNF449	3.088
C18orf25	2.494	DPPA4	-6.532	HSPA9	-2.189	MAP2K4	2.246	PARPBP	3.241	SAMSN1	3.934	TLR1	4.342	ZNF451	-2.499
C19orf12	2.267	DRAXIN	2.475	HSPA1A/H	2.904	MAPK1IP1	-6.470	PASK	-6.561	SAPCD2	5.150	TLR2	-2.032	ZNF462	2.301
C19orf38	-3.131	DRICH1	-2.265	HSPB1	-3.752	MAPKAPK	2.526	PBK	12.423	SAR1B	3.147	TLR9	2.279	ZNF468	3.218
C1GALT1(	10.608	DSCC1	3.094	HSPB11	2.465	MARCH3	-2.304	PCK2	2.327	SARDH	-2.975	TM2D1	2.146	ZNF470	2.514
C1orf233	2.174	DTL	5.551	HSPE1	2.216	MARVELD	-2.045	PCMT1	2.006	SARNP	3.001	TM2D2	2.385	ZNF480	2.264
C20orf196	2.249	DUSP1	-168.188	HYLS1	9.987	MAT2A	3.392	PCNA	6.500	SAT1	-4.970	TM6SF1	2.295	ZNF484	2.292
C2CD3	-19.866	DUSP2	-14.761	ID1	-9.785	MAX	-3.031	PCP2	-2.217	SATB1-AS	-2.784	TMA16	2.883	ZNF485	2.510
C2orf40	-5.888	DUSP10	-5.685	IDH1	2.893	MB21D1	2.771	PCSK5	-3.051	SBDS	-6.572	TMBIM4	2.600	ZNF501	2.529
C2orf44	3.680	DYNC1I2	2.010	IDS	-6.846	MC1R	-2.521	PDCD6	-2.228	SBDSP1	-6.433	TMCO1	4.299	ZNF502	2.937
C2orf47	2.093	DYNLL1	4.796	IDUA	-2.007	MCAM	-2.807	PDCD10	3.726	SCAI	-2.784	TMEM2	-2.252	ZNF507	2.255
C3orf14	2.554	DYNLL2	-6.988	IER5	-2.288	MCAT	2.012	PDE4D	-5.619	SCARNA5	-2.706	TMEM57	-2.452	ZNF511	2.069
C3orf18	4.407	E2F6	2.305	IFIT1B	-10.153	MCEMP1	-9.880	PDE6G	2.706	SCARNA9	-2.730	TMEM60	5.340	ZNF516	-2.425
C4orf3	2.174	E2F8	4.513	IFITM1	2.003	MCM3	2.019	PDIK1L	2.022	SCML4	-2.810	TMEM88	-11.843	ZNF527	2.546
C5AR1	-18.514	EBLN2	-2.210	IFRD1	-4.631	MCOLN2	3.754	PDP2	2.109	SCO1	2.185	TMEM128	2.491	ZNF530	2.307
C5orf15	2.098	EBPL	2.758	IGF2	-3.730	MCOLN3	2.156	PDXDC1	-2.114	SCPEP1	-2.436	TMEM165	2.139	ZNF541	3.043
C5orf30	4.207	ECHDC2	-2.888	IGFBP2	4.299	MCRS1	2.473	PDZD11	2.311	SCRN1	-2.745	TMEM168	2.146	ZNF544	2.430
C6orf25	-2.056	EDARADD	2.342	IGHG1	2.996	MDC1	2.065	PECR	2.635	SCRN3	2.540	TMEM177	4.334	ZNF547	2.246
C6orf203	2.657	EED	3.074	IGHV1-69	2.811	MDFIC	3.219	PELI1	-4.642	SDE2	-8.666	TMEM208	2.483	ZNF555	4.056
C8G	-3.365	EEF1E1	2.950	IGKC	-18.963	MDH1	2.671	PER1	-16.749	SDHAF1	3.361	TMEM223	2.145	ZNF561	2.942
C8orf76	2.378	EFNA1	-8.713	IGLC1	-4.635	MDK	-3.235	PET100	2.210	SDHAF4	2.215	TMEM258	2.042	ZNF562	2.565
C9orf40	3.266	EGFL6	3.098	IGLJ3	-9.146	MDM2	2.937	PEX3	2.689	SDHC	2.457	TMEM126	2.681	ZNF564	3.371
C9orf156	3.124	EGLN3	11.092	IGLL1/IGL	-23.030	MDM4	-2.253	PEX11B	2.272	SDHD	2.547	TMEM14A	3.196	ZNF566	2.082
CA1	-9.857	EIF5	-6.033	IGSF11	2.315	MDS2	-2.260	PF4	-13.636	SDR42E1	4.420	TMEM167	-2.010	ZNF569	2.184
CA5B	-9.008	EIF2B2	2.016	IGSF9B	2.238	MED7	2.513	PGK1	2.009	SEC14L1	-2.493	TMEM39A	2.288	ZNF572	2.769
CABYR	-3.002	EIF4E	2.473	IKBIP	2.860	MED8	2.917	PGLYRP1	-3.577	SEC14L2	-2.387	TMEM8B	-2.497	ZNF576	2.813
CACHD1	-2.003	EIF4G1	2.210	IKBKE	2.352	MED9	2.220	PGM3	2.116	SEC23B	2.015	TMIE	-2.380	ZNF584	-6.904
CALM1 (in	2.872	EIF4G3	-3.232	IKZF2	-2.795	MED11	2.605	PGRMC2	-2.859	SEC61B	2.648	TMSB10/T	2.115	ZNF593	3.294
CAMK1D	-3.110	ELF1	-2.945	IKZF4	5.465	MED18	2.165	PHACTR1	-3.752	SECTM1	-7.960	TNFAIP3	-67.242	ZNF594	2.106
CAMP	-12.616	ELL2	-10.155	IKZF5	-3.806	MED20	4.884	PHC1	-2.149	SEL1L3	2.927	TNFAIP8L	9.642	ZNF613	7.749
CAMSAP2	-2.318	ELMOD2	3.340	IL13	-2.181	MED21	2.269	PHF1	-2.711	SELL	-3.095	TNFRSF1(	-2.796	ZNF614	4.406
CANT1	2.110	EMC2	3.625	IL32	6.932	MED26	-3.437	PHF13	-2.690	SELT	2.132	TNFSF4	2.009	ZNF615	3.946
CAPN5	-3.215	EMG1	4.208	IL10RA	2.413	MED30	-2.141	PHF23	3.969	SEMA4D	-4.847	TNFSF10	5.042	ZNF616	3.988
CAPN12	4.063	EMP3	-2.180	IL15RA	3.464	MEF2B	-2.147	PHF21A	-2.638	SENP1	2.102	TNFSF12	2.025	ZNF628	-3.449
CAPZA1	2.087	ENO1	2.498	IL1B	-24.810	MEF2C	-4.521	PHF5A	2.209	SEPN1	-2.764	TNFSF14	16.985	ZNF629	2.216
CARD6	3.243	ENPP4	2.148	IL27RA	2.437	MEF2D	-3.229	PHGDH	3.288	SERPINA1	-14.509	TNIK	-2.157	ZNF639	-2.298
CARNS1	-3.399	EPB42	-3.098	IL2RA	12.801	MEGF10	-2.074	PHLDA2	-7.679	SERTAD1	-4.123	TNK1	-2.437	ZNF644	2.023
CARS	2.221	EPHB6	-2.006	IL6ST	-7.030	MELK	7.008	PHLDA3	3.384	SETD2	-2.243	TNNC2	-2.483	ZNF649	3.513
CASC5	8.197	EPHX3	-2.470	IL7R	-8.476	MEOX1	2.180	PHLDB3	-2.306	SETD7	-3.661	TNRC6B	-2.275	ZNF653	2.371
CASP3	7.221	EPN2	-3.000	IL9R	2.645	MESDC1	2.560	PI4K2B	3.087	SF1	-3.215	TOB1	-8.986	ZNF658	3.484
CASP8AP	2.167	ERCC6	2.254	IMMP1L	2.288	METRN	-3.224	PI4KAP1	-2.220	SF3B3	3.064	TOLLIP-AS	-2.174	ZNF682	2.363
CASS4	-2.390	EREG	-2.053	IMMT	2.012	METRNL	-11.817	PIAS2	-2.728	SF3B5	2.011	TOMM5	2.157	ZNF684	2.021
CBLL1	-2.028	ERGIC2	3.493	IMP3	3.008	METTL1	2.418	PIGA	-5.019	SFN	-2.638	TOP1MT	-2.642	ZNF691	2.100
CBR3	9.861	ERH	2.457	IMPA1	3.640	METTL14	2.209	PIGF	2.095	SFPQ	-2.874	TOP2A	11.016	ZNF696	3.095
CBS/LOC	7.411	ERP44	2.486	IMPAD1	2.085	METTL18	3.813	PIK3IP1	-4.480	SFT2D3	2.523	TOX2	-19.319	ZNF700	2.268
CBWD2	2.100	ESCO2	3.108	ING1	-2.468	MEX3B	-4.179	PIM3	-3.754	SGK1	-6.691	TP53RK	2.056	ZNF701	2.784
CBX4	-4.469	ESRRA	2.329	ING2	-2.973	MFAP1	4.227	PIN4	2.173	SGOL1	2.635	TPP2	-9.334	ZNF703	-2.162
CCDC51	3.528	ETF1	-2.123	INIP	2.070	MFAP3	3.776	PLAG1	-2.411	SGOL2	4.262	TRA2B	-9.598	ZNF709	2.119
CCDC53	2.145	ETNK1	-3.790	INPP4A	-2.169	MFSD5	2.656	PLAUR	-9.061	SH2D1B	-19.601	TRABD2A	-2.559	ZNF711	-3.237
CCDC58	2.877	EXO1	4.196	INSIG2	3.342	MGAT3	16.482	PLBD1	-14.274	SH3BP5	6.203	TRAF4	-3.734	ZNF720	-2.304
CCDC59	-2.537	EXO5	2.444	INTS7	2.068	MGAT4A	-6.051	PLCB1	-2.318	SH3TC1	-2.007	TRANK1	-2.093	ZNF730	2.317
CCDC71	2.236	EXOC2	2.005	IPCEF1	-2.509	MGEA5	-2.133	PLCXD2	-3.768	SHFM1	2.884	TRAT1	4.141	ZNF761	2.242
CCDC77	2.292	EXOC7	-2.941	IRAK3	-4.181	MGME1	4.230	PLEKHF2	-2.134	SIT1	4.010	TREM1	-5.867	ZNF765	3.184
CCDC82	2.308	EXOSC3	3.193	IRF4	2.294	MGST1	-3.229	PLEKHM1	-3.592	SKA2	2.918	TRERF1	-2.766	ZNF790	3.320
CCDC136	-4.598	EYA3	3.596	IRF9	2.135	MIAT	3.013	PLEKHO1	2.449	SKA3	3.610	TRIM13	4.576	ZNF813	2.525
CCDC85C	2.236	F13A1	-5.741	IRS2	-9.143	MID1IP1	2.633	PLIN2	-2.969	SKI	-2.781	TRIM27	2.074	ZNF816	6.208
CCDC88C	-2.855	FADD	3.617	ISCA1	-3.090	MIDN	-6.085	PLK2	-9.834	SLAMF1	3.281	TRIM32	2.888	ZNF823	3.764
CCL5	7.876	FAM110A	2.947	ISCA2	2.632	MIEF1	2.002	PLK4	3.745	SLC11A1	-2.653	TRIM47	5.034	ZNF831	-2.096
CCL16	-3.152	FAM117B	-5.873	ITGA7	-3.612	MIER1	-2.056	PLP2	2.780	SLC16A5	-2.037	TRIM58	-5.249	ZNF845	3.854
CCL4L1/C	-2.754	FAM126A	2.552	ITGB3	-2.131	MIF	2.027	PLXDC1	-13.396	SLC1A4	2.378	TRIM59	3.251	ZNF850	3.072
CCNA2	6.531	FAM127B	2.505	ITGB7	3.151	MINOS1-N	2.442	PLXNA1	-2.821	SLC1A5	3.632	TRIM52-A	-2.402	ZNF137P	2.218
CCNB1	7.496	FAM129A	8.144	ITGB2-AS	-3.600	MINPP1	2.039	PLXNB2	2.355	SLC24A4	-2.013	TRIP13	5.656	ZNF33A	2.332

CCNB2	9.029		FAM129B	3.816		ITGB3BP	2.024		mir-146	12.099		PMAIP1	-4.223		SLC25A17	2.116		TRMT5	3.053		ZNF37A	2.450
CCNE2	4.051		FAM160B1	-2.361		ITPKB	-17.037		MIR181A1	-5.051		PMEPA1	-2.620		SLC25A24	5.166		TRMT12	2.902		ZNF674-A	-6.566
CCNK	-2.129		FAM160B2	-2.345		ITPRIP	-13.069		MIR22HG	-2.475		PMS1	-3.567		SLC25A33	-2.100		TRMT44	-2.016		ZNF75A	3.071
CCNL1	-6.784		FAM173A	-3.266		IVD	2.005		MIR4697H	3.988		PNP	2.949		SLC25A34	-2.059		TRMT10A	2.067		ZNF75D	3.273
CCNT1	-3.272		FAM175B	2.206		JADE2	-2.017		MIR503HG	2.330		PNPLA8	-2.336		SLC25A36	-2.032		TRNT1	2.045		ZNF780B	2.229
CCR2	18.374		FAM183B	-2.296		JAK2	3.093		MIR646HG	-5.040		PNRC1	-3.487		SLC25A37	-3.738		TRO	-3.343		ZNFX1	-2.582
CCR5	2.785		FAM185A	2.098		JAKMIP1	4.305		MIS12	2.128		POC5	2.329		SLC2A3	-5.926		TRPM2	-3.907		ZNHIT2	2.190
CCR9	-10.048		FAM19A1	2.464		JARID2-A	-3.112		MIS18A	2.228		POGZ	-2.185		SLC2A14	-8.194		TRUB2	2.415		ZNHIT3	2.081
CD14	-21.130		FAM200A	2.241		JDP2	-3.714		MKI67	5.974		POLD3	2.299		SLC35A5	2.035		TSC22D3	-42.455		ZSCAN9	2.034
CD33	-2.534		FAM209A	-2.721		JMJD6	-3.106		MLEC	2.625		POLR1C	3.365		SLC35E1	-2.579		TSEN15	2.156		ZSCAN21	2.001
CD36	-6.314		FAM216A	2.443		JMY	-8.824		MLXIP	-2.234		POLR2B	2.489		SLC35F3	26.433		TSG101	2.000		ZSCAN32	2.913
CD44	-6.180		FAM226A	-3.394		JOSD1	-3.506		MLYCD	2.591		POLR3B	2.399		SLC39A8	3.436		TSPAN6	-2.437		ZSWIM3	3.094
CD52	2.166		FAM229A	-2.564		JUN	-71.775		MMP25	6.059		POP1	3.372		SLC41A1	-2.292		TSPAN18	3.277		ZSWIM8-A	-2.493
CD68	-2.916		FAM35A	2.161		JUNB	-19.439		MMS22L	2.041		POP4	3.114		SLC44A1	-2.409		TSPYL2	-8.270		ZXDB	-2.197
CD69	-22.179		FAM45A	2.880		JUND	-7.064		MNDA	-5.252		PPA2	3.669		SLC4A4	5.410		TTC16	-2.357			
CD70	9.232		FAM46C	-5.721		KATNA1	2.803		MNT	-2.203		PPBP	-7.108		SLC4A11	2.488		TTC28	-3.036			
CD93	-2.747		FAM53C	-5.749		KATNAL2	-2.154		MOAP1	-2.832		PPIAL4G	2.010		SLC6A13	-3.227		TTC9C	2.446			
CD163	-7.779		FAM57A	3.133		KBTBD3	2.544		MOB4	2.521		PPIL1	8.460		SLC7A1	3.154		TTF2	2.349			
CD302	-3.246		FAM69A	5.345		KBTBD7	2.257		MOB3A	2.290		PPM1A	-2.659		SLC7A7	-7.883		TTK	4.972			
CD2BP2	2.397		FAM72A	5.166		KBTBD11	-5.959		MORC2	2.866		PPM1B	-2.659		SLC8A1-A	3.978		TTN	-2.496			

Table S3. List of the antibodies used for flow cytometry. (From 37).

Antibody used	Clone	Company	Antibody used	Clone	Company
CCR2/PE	K036C2	BioLegend	CD45RA/PE-Cy7	HI100	BD
CCR7/Fitc	G043H7	BD	CD45RA/BV510	HI100	BioLegend
CCR7/PE	G043H7	R&D	CD49d/PE	9F10	eBioscience
CCR7/BV605	G043H7	BioLegend	CD62L/PE	DREG-56	BD
CCR9/PE-Cy7	L053E8	BioLegend	CD62L/PE	DREG-56	BD
CD11a/PE	HI111	BD	CD69/eFluor 450	FN50	eBioscience
CD11b/PE	ICRF44	BD	CD70/APC	113-16	BioLegend
CD120b/APC	22235	R&D	CD8/Fitc	SK1	BD
CD122/APC	TU27	BioLegend	CD8/Pacific Blue	RPA-T8	BD
CD127/PE	A019D5	BioLegend	CD95/APC	DX2	BD
CD14/Pacific Blue	TuK4	Invitrogen	CXCR3/APC	1C6/CXCR3	BD
CD14/PerCP	134620	R&D	CXCR4/APC	12G5	eBioscience
CD16/Pacific Blue	3G8	Invitrogen	gp130/PE	28126	R&D
CD16/PerCP	245536	R&D	Granzyme B/Fitc	GB11	BD
CD19/Pacific Blue	SJ25-C1	Invitrogen	IL-15Ra/PE	JM7A4	BioLegend
CD19/PerCP	4G7-2E3	R&D	Integrin b7/PE	473207	R&D
CD25/APC-Cy7	M-A251	BD	LT-a/PE	359-81-11	BioLegend
CD27/APC	57703	R&D	LY-9/PE	hIY-9.1.25	BioLegend
CD3/PE	OKT3	BioLegend	PD-1/Fitc	MIH4	BD
CD3/V500	UCHT1	BD	Perforin/Fitc	Dg9	eBioscience
CD31/APC-eFluor 780	WM-59	eBioscience	PTK7/PE	188B	Miltenyi
CD4/Fitc	SK3	eBioscience	TNFSF10/PE	RIK-2	BioLegend
CD44/PE	BJ18	BioLegend	TNFSF14/APC	115520	R&D
CD45RA/Fitc	HI100	BD			

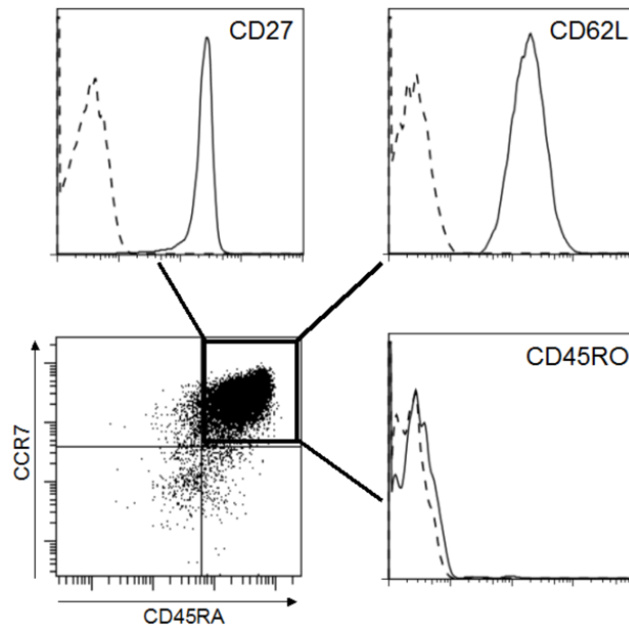

Fig. S1. The expression of CD27, CD45RO and CD62L in T_{Nrev}. The expression is shown for the cells having undergone phenotype reversion in Fig. 1A, right panel. Dashed lines show the profiles of isotype controls.
(From 37).

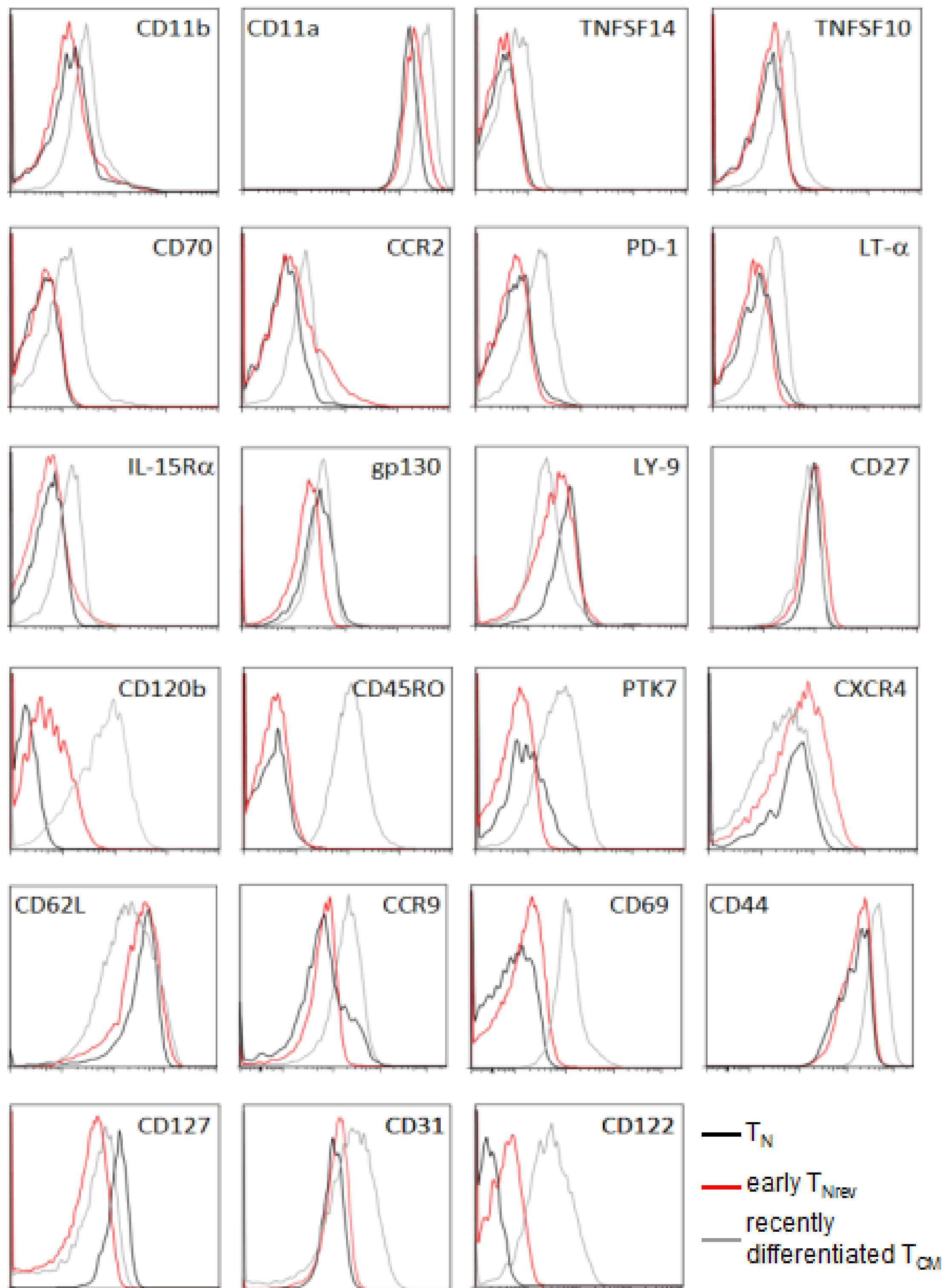

Fig. S2. Extended phenotypic screening for putative markers of T_{Nrev}. The membrane expression of non-discriminatory markers of T_N, recently reverted T_{Nrev} and recently differentiated T_{CM} is shown. Single representative experiment out of three. (From 37).

4. CORD BLOOD T CELLS RETAIN EARLY DIFFERENTIATION PHENOTYPE SUITABLE FOR IMMUNOTHERAPY AFTER TCR GENE TRANSFER TO CONFER EBV-SPECIFICITY

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Acknowledgment of collaborative work

GF designed and performed most of the experiments, interpreted the data and wrote the manuscript; YZ generated the TCR construct and advised on experiments; GA performed the experiments on telomers and telomerase; MR advised on experiments; PML advised on experiments; PM advised and edited the manuscript; SPL generated the TCR construct, advised on experiments and revised the manuscript; FEC advised on designing the experiments and interpreting the data, and revised the manuscript.

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See Appendix

4.1. ABSTRACT

Adoptive T-cell therapy can be effective for Epstein-Barr virus (EBV)-associated post-transplant lymphoproliferative disease and melanoma. Transducing high affinity TCR genes into T lymphocytes is an emerging method to improve potency and specificity of tumor-specific T cells. However, both methods necessitate *in vitro* lymphocyte proliferation, generating highly differentiated effector cells that display reduced survival and anti-tumor efficacy post-infusion. TCR-transduction of naïve lymphocytes isolated from peripheral blood is reported to provide superior *in vivo* survival and function. We utilized cord blood (CB) lymphocytes, which comprise mainly naïve cells, for transducing EBV-specific TCR. Comparable TCR expression was achieved in adult and CB cells, but the latter expressed an earlier differentiation profile. Further antigen-driven stimulation skewed adult lymphocytes to a late differentiation phenotype associated with immune exhaustion. In contrast, CB T cells retained a less differentiated phenotype after antigen stimulation, remaining $CD57^-$ but were still capable of antigen-specific polyfunctional cytokine expression and cytotoxicity in response to EBV antigen. CB T cells also retained longer telomeres and in general possessed higher telomerase activity indicative of greater proliferative potential. CB lymphocytes therefore have qualities indicating prolonged survival and effector function favourable to immunotherapy, especially in settings where donor lymphocytes are unavailable such as in solid organ and CB transplantation.

4.2. INTRODUCTION

Epstein-Barr Virus (EBV)-associated post-transplant lymphoproliferative disease (PTLD) complicates up to 13% of solid organ transplantation (SOT) (1). Anti-CD20 immunotherapy is an effective first line treatment but around 40% of cases remain refractory. Cord blood (CB) transplantation is also associated with significant EBV and Cytomegalovirus (CMV) reactivation and PTLD. Adoptive cellular immunotherapy (ACT) using third party partially HLA-matched EBV-specific T cells is an effective treatment for PTLD in both settings (2-4). However, for PTLD following SOT only 50% respond to ACT (2) possibly because the polyspecific T cells generated *in vitro* using an EBV-transformed B-lymphoblastoid cell line (LCL) contain too few effectors specific for the limited set of EBV antigens expressed by the tumor.

An alternative approach to rapidly generate large numbers of potent and specific effectors is to engineer T cells to express an appropriate antigen-specific TCR or a chimeric antigen receptor (5, 6). This has been used successfully to treat cancers such as melanoma (7), where naturally occurring tumor-specific T cells are rare and of low avidity (8-10). Using retroviral vectors, human T cells can be reliably transduced with TCR genes enabling them to recognize viral or tumor antigens. Adoptive transfer of engineered T cells is currently undergoing clinical trials with encouraging results (11-13). ACT studies indicate that anti-tumor response is linked to long term *in vivo* persistence of infused cells (14-16). The factors influencing *in vivo* persistence of lymphocytes are not fully understood, but evidence suggests that the differentiation status of the T-cell is critical. Less differentiated naïve (T_N) and central memory (T_{CM}) T-cell subsets display superior proliferation, persistence and anti-tumor responses following infusion when compared to the more differentiated effector memory (T_{EM}) subset (17-19). This raises an

important issue for ACT using genetically engineered T cells because *in vitro* activation of adult lymphocytes, required for retroviral transduction, drives the majority of peripheral blood-derived T cells into highly differentiated effector. Thus current approaches using transduced T cells may be suboptimal because the majority of cells (20, 21) infused will be differentiated and may therefore be of limited efficacy *in vivo*.

The challenge for ACT with genetically engineered T cells, or with any protocol involving cell expansion, is therefore to generate cells with a minimally differentiated phenotype. Recent studies (20, 21) suggest that $CD8^+$ T_N lymphocytes selected from adult peripheral blood (PB) are optimal for this purpose because, in contrast to T_{CM} and T_{EM} cells, they display minimal differentiation following TCR transduction. Human umbilical CB T cells, unlike adult-derived PB lymphocytes, are mostly T_N. It is therefore reasonable to speculate whether CB might be an alternative source of T-lymphocytes for genetic engineering. As both solid organ and CB transplant recipients cannot access lymphocytes from the original donors, third party allogeneic CB is a convenient alternate source of lymphocytes for ACT against EBV-PTLD. Such cells can also be used in lymphopenic cancer patients where autologous lymphopheresis is not possible

This study utilizes cryopreserved CB units from an unrelated cord blood bank and assesses the feasibility of using cord T cells to transduce EBV-specific TCR, and to analyze their functional capacity for *in vivo* use in immunotherapy.

4.3. MATERIALS AND METHODS

4.3.1. CELL ISOLATION AND CULTURE.

Frozen umbilical CB units, unsuitable for transplantation, were provided by NHSBT Cord Blood Bank, UK. Units were thawed in cold RPMI 1640 (Sigma-Aldrich, St. Louis, USA) plus 10 % fetal calf serum (FCS) (PAA, Pasching, Austria). After washing, mononuclear cells were isolated using Ficoll. Adult PB mononuclear cells (PBMC) were isolated from apheresis from healthy platelet donors (22) collected at the Blood Donor Centre, where the mean donor age was 42 years. The study was approved by the West Midlands Research Ethics Committee (05/Q2706/91). Dendritic cells (DCs) were generated from adherent mononuclear cells after incubating in plates for 2 hours. Adherent cells were cultured in medium supplemented on days 0, 3 and 6 with 50ng/ml GMCSF and 500 U/ml IL-4. On day 6, DCs were matured by adding 2 ng/ml IL-1β, 1000 U/ml IL-6, and 10 ng/ml TNFα (R&D Systems, Minneapolis, USA) (23). DCs were harvested after a further 24/48 hours. To confirm maturation, they were stained for CD14, CD83, CD86 and HLA Class II antigens. DCs were pulsed with the SSCSSCPLSK (SSC) peptide epitope (24) at a concentration of 10 μg/ml for 2 hrs, washed and used together with 100 U/ml IL-2 to stimulate transduced T cells at a responder:stimulator ratio of 20:1. Cell counts were performed using ABX-Pentra 60 (Horiba, Kyoto, Japan).

4.3.2. LYMPHOCYTE ACTIVATION AND TCR GENE TRANSDUCTION.

CB mononuclear cells (CBMC) or PBMC, were resuspended in RPMI containing 10% FCS, 1% pooled human AB serum (TCS Biosciences, Buckingham, UK), 2 mM L-

glutamine, 100 mg/ml streptomycin, and 100 IU/ml penicillin and activated with 30 ng/ml anti-CD3 antibody (OKT3) plus 600 U/ml IL-2 (Chiron, Emeryville, USA). Cells were transduced with retrovirus 48hours later. The retrovirus used was the pMP71-PRE vector (25) (provided by C. Baum, Hannover, Germany) into which we had inserted genes encoding TCR α and β chains isolated from an EBV-specific $CD8^+$ T-cell clone that targets the HLA A*1101-restricted epitope SSC derived from the viral protein LMP2 (manuscript in preparation, *Zheng Y, Lee SP et al.*). To generate the retrovirus, Phoenix amphotropic packaging cells (26) were transfected with the pMP71-PRE vector using FuGENE HD (Roche, Basel, Switzerland). After 48 hours the retroviral supernatant was harvested. Pre-activated cells were seeded at 4-6 x 10^6 cells/well in 1ml RPMI onto 6-well plates coated with retronectin (Takara, Shiga, Japan). Retroviral supernatant (1.5ml/well) or medium alone (mock-transduced) was added to each well and centrifuged for 1 hour x 800g at 30ºC. Medium supplemented with IL-2 (100 U/ml) was added three times weekly. Cells from six CB and six adult PB samples were assayed. Seven and 15 days after transduction, T cells were stimulated with SSC peptide-pulsed DCs. Sixteen days after transduction, $CD8^+$ lymphocytes were isolated using immunomagnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

4.3.3. FLOW CYTOMETRY.

Transduced lymphocytes were identified using HLA A*1101:SSC peptide-specific pentamers (Proimmune, Oxford, UK). Cells were also co-stained with fluorochrome-conjugated antibodies: anti-CCR7 (FITC; R&D), anti-CD4 (PerCP-Cy5.5 or PE-Cy7) (eBioscience, San Diego, USA), anti-CD3 (PE or APC-H7), anti-CD8 (APC or APC-H7),

anti-CD27 (APC), anti-CD45RA (PE-Cy7), anti-CD62L (PE) (all from BD), and anti-CD57 (APC; BioLegend, San Diego, CA). 7-aminoactinomycin D (7AAD from BD) was used as a viability marker.

For intracellular staining, cells were fixed and permeabilized using the FIX&PERM kit (ADG, Kaumberg, Austria), followed by anti-Perforin FITC (eBioscience).

For intracellular cytokine staining, cells were first stimulated with either anti-CD3 plus IL-2 or autologous peptide-pulsed DCs, and 1 hour later Monensin (Golgi Stop; 1 μl/ml; BD) was added. After overnight incubation cells were then fixed and permeabilized, and stained with anti-IL-2 FITC, anti-IFNγ APC and anti-TNFα PE-Cy7 (BD).

Proliferation was evaluated by staining cells for 2 min with 1 μM carboxyfluorescein succinimidyl ester (CFSE) prior to activation.

The enumeration of cells in the different phases of cell cycle or in apoptosis was performed using propidium iodide (PI) (27). Briefly, the cell pellet was incubated for 30 seconds in 200μl 0.1% Triton X100 in PBS. Afterwards, the same volume of PBS plus PI 50 μg/ml and RNAse 500 μg/ml was added, and samples were analyzed after 10 min at RT. Data were acquired using a FACSCanto II flow cytometer (BD).

4.3.4. CYTOTOXICITY ASSAY

Cytotoxicity of transduced T cells was assessed in a standard 5 hour chromium release assay at known effector:target ratios using 2500 target cells/well. HLA A*1101-transduced T2 cells (28) were used as targets and were pulsed with the SSC peptide or another A*1101-restricted peptide epitope (IVTDFSVIK)(29) as a control. Percentages of specific lysis and lytic units (LU) per 10^6 effector cells were calculated. One lytic unit was defined as the number of effectors required to achieve lysis of 50% of targets (30).

4.3.5. TELOMERE LENGTH MEASUREMENT

Telomere length measurements using automated multicolor flow-fluorescence in situ hybridization (flow FISH) was performed by Repeat Diagnostics Inc. (North Vancouver, Canada). as described by Baerlocher et al. (31)

4.3.6. TELOMERASE REPEAT AMPLIFICATION ASSAY

Telomerase activity in extracts from cultured lymphocytes was measured using the telomerase detection assay kit (TRAPeze® telomerase Detection, Millipore) according to manufacturer's instructions.

4.3.7. STATISTICS

The statistical analyses were performed using paired, two-tailed t test.

4.4. RESULTS

4.4.1. ACTIVATED CB-DERIVED T-LYMPHOCYTES HAVE GREATER PROLIFERATIVE CAPACITY COMPARED TO ACTIVATED ADULT T CELLS

Initially we determined the relative proportions of naive and memory T cells within CB and compared this to blood from adult donors. T-lymphocytes were categorized into: T_N: $CCR7^+/CD27^+/CD45RA^+/CD62L^+$; T_{CM}: $CCR7^+/CD27^+/CD45RA^-/CD62L^+$; T_{EM}: $CCR7^-/CD27^+/CD45RA^-/CD62L^-$; or T_{EMRA}: $CCR7^-/CD27^-/CD45RA^+/CD62L^-$ (6).

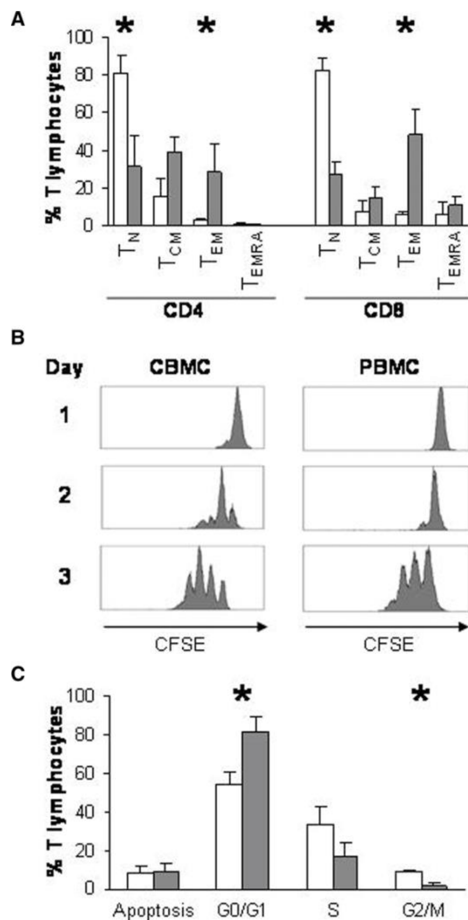

Figure 4.1. Lymphocytes from cord blood display increased proliferative activity in comparison to lymphocytes from adult blood. (**A**) The distribution, evaluated by flow cytometry, of CD4 and CD8 lymphocytes into T_N, T_{CM}, T_{EM} and T_{EMRA} subsets is shown for CBMC (white bars) and PBMC (grey bars). Asterisks indicate $P<0.05$. (**B**) CFSE-stained cells were activated with anti-CD3 plus IL-2, and proliferation was assessed at the indicated time points. One representative experiment out of six is shown. (**C**) Lymphocytes, either from CBMC (white bars) or PBMC (grey bars) were activated, and the distribution of cells in the different phases of the cell cycle was measured 48 hrs later by propidium iodide uptake. The percentage of cells in apoptosis or in different phases of the cell cycle is indicated. Asterisks indicate $p<0.05$.
(From 57).

CCR7 and CD45RA provided sufficient discrimination among the subsets and therefore results based on expression of these two markers are shown hereafter. Phenotypic analysis confirmed that a mean of 81% of T cells in CB samples (n=6) displayed a T_N phenotype in contrast to healthy adult blood donors (n=6) where T_N constituted a mean of 29% (Figure 4.1A). The memory cell subset within CB was comprised predominantly of T_{CM} with very few T_{EM}.

The proliferation prior to retroviral transduction was monitored by the dilution of CFSE over the next 3 days. This showed an increased proliferation rate within CB cells compared to adult T cells (Figure 4.1B) and was confirmed with PI staining 48 hours after activation which showed more than twice as many CB cells in the S to M phases of cell cycle compared to cells from adult donors. Importantly, this difference was not due to cell death as the proportion of apoptotic cells was the same in both cultures (Figure 4.1C)

4.4.2. CORD AND ADULT LYMPHOCYTES SHOW COMPARABLE LEVELS OF TCR EXPRESSION FOLLOWING RETROVIRUS-MEDIATED GENE TRANSFER

For the transduction of EBV-specific TCR genes, CBMC and adult PBMC were activated for two days and then infected with the retroviral vector encoding the TCR. Expression of the transduced TCR was determined using HLA-A*1101:SSC pentamers (Figure 4.2A). The TCR transduction efficiency was similar for both cord and adult lymphocytes (Figure 4.2B). Pentamer staining of CB $CD8^+$ and $CD4^+$ T cells showed mean transduction efficiencies of 9.8% and 5.6% respectively whereas for adult $CD8^+$ and $CD4^+$ cells they were 10.7% and 6.1% respectively. There was no difference in the mean fluorescence

intensity of pentamer staining of T cells from cord or adult blood, indicating comparable levels of surface TCR expression (Figure 4.2C).

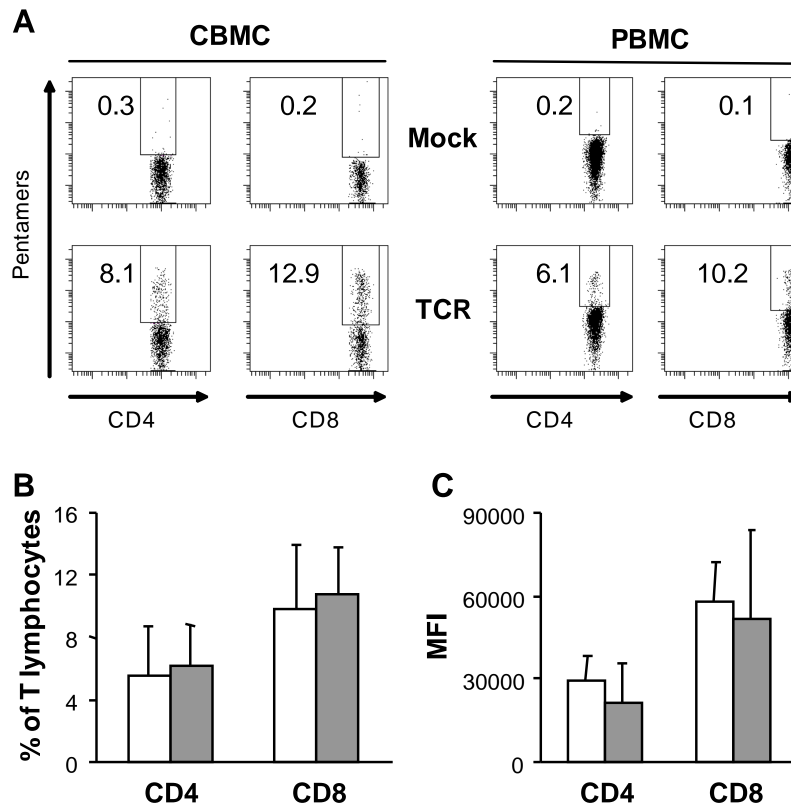

Figure 4.2. Cord blood lymphocytes can be transduced as effectively as lymphocytes from adult blood. (**A**) The results from a representative transduction of cord blood and adult blood lymphocytes with the EBV-specific TCR are shown at day 6 post-activation. The transduction efficiency was assessed by staining with a specific pentamer using mock-transduced T cells as controls. The proportions of pentamer-stained cells amongst $CD4^{+}$ and $CD8^{+}$ T-cell subsets are indicated. One representative experiment out of six is shown. (**B**) The mean percentage (+ SD) of transduced T-lymphocytes are shown for CBMC (n=6; white bars) and PBMC (n=6; grey bars). (**C**) The mean fluorescence intensity (MFI) of transduced T-lymphocytes derived from CBMC (n=6; white bars) and PBMC (n=6; grey bars) that stained positive with the pentamer.
(From 57).

4.4.3. TCR-TRANSDUCED CB T CELLS ACQUIRE A PREDOMINANTLY CENTRAL MEMORY PHENOTYPE

We then explored how retroviral transduction affected the differentiation status of CB and adult T cells. Amongst transduced CB cells, 60% were T_{CM} and most of the remaining cells were T_N (Figures 4.3A,B).

In contrast, the majority of transduced adult T cells had a T_{EM} phenotype. This pattern was similar for both $CD4^+$ and $CD8^+$ T cells and for those T cells that failed to express the TCR (Figure 4.3A,B).

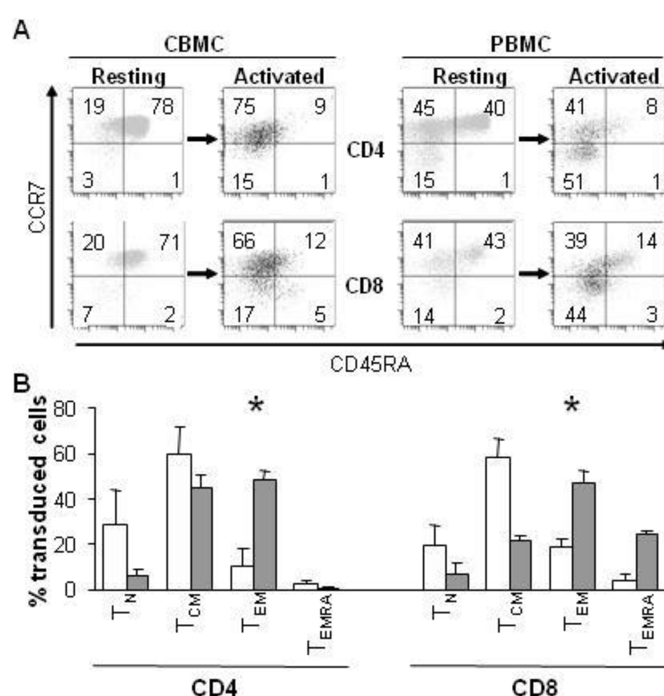

Figure 4.3. After activation and TCR transduction, cord blood lymphocytes display a less differentiated phenotype compared with adult T-lymphocytes. (**A**) The expression of CCR7 and CD45RA before cells were activated with anti-CD3 and IL2 and then transduced compared with the same cells 6 days later. Black dots indicate transduced lymphocytes, grey dots non-transduced lymphocytes. One representative experiment out of six is shown. (**B**) The distribution of transduced $CD4^+$ and $CD8^+$ among T_N, T_{CM}, T_{EM} and T_{EMRA} subsets on day 6 is shown as mean (+ SD) percentages for CBMC (n=6; white bars) and PBMC (n=6; grey bars). Asterisks indicate $p<0.05$.
(From 57).

4.4.4. TCR-TRANSDUCED CB CD8+ T CELLS PROLIFERATE AFTER EXPOSURE TO ANTIGEN BUT ARE LESS DIFFERENTIATED THAN ADULT T CELLS

The ultimate aim of these studies would be to use TCR-transduced CB T cells in ACT. We therefore studied the proliferation, differentiation phenotype and cytokine production of such cells after prolonged *in vitro* culture and exposure to antigen.

TCR-transduced CB and adult T cells were expanded using two rounds of stimulation with SSC peptide-pulsed autologous DCs. After 23 days of culture, both CB and adult T cells had expanded 50-fold with cell numbers continuing to rise (Figure 4.4A).

Preferential expansion of transduced cells occurred so that by day 23 they comprised 50% of the $CD8^+$ T-cell population (Figure 4.4B).

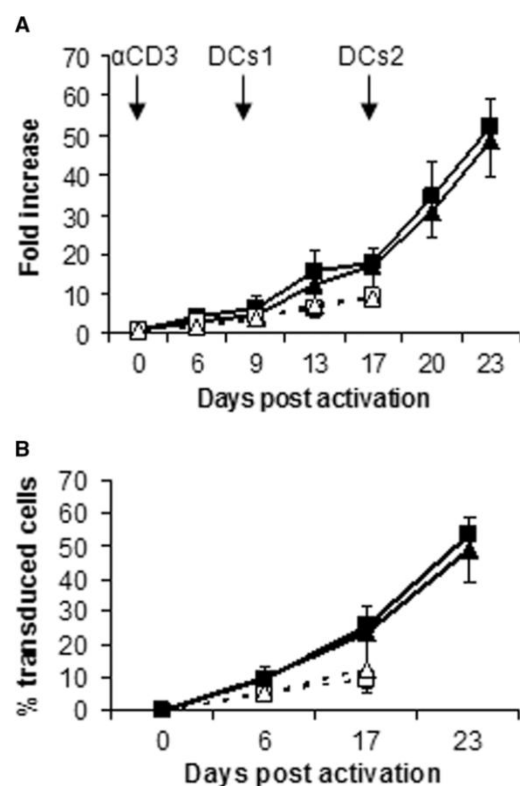

Figure 4.4. After activation and transduction cord blood T-lymphocytes proliferate as effectively as T-lymphocytes from adults. (**A**) Graph showing the mean (+ SD) fold increase in cell number of lymphocytes after activation with anti-CD3, retroviral transduction and two re-stimulations with peptide-pulsed DCs for $CD8^+$ (continuous lines, filled symbols) and $CD4^+$ (dotted lines, open symbols) T-lymphocytes from CBMC (n=6; squares) and PBMC (n=6; triangles). (**B**) The expansion of transduced cells is shown as the mean percentage (+ SD) of pentamer-stained cells within $CD8^+$ (continuous lines, filled symbols) and $CD4^+$ (dotted lines, open symbols) T-cell subsets of lines generated from CBMC (n=6; squares) and PBMC (n=6; triangles).
(From 57).

Comparing the differentiation status of transduced CB and adult T cells after stimulation with peptide-pulsed DCs we observed consistent differences especially within the $CD8^+$ T-cell subset (Figure 4.5A,B).

Transduced $CD4^+$ CB T cells were initially dominated by T_N and T_{CM}, the latter twice as common, whilst adult T cells were dominated by equal proportions of T_{CM} and T_{EM} with minimal T_N. However, following stimulation with antigen the T_{EM} expanded markedly in both populations whilst T_{CM} proportions fell.

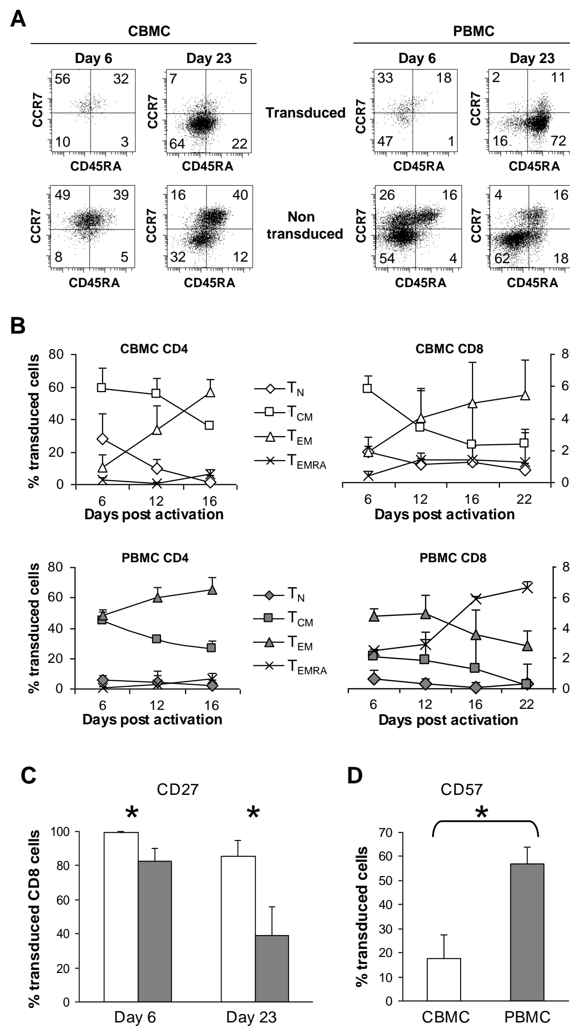

Figure 4.5. After antigen stimulation TCR-transduced CBMC retain a less differentiated phenotype than TCR-transduced PBMC. (**A**) The changes in phenotype of transduced and non-transduced cells from the same culture are shown at two time points (day 6 and day 23 post-activation). Representative data from a single CBMC- and PBMC-derived culture are shown. (**B**) The relative proportion of TCR-transduced T_N, T_{CM}, T_{EM} and T_{EMRA} cells within the $CD4^+$ and $CD8^+$ subsets from CBMC (n=6, open symbols, upper panel) and PBMC (n=6; closed symbols, lower panel). Data shown are the mean percentage (+ SD). (**C**) The mean percentage of transduced $CD8^+$ lymphocytes expressing CD27 at the indicated time points is shown for CBMC (n=6; white bars) and for PBMC (n=6; grey bars). Asterisks indicate P<0.05. (**D**) The expression of CD57 on the same cell lines at day 13 post-activation. Asterisks indicate $p<0.05$.
(From 57).

After 16 days the final product was comparable in both cultures with means of 55-65% T_{EM} cells.

However, the differentiation status of $CD8^+$ T cells during expansion was markedly different between cord and adult blood. $CD8^+$ T cells in CB showed a memory cell transition that was similar to $CD4^+$ CB T cells, with the proportion of T_{CM} decreasing and T_{EM} increasing until they dominated the population reaching a mean of 54.8% at the end of the culture period. Very few T_{EMRA} were observed. In contrast, adult T cells showed a sharp decline in the proportion of both T_{CM} and T_{EM} subsets during expansion, and a marked increase in T_{EMRA} which comprised a mean of 66.2% of the final product.

At the end of the culture period, whilst 32.1% of CB $CD8^+$ T cells retained the least differentiated T_{CM} or T_N phenotype, these two subsets accounted for only 5.4% of adult $CD8^+$ cells. Note that only the transduced lymphocytes moved towards a more differentiated phenotype indicating that differentiation was driven by antigen-specific recognition (Figure 4.5A). Transduced CB $CD8^+$ T cells therefore display a less differentiated phenotype compared to adult T cells throughout the *in vitro* culture period although this difference is not seen within the $CD4^+$ population.

In addition, after 23 days of *in vitro* expansion, CD27, a marker of less differentiated T cells, was expressed on 85.5% of transduced CB $CD8^+$ cells but only 39.1% of transduced adult $CD8^+$ cells (Figure 4.5C). Similarly, after 13 days of *in vitro* expansion, CD57, a marker of replicative senescence and antigen-induced apoptotic death of T cells (32), was expressed on only 17.8% of transduced $CD8^+$ CB T cells but 56.9% of adult $CD8^+$ cells (Figure 4.5D).

4.4.5. TELOMERE LENGTH DYNAMICS AND TELOMERASE ACTIVITY IN TCR-TRANSDUCED CB AND ADULT T CELLS.

TCR-transduced adult and CB T cells were taken at day 0 (pre-stimulation) and day 21 (4 days after the last stimulation) and assayed for telomere length (Figure 4.6A).

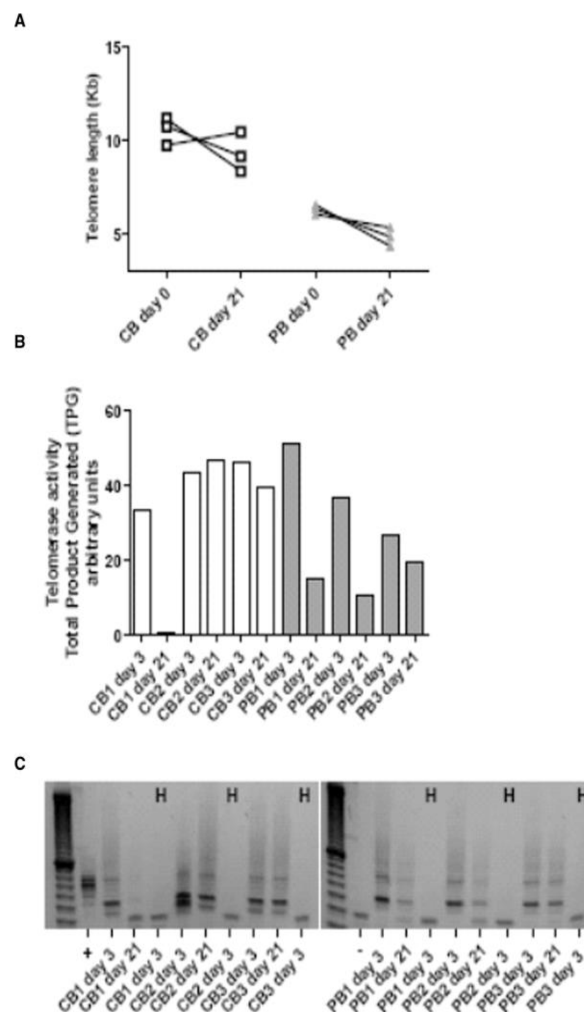

Figure 4.6. TCR-transduced CBMC have longer telomere length than TCR-transduced PBMC and generally retain elevated telomerase activity after antigen stimulation. (A) Changes in telomere length measured by Flow-FISH (Kb) of transduced cells from three CBMC and three PBMC cultures are shown at two time points (start of culture day 0 and day 21 post activation). (B) Corresponding telomerase activity from cell extracts of the same three CBMC and three PBMC cultures harvested at day 3 and day 21 after initial antigen stimulation. Data shown are the total TRAP assay telomerase products generated (TPG) from $2x10^4$ cell equivalents measured by densitometry and expressed as arbitrary units. (C) Representative telomerase activity gels including assay controls as well as enzyme heat inactivated controls (H).
(From 57).

Both adult and CB samples (with the exception of CB1) showed comparable rates of telomere length decline over 21 days of culture, consistent with expected shortening following cell proliferation, but telomeres were consistently longer in CB cells. Telomerase activity was assessed at day 3 after stimulation and again at day 21 (Figure 4.6B, C). Two out of three CB cells maintained relatively high levels of telomerase activity, indicative of greater proliferative potential, whereas all adult T cells showed a decline in telomerase activity to relatively low levels at day 21. Interestingly CB 1 lost telomerase activity by day 21, and was the only sample to show an increase in telomere length during the culture period.

4.4.6. TCR-TRANSDUCED CB T CELLS MEDIATE MULTICYTOKINE PRODUCTION AND ANTIGEN-SPECIFIC CYTOLYSIS

To investigate the effector function of the transduced cells we determined their cytokine production profile. T cells which secrete multiple cytokines are considered polyfunctional and this correlates with optimally functioning effector cells (33, 34). As such, the cultured cells were analyzed for production of IFNγ, IL-2 and TNFα on day 1 (24-hours after stimulation with anti-CD3) and on day 10 (24-hours after stimulation with peptide-pulsed DCs) (Figure 4.7A). On day 1 IFN γ secretion was negligible in CBMC but detectable in PBMC. However, after restimulation, the proportion of IFN γ–secreting $CD8^+$ cells in both CBMC and PBMC increased to approximately half of both populations. Polyfunctional CBMC secreting more than one cytokine also increased several fold upon restimulation, exceeding multi-cytokine secretion by PBMC.

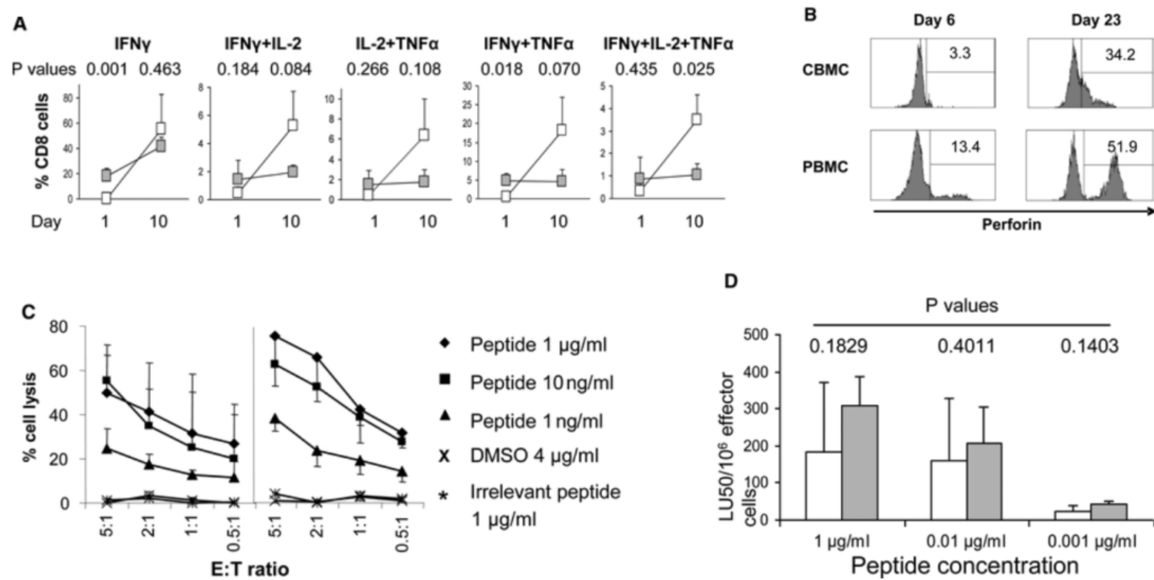

Figure 4.7. TCR-transduced cord blood T-lymphocytes can secrete cytokines and mediate cytolytic function. (A) Intracellular staining for IFNγ, IL-2 and TNFα was performed 24 hours after activation with anti-CD3 (Day 1) and 24 hours after the first restimulation with autologous peptide-pulsed DCs (Day 10). Mean percentage plus standard deviation of $CD8^+$ T lymphocytes staining for IFNγ and for the different combinations of cytokines are displayed for CBMC (n=3, white symbols) and for PBMC (n=3, grey symbols). The percentage of cells staining for all three cytokines was evaluated by first gating on double positive IFNγ/TNFα staining cells and then analysed for IL2 expression. (B) Intracellular perforin was assessed by flow cytometry on days 6 and 23 post-activation. The percentages of perforin-positive cells are indicated. One representative experiment of three is shown. (C) $CD8^+$ T-lymphocytes from CBMC (left panel) and from PBMC (right panel) were assayed for specific cell lysis activity against HLA A*1101-transduced T2 cells at the effector to target (E:T) ratios indicated. The ^{51}Cr release assay was performed in the presence of an irrelevant peptide, DMSO or different concentrations of the cognate SSC peptide. Results show the mean % specific lysis plus standard deviation from 3 separate experiments. (D) The same data are expressed as mean lytic units with grey bars for PBMC and white bars for CBMC.
(From 57).

Compared to PBMC, the proportion of $CD8^+$ CBMC secreting all three cytokines was significantly greater (*p= 0.025*). This pattern likely reflects the earlier differentiation phenotype of CB T cells and the need for antigen re-challenge before full effector function is activated. Antigenic stimulation of transduced $CD8^+$ T cells from both cord

and adult blood led to increased expression of intracellular perforin (Figure 4.7B) a marker of cytotoxic potential. Cytotoxic function was assessed using a chromium release assay. Lysis of HLA-A*1101-transfected T2 cells pulsed with SSC peptide was observed at relatively low E:T ratios and decreased with titration of the peptide (Figure 4.7C). The results were expressed in equivalent lytic units (Figure 4.7D) and compared for cord and adult TCR-transduced $CD8^+$ lymphocytes. Although there was a trend towards increased cytotoxicity with adult effector cells, reflecting the increased percentage of T_{EMRA} and perforin-positive cells within this population (Figures 4.5B and 4.7B), this difference was not statistically significant (Figure 4.7D).

4.5. DISCUSSION

The clinical efficacy of adoptively transferred T-lymphocytes correlates with their ability to persist *in vivo* (14). Several studies indicate that *in vivo* persistence correlates with a less differentiated T-cell phenotype (7, 12, 15-17, 19, 35, 36) and more recent work suggests T_N may be optimal in this setting, especially where retroviral transduction of T cells is required to engineer the appropriate antigenic specificity (20, 21). For this reason we studied CB as a potential source of T cells for TCR engineered effectors since the vast majority of these cells are T_N.

Using a protocol adopted for clinical trials (11, 12, 37) we found that retroviral transduction of CB and adult T cells led to comparable EBV-TCR expression (Figure 2) in both $CD8^+$ and $CD4^+$ CB T cells. This is important, as TCR transduced $CD4^+$ T cells can provide helper functions *in vivo* to maintain an effective $CD8^+$ T-cell response, as well as mediate direct anti-tumor effects (38, 39).

Immediately post-transduction, the differentiation phenotype of CB T cells differed from that of adult T cells, shifting from T_N to a predominantly T_{CM} rather than T_{EM} phenotype. Moreover, phenotypic differences between the two cell sources were maintained throughout the *in vitro* culture. Within $CD8^+$ T cells CB cells differentiated predominantly to T_{EM} by day 23 whereas adult $CD8^+$ T cells shifted to a T_{EMRA} phenotype typical of late differentiated cells. It is not clear to what extent the increased proportion of more differentiated T cells was due to increased proliferation of these cells or maturation of T cells from subsets with a less differentiated phenotype. The relative 'youth' of expanded CB lymphocytes when compared to adult T cells was supported by reduced expression on cord $CD8^+$ cells of CD57 , a marker of replicative senescence and antigen-induced apoptotic death of T cells (32). Furthermore, 85.5% of transduced $CD8^+$

CB T cells retained expression of CD27, a marker recently identified as predictive of clinical response following infusion of T cells to treat melanoma and CMV (15, 36).

Telomere shortening is associated with lymphocyte differentiation eventually leading to senescence and apoptosis (40) and has been observed with cell culture *in vitro* and with age *in vivo* (41-43). Telomerase maintains telomere length and supports proliferative potential but cannot fully prevent telomere shortening (44). Ectopic telomerase expression supports extended lymphocyte proliferation *in vitro* (45). In our study (Figure 4.6), CB T cells had longer telomeres than adult T cells and this difference was maintained after 3 weeks of antigen-driven *in vitro* expansion. Similarly, in 2/3 CB samples, high telomerase activity was maintained over the same culture period, whereas this activity decreased in 3/3 adult T-cell samples. In one CB culture there was telomere elongation but suppressed telomerase activity at day 21. We speculate this may be related to a negative feedback mechanism that prevents uncontrolled telomere elongation by telomerase and warrants further investigation (46). Taken together, our results indicate that in contrast to TCR-transduced adult T-lymphocytes, CB T-lymphocytes have longer telomeres and generally maintain higher levels of telomerase activity during culture, supportive of greater proliferative and survival potential *in vivo* (19).

Transduced CB cells expanded well *in vitro* (Figure 4.4) and were capable of multiple cytokine production and cytotoxic activity following antigen-specific stimulation (Figure 4.7). Nevertheless, they expressed lower levels of perforin and, though not statistically significant, there was a trend towards reduced cytotoxic function in CB T cells *in vitro* when compared to adult T cells (Figure 4.7). This mirrors that seen in mouse studies where less differentiated T cells display reduced cytotoxic function *in vitro* compared with more differentiated effectors. However, in the same study, the less differentiated T cells possessed more potent anti-tumor activity *in vivo* probably reflecting the reduced

proliferative and survival potential of more differentiated cells (17). By analogy, our results suggest that the less differentiated CB T cells, which may have reduced cytotoxic function *in vitro*, may prove more effective *in vivo* than adult T cells, although further studies are required to confirm this. In this work HLA A11-restricted EBV-specific TCR was used to explore the function of TCR-transduced CB T cells, but further studies are required to confirm that these properties are generally applicable to any TCR.

When we explored activation of CB T cells as a necessary step for retroviral transduction, we found CB T cells initially proliferated more rapidly than adult T cells (Figure 1B,C). This contradicts some reports that CB cells have increased propensity to apoptosis following activation (47, 48). It is unclear whether increased proliferation of CB cells reflects the differing proportions of naive and memory cells within cord and adult blood, or whether cord T_N have a distinct response to mitogenic stimulation. Recent work suggests that the development of the immune system occurs in distinct waves derived from different stem cell populations, and that fetal lymphopoiesis differs from adult lymphopoiesis with enhanced proliferation after exposure to allo-stimulation (49). CB is at the transition between fetal and adult haematopoiesis, and the greater proliferation we observed in CB T cells may be a reflection of this.

The clinical implication of this study is the possibility of CB providing potent third party T cells with good replicative and functional reserve for TCR engineering. Third party ACT is effective in transplant settings where matched donor lymphocytes are unavailable. Third party, partially HLA-matched EBV-specific T-cell lines have demonstrated safety and efficacy in eradicating PTLD following SOT with minimal GVHD risk (2, 3). *In vitro* expanded third party CMV-specific T cells have also been given successfully to a CB transplant patient with CMV encephalitis with no adverse effects (50). Immunotherapy with CB T-lymphocytes may also be appropriate for cancer

patients whose prior treatment with radio/chemotherapy and age-related thymic involution have rendered them lymphopenic with reduced numbers of T_N and T_{CM} subsets and CD27 expression (51, 52). Such cells may not have the capacity for *in vivo* persistence and clinical efficacy. CB T cells may also benefit patients with primary T-cell dysfunction and where autologous PBMC are difficult to handle *ex vivo* (eg. HIV-infected blood). Notwithstanding the potential benefits, TCR gene transfer with third party T cells carries significant theoretical risks. Although GVHD risk is reduced in CB transplantation (53, 54), introducing TCRs could induce heterologous immunity including the risk of graft-versus-graft effects in SOT recipients as the introduced TCR may display unanticipated alloreactivity to normal cells (55). The HLA A11-restricted EBV-specific TCR described here is not known to cross-react with other antigens, but some EBV-specific TCRs recognise particular alloantigens (56). Transduced cells should therefore be checked for reactivity to patient or transplant donor cells before infusion. Since TCR-transduced CB T cells may have greater proliferative capacity, fewer cells may need to be infused compared with adult T cells thus reducing the risk of GVHD. Selective enrichment of TCR-transduced T cells with HLA:peptide multimers could reduce the required dose still further.

In summary, we have demonstrated that human CB lymphocytes have qualities suited for adoptive therapy using retrovirally-transduced T cells since they can be engineered to express high avidity functional TCR whilst maintaining an early differentiation phenotype that could lead to long term *in vivo* persistence after infusion.

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5. CONCLUSIONS

AND PERSPECTIVES

5.1. GENERAL CONCLUSIONS

We have demonstrated in vitro that human CB $CD8^+$ T lymphocytes are potentially better suited than analogous cells from peripheral blood of adult donors for adoptive therapy using TCR-transduced T cells, since they maintain an earlier differentiation phenotype that could lead to long term *in vivo* persistence after infusion.

This consideration can be extended to T lymphocytes transduced with CAR, an approach that is increasingly used in ATCT. In this regard, EBV-specific T cells engineered with an anti-CD30 CAR have been generated for the treatment of HL and proved effective in a mouse model (Savoldo et al., 2007). Using CB as source of T lymphocytes for CAR transduction should allow for a longer *in vivo* survival of the infused cells. Indeed, it has been demonstrated that CB T lymphocytes after massive expansion retain a predominantly T_{CM} phenotype and, after transduction of CD19-specific Cpost-thymic AR and IL-2 transgenes, prolong the overall survival in a mouse model of B-cell acute lymphoblastic leukemia (Pegram et al., 2015).

It is of note that almost half of the CB units collected by public CB banks for HSCT are not suitable for transplant use, due to insufficient hematopoietic stem cells dose (Querol, 2012). These CB units, which are currently useless, might provide an abundant source of T lymphocytes for ATCT.

It is now 30 years since the first CB transplant, performed on 6 October 1988 (Gluckman et al., 1989), and this approach has become a safe and dependable alternative donor graft source in allogeneic HSCT when better sources are not available. In particular, CB transplants show less graft versus host disease and better tolerance to HLA mismatches (Gluckman et al., 1997; Eapen et al., 2007). This implies that CB T cells are less effective in mounting an immune response or more tolerogenic than their adult counterpart. We

have demonstrated that after the activation needed for gene transduction, CB T cells retain a less differentiated phenotype and show a delay in displaying effector functions, compared to PB T cells which quickly become T_{Eff}. This delay might contribute to the reduced alloreactive response observed in CB transplants, and is explained with the fact that T cells in CB are mainly naive while those in adult PB are largely memory cells. However, differences exist also between T_N cells from the two sources. It has been recently demonstrated that $CD8^+$ T_N cells are less cytotoxic than the same cells from adult PB and display a genetic profile biased toward the innate immune response (Galindo-Albarran et al., 2016). This might contribute to explain why CB transplants have a reduced allogeneic response, compared to transplants from adult donors. Moreover, the $CD4^+$ T_N pool in PB seems to be composed of two subsets, one of classical naive cells and one, likely resulting from the homeostatic peripheral expansion of the latter cells, characterised by decreased content in T cell receptor excision circles and reduced expression of CD31 (Kimmig et al., 2002). On our part, we found that T_N lymphocytes from CB proved to be more effective than the same cells from PB in generating T_{Nrev}, demonstrating that the TN lymphocytes from the two sources also differ at the functional level. On the basis of these considerations, we should regard CB T cells as true naïve cells and consider the phenotypically naïve cells from PB as a disomogeneous cell pool which encompasses true naïve cells, cells having ondergone homeostatic peripheral expansion, T_{SCM}, T_{MNP} and T_{Nrev}.

Our results show that CB might be a better source of T_N than PB in order to generate $T_{Nrev/SCM}$ cells. In this regard, the identification of the mechanism that allows for the reversion of the phenotype in recently differentiated memory $CD8^+$ T cells provides a powerful tool for the *in vitro* generation of cells endowed with optimal proliferative and differentiative potential and therefore best suited for ATCT. Although T_{SCM} can be

generated *in vitro* from T_N cells without undergoing phenotype reversion (Sabatino et al., 2016), it is not known if the co-stimuli used, i. e. IL-7, IL-21 and the glycogen synthase-3β inhibitor TWS119, are more or less effective than IL-2 in inducing a proliferative response. Indeed the comparison of the two techniques should be carried out, in order to identify the best method to produce T_{SCM}-like cells.

The quality of the infused cells is a parameter that has to be taken into account whenever a cell preparation is to be used for ATCT, including the products of banks for "off the shelf" lymphocytes. These banks provide EBV-specific T-cell lines (reviewed in O'Reilly et al., 2016) which, although proven effective (Haque et al., 2007; Leen et al., 2013), might not provide the optimal *in vivo* persistence, due to the prolonged *in vitro* manipulation required to generate an established T-cell line. Instead, the establishment of a registry of HLA-typed donors with high levels of circulating EBV-specific T lympocytes, as proposed in this thesis and by others (Eiz-Vesper et al., 2012; Sukdolak et al., 2013), should allow for the *ex vivo* selection of minimally manipulated cells, likely better suited for ATCT.

5.2. FUTURE PERSPECTIVES

Although ATCT has demonstrated its therapeutic effect, there are several fields where there is still space for improvement. However, an exhaustive analysis of all the possible developments is beyond the scope of this thesis, so I will briefly address three fields that in my opinion might be of interest.

5.2.1. MANIPULATING THE HOMING OF THE INFUSED CELLS

The administration route of the infused cells can greatly affect the outcome of ATCT. Using an orthotopic mouse model of human mesothelioma, it was demonstrated that the local administration of mesothelin-specific CAR-engineered T lymphocytes had a clear advantage on the systemic route, which required many more cells to achieve the same remission rate (Adusumilli et al., 2014).

An optimal homing to the tumor tissue can be better achieved by transducing the cells to be infused with the receptor(s) for the chemokine(s) expressed by the tumor cells. It has already been demonstrated, in a mouse model of HL, that CD30-specific CAR-engineered T lymphocytes are more effective at infiltrating and killing when they co-express CCR4 (Di Stasi et al., 2009).

A similar approach might be followed for other chemokines. In HL the chemokine CXCL10 is expressed predominantly in the EBV-positive cases (Maggio et al., 2002), and most of NPC case express RANTES (Buettner et al., 2007). The co-transduction of the respective receptors, CXCR3, and CCR1, CCR3 and CCR5 may be an option to be considered.

Other possible cytokines which might be investigated are CCL17 and CCL22, which are known to be induced by the EBV protein LMP1 (Nakayama et al., 2004).

5.2.2. ENGINEERING THE INFUSED CELLS TO RESIST THE IMMUNOSUPPRESSIVE TUMOR ENVIRONMENT

Tumors escape immune surveillance by means of a number of different mechanisms (reviewed by Camisaschi et al, 2016), which affect, at least in part, the efficacy of ATCT. Strategies have therefore been envisaged to target a number of tumor-associated immune escape mechanisms.

Particular interest has been focused on TGFβ, due to its pleiotropic effects on supporting tumor growth (Yang et al., 2010). It was found that transduction of T lymphocytes with a truncated form of the dominant-negative TGFβ receptor type II (DNR) renders the cells insensitive to TGFβ (Foster et al., 2008). On these basis a trial on HL and NHL was conducted, which demonstrated an improvement in clinical response when the EBV-specific T cells also expressed the truncated DNR (Bollard et al., 2012). Furthermore, a recombinant receptor was created, encompassing the extracellular portion of the TGFβ receptor type II and the intracellular domain of the toll-like receptor 4. In this way, the transduced T cells responded to TGFβ by activating and proliferating (Watanabe et al., 2013).

Another approach aims at targeting the tumor-associated cells exerting an immune suppressive activity. Tumor-specific $CD8^+$ T cells engineered to secrete IL-12 were found to re-program DCs, macrophages and myeloid-derived suppressor cells (MDSCs), ultimately improving ATCT in a mouse model of melanoma (Kerkar et al., 2011).

Also granulocytes may be regarded as potential targets: they represent a significant component of tumor-infiltrating leukocytes in NPC, where they are recruited via release of IL-8 by tumor cells (Hsu et al., 2008). Although granulocytes are endowed with strong immune suppressive potential, in this tumor type they seem to play an anti-tumor role by

promoting an acute inflammatory response and providing immune stimulation, both mediated by tumor-derived IL-1β (Chen et al., 2012). Their role in EBV-associated tumors should be further investigated.

5.2.3. COMBINING ATCT WITH OTHER TREATMENTS

ATCT, used so far as single therapy, might get advantage from the association with other treatments, either traditional or new.

The simplest approach consists in combining ATCT with standard chemotherapy. Although the results need to be confirmed in a randomized trial, a phase 2 trial on 35 EBV-positive NPC patients showed that carboplatin plus gemcitabine in association with infusion of EBV-specific CTLs achieved a response rate not achieved by previous trials with standard chemotherapy (Chia et al., 2014). Encouraging results have also been attained in a mouse model by combining ATCT with doxorubicin, probably due to the cytotoxic effect of the drug on MDSCs (Alizadeh et al., 2014).

A more innovative approach involves the targeting of molecules down-regulating the immune response, such as those involved in immune checkpoints. MoAbs directed against immune checkpoints are currently used in specific tumor types, and the association of an anti PD-1 MoAb was found to significantly increase the response to the anti-CTLA-4 MoAb Ipilimumab in melanoma patients (Postow et al., 2017). Nivolumab, an anti PD-1 MoAb, has been proposed for the treatment of HD, since in this tumor the expression of PD-1, and of its ligand PD-L1 as well, is often increased (Ansell, 2017). Actually, phase I/II trials with nivolimumab and pembrolizumab, an anti PD-L1 MoAb,

are under way in NPC. Noteworthy, the effect of ATCT in a mouse tumor model was enhanced by the concomitant administration of an anti-PD-1 antibody (John et al., 2013). Improved therapeutic effects might be obtained using drugs targeting single molecules involved in tumor growth. In a xenograft model of *BRAF*-mutated human melanoma, the B-Raf inhibitor PLX4720 enhanced the effect of ATCT, likely reducing the production of vascular endothelial growth factor by tumor cells (Liu et al., 2013). In a similar melanoma model the effect of ATCT was improved by the administration of the colony stimulating factor 1 inhibitor PLX3397, which blocked the recruitment at the tumor site of M2 macrophages and MDSC (Mok et al., 2014).

An alternative strategy might aim at making EBV-associated malignancies more sensitive to ATCT via the induction of the expression of immunodominant epitopes, which are a prerogative of lytic cycle antigens not expressed in type1 and type 2 latency tumors. Butyrate derivatives induce the expression of lytic antigens in LCLs and, in combination with ganciclovir, induced partial or complete remission in 10 out of 15 patients with EBV-positive lymphomas refractory to conventional therapy (Perrine et al., 2007). Moreover, it has been found in needle aspiration specimens from BL patients that treatment with cyclophosphamide results in the expression of lytic genes in tumor cells (Tang et al., 2010). It might be worth thinking of a trial combining these drugs with ATCT.

In perspective, the use of microRNAs for enhancing and fine tuning the activity of infused T lymphocytes was also suggested (Ji et al., 2016)

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APPENDICES

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Cord Blood T Cells Retain Early Differentiation Phenotype Suitable for Immunotherapy After TCR Gene Transfer to Confer EBV Specificity

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Adoptive T cell therapy can be effective for Epstein–Barr virus (EBV)-associated posttransplant lymphoproliferative disease and melanoma. Transducing high-affinity TCR genes into T lymphocytes is an emerging method to improve potency and specificity of tumor-specific T cells. However, both methods necessitate *in vitro* lymphocyte proliferation, generating highly differentiated effector cells that display reduced survival and antitumor efficacy postinfusion. TCR-transduction of naive lymphocytes isolated from peripheral blood is reported to provide superior *in vivo* survival and function. We utilized cord blood (CB) lymphocytes, which comprise mainly naive cells, for transducing EBV-specific TCR. Comparable TCR expression was achieved in adult and CB cells, but the latter expressed an earlier differentiation profile. Further antigen-driven stimulation skewed adult lymphocytes to a late differentiation phenotype associated with immune exhaustion. In contrast, CB T cells retained a less differentiated phenotype after antigen stimulation, remaining CD57-negative but were still capable of antigen-specific polyfunctional cytokine expression and cytotoxicity in response to EBV antigen. CB T cells also retained longer telomeres and in general possessed higher telomerase activity indicative of greater proliferative potential. CB lymphocytes therefore have qualities indicating prolonged survival and effector function favorable to immunotherapy, especially in settings where donor lymphocytes are unavailable such as in solid organ and CB transplantation.

Key words: Epstein–Barr virus, gene transfer, T cell receptor, T cell therapy, T lymphocytes

Abbreviations: 7AAD, 7-aminoactinomycin; ACT, adoptive cellular immunotherapy; CB, cord blood; CBMC, cord blood mononuclear cells; CFSE, carboxyfluorescein succinimidyl ester; CMV, cytomegalovirus; DCs, dendritic cells; DMSO, dimethyl sulfoxide; EBV, Epstein–Barr virus; FCS, fetal calf serum; GMCSF, granulocyte-macrophage colony-stimulating factor; GVHD, graft versus host disease; IFNγ, interferon gamma; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered solution; PI, propidium iodide; PTLD, post-transplant lymphoproliferative disease; SOT, solid organ transplantation; T_N, naive T lymphocytes; T_{CM}, central memory T lymphocytes; T_{EM}, effector memory T lymphocytes; T_{EMRA}, effector memory CD45RA+ T lymphocytes; TNFα, tumor necrosis factor alpha.

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