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# PRECLINICAL MODELING HIGHLIGHTS THE THERAPEUTIC POTENTIAL OF THE ADOPTIVE TRANSPLANT OF GENE CORRECTED T CELLS AND HEMATOPOIETIC STEM CELLS IN X-LINKED HYPER-IGM IMMUNODEFICIENCY

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# 1.INTRODUCTION

## 1.1 Gene therapy in hematopoietic stem cells

Hematopoietic stem cell (HSC) transplantation (HSCT) consists in the infusion of hematopoietic stem and progenitor cells (HSPCs), that "home" to the recipient's hematopoietic microenvironment, to establish marrow and immune function in patients with a variety of hematologic malignancies (e.g., leukemia, lymphoma, and myeloma) and acquired (e.g., aplastic anemia) and inherited (thalassemia, sickle cell anemia, and severe combined immunodeficiency) disorders. HSCT is also used in the support of patients undergoing high-dose chemotherapy for the treatment of certain solid tumors for whom hematologic toxicity would otherwise limit drug administration (germ cell tumors, soft tissue sarcomas, and neuroblastoma) (Galgano & Hutt 2017). There are three different categories of transplantation, autologous, allogeneic and syngeneic and all the categories consists in the harvest of the stem cell, conditioning of the patient, aplasia and engraftment until the recovery of the hematopoietic function. In the autologous HSCT patients receive a high dose chemotherapy followed by the reinfusion of their own HSC previously cryopreserved. There is no risk of rejection or graft versus host disease (GvHD) and graft failure can occur rarely. It is used as an alternative treatment modality for lymphomas, multiple myeloma, and a few solid tumors and for inherited malignant disorders. In allogeneic transplantation, instead, the patients receive HSPCs, after conditioning, from a related (family member) or unrelated (present in a Bone Marrow Donors Worldwide" registry database (BMDW) donor, who can be fully human leukocyte antigen (HLA)-matched or partially in the case of unavailability. HLA-matched sibling donors are preferred for allogeneic transplants; however, less than 30% of patients have a suitable donor. The ideal donor is a geno-identical twin (syngeneic transplantation). Allogeneic HSCT in fact, is a highly risk procedure,

particularly when the human leukocyte antigen (HLA) complex is mismatched between patient and donor, as it happens in most of the cases. Transplantation with a mismatched HLA complex decreases the efficiency of engraftment and favours the development of graft-versus-host disease (GvHD), then resulting in higher mortality and morbidity (Copelan, 2006; Naldini, 2015). Thus, patients affected by haematological disorders, but also carrying primary immunodeficiencies (PID) or leukodystrophies, can benefit from HSC gene therapy (Qasim et al., 2009; Naldini, 2015). Currently, many HSC gene therapy clinical trials have been completed and some others are still in progress. Lentiviral vectors are used for gene transfer into HSCs to treat different diseases, e.g. Wiskott-Aldrich syndrome (Aiuti et al., 2013; NCT01515462), β-Thalassaemia major (NCT02151526) and metachromatic leukodystrophy (Biffi et al., 2013; NCT01560182) (Naldini, 2015). In 2016, StrimvelisTM, a gene therapy developed at San Raffaele Telethon Institute for Gene Therapy by GlaxoSmithKline (GSK) through a collaboration with Fondazione Telethon and Ospedale San Raffaele, receives European marketing authorization to treat adenosine deaminase severe combined immunodeficiency disorder (ADA-SCID), thus underscoring the success of HSC gene therapy.

### 1.1.1 HSC sources

Hematopoietic stem cells can be derived from the bone marrow (BM), peripheral blood after mobilization (PBSC) and umbilical cord blood (UCB). The bone marrow (BM) represent the first identified source of stem cells. However, it has almost been replaced by peripheral blood stem cells (PBSC), the most widely used stem cell source in both auto- and allo-HSCT over the last decade. HSCT performed with PBSC allows a relatively more rapid recovery of hematopoiesis than BM and increases the overall survival in high-risk hematological malignancies. The disadvantage is an increased risk of chronic GvHD in the allogenic HSCT because of an increased number of circulating T cells. The main limitations of allogeneic BMT are the lack of suitable HLA-matched donors. In this case, mismatched donors represent an alternative, that however increases failures,

GvHD and delayed immune reconstitution. More recently, the cord blood (CB) has been shown to be a good alternative source of HSC. CB has fewer mature T lymphocytes compared with peripheral blood, thus making a UCB transplantation (UCBT) with a greater degree of HLA mismatch possible. [5] (Stanevsky et al., 2009). Also, compared with adult cells CB HSC are enriched in the most primitive stem cells, producing *in vivo* long-term repopulating stem cells. In particular, it has been shown that hematopoietic stem and progenitor cells (HSPCs) derived from CB have greater telomere length which confers a better proliferation potential [6](Gammaitoni et al., 2004). The collecting procedure of cord blood is simple and has no medical risk to the mother or newborn baby. Moreover, Broxmeyer and colleagues, the first to analyse the features of CB derived HSC, noted that these cells could be successfully frozen, stored and thawed without major loss. For all these reasons CB represent a valid alternative for HSCT. Nowadays there are banks collecting CB unrelated donors that rely of specific criteria of CB collection, banking, processing and cryopreservation for delivering units for the treatment of various hematologic malignant and non-malignant diseases (Panch et al., 2017, Gluckman et al., 2005). However, there are certain limitations with CBT, the first of which is the limited volume of blood that can be collected from a single umbilical cord. Consequently, a limited number of stem cells can be derived from a single cord blood collection and this limitation is associated with the delayed engraftment and a higher risk of graft failure. The limited cell dose is of particular importance in adult recipients, because of their greater body weight (Stanevsky et al., 2009). Furthermore, CB is not only low in T cell numbers, but the few T cells in that source are also naïve, thus making them ineffective in combating viral infections post-transplant. This translates into a higher morbidity and mortality from infections (especially from CMV, adenovirus (Adv), and EBV) in the post-transplant period (Emiloju et al., 2019).

### 1.1.2 The hematopoietic system

The hematopoietic system supplies every cell in our body with a vast array of mature blood cells that facilitate oxygen transport, immunity and tissue remodeling (Boulais and Frenette, 2015). At the beginning of the 20th century, A. Maximov, a Russian biologist theorized, for the first time, that hematopoiesis is organized as a cellular hierarchy derived from a common progenitor. Later this observation, was proofed by Till and McCulloch, who showed the existence of this common and multipotent precursor, the hematopoietic stem cell (HSC). They showed that ten days after transplanting limiting number of syngenic bone marrow (BM) cells into recipient mice, in the spleen of the mice there were cellular colonies that revealed a very small sub-population of the donor BM cells that possessed two main properties: the ability to generate multiple types of myeloerythroid cells, and the ability to self-replicate (Becker et al., 1963, Mcculloch et al., 1961)

Thus, HSC are defined by three main different features:

i. homeostatic control, i.e. the ability to balance self-renewal capacity and differentiation potential according to both intrinsic and extrinsic stimuli in order to support hematopoietic system homeostasis (Matsumoto and Nakayama, 2013);
ii. self-renewal capacity; i.e. the ability to give rise to HSC itself without differentiation while maintaining the proliferation and multipotent potential;
iii. differentiation, i.e. the ability to give rise to progressively more committed type of cells, which lack of stem cell properties and gain highly specialized functions (Matsumoto et al., 2013).

Stem cell quiescence has been postulated to be important to preserve adult stem cell function and prevent their exhaustion. It is thought to protect stem cells from most proliferative stimuli, including genetic alterations, that induce acquisition of mutations, leading to their malignant transformation to putative cancer stem cell (Wilson et al., 2008). Only 0,01% of cell in the BM is an HSC (Walesek et al.,

2012), and consequently many are in a state of quiescence that protects them from genotoxic insults (Boulais and Frenette, 2015). Cycling frequency increases as HSCs gradually differentiate (Pietrzyk et al., 1985; Bradford et al., 1997). The reversible or transitory absence of cycling, and thus of cell division, is defined as quiescence and it corresponds to the $G_0$ state. As opposed to somatic cells that progress through the different cell cycle states, HSCs have the ability to withdraw from the cell cycle and enter the G0 phase (Wilson et al., 2008; Nakamura-Ishizu et al., 2014). Three different subpopulations of HSCs have been proposed based on their self-renewal or repopulation potential (Beveniste et al., 2010; Copley et al., 2012): short-term HSC (ST- HSC), intermediate-term HSC (IT-HSC) and long-term HSC (LT-HSC) (Figure 1.1). Measured by competitive bone marrow transplantation with myeloablative conditioning, LT-HSC have the highest repopulation potential (and ST-HSC the lowest) and likely the lowest rate of cell cycling (i.e. LT-HSC are the most quiescent HSC) as quiescence and self-renewal are thought to be positively correlated (Nakamura-Ishizu et al., 2014). ST-HSC immediate progeny are multipotent progenitors (MPP) cells, which have lost almost totally the ability to self-renewal, but are still able to differentiate into different lineage committed cells, thus maintaining multipotency (Seita and Weissman, 2010). MPP cells, on their turn, can differentiate in oligopotent progenitors: granulocyte-macrophage- lymphocyte progenitor cells (GMLPs) and common myeloid progenitors (CMP) can give rise to only a restricted number of lineages. GMLPs differentiate in common lymphoid progenitors (CLP) or granulocyte-macrophage progenitor cells (GMPs), while CMPs differentiate in megakaryocyte-erythrocyte progenitors (MEP) or GMP. MEPs, GMPs and CLPs differentiate into lineage restricted committed progenitors that differentiate into their final blood effector cells (i.e. platelets and red blood cells for MEPs, different granulocytes and monocytes for GMPs and B-cells, T-cells and NK-cells for CLPs. Dendritic cells can be formed from either GMPs or GMLPs; Rossi et al., 2012). This highly hierarchical system is able to quickly produce many effector cells from a single

HSC division, because committed progenitors replicate themselves frequently before differentiating (Alberts et al., 2002).

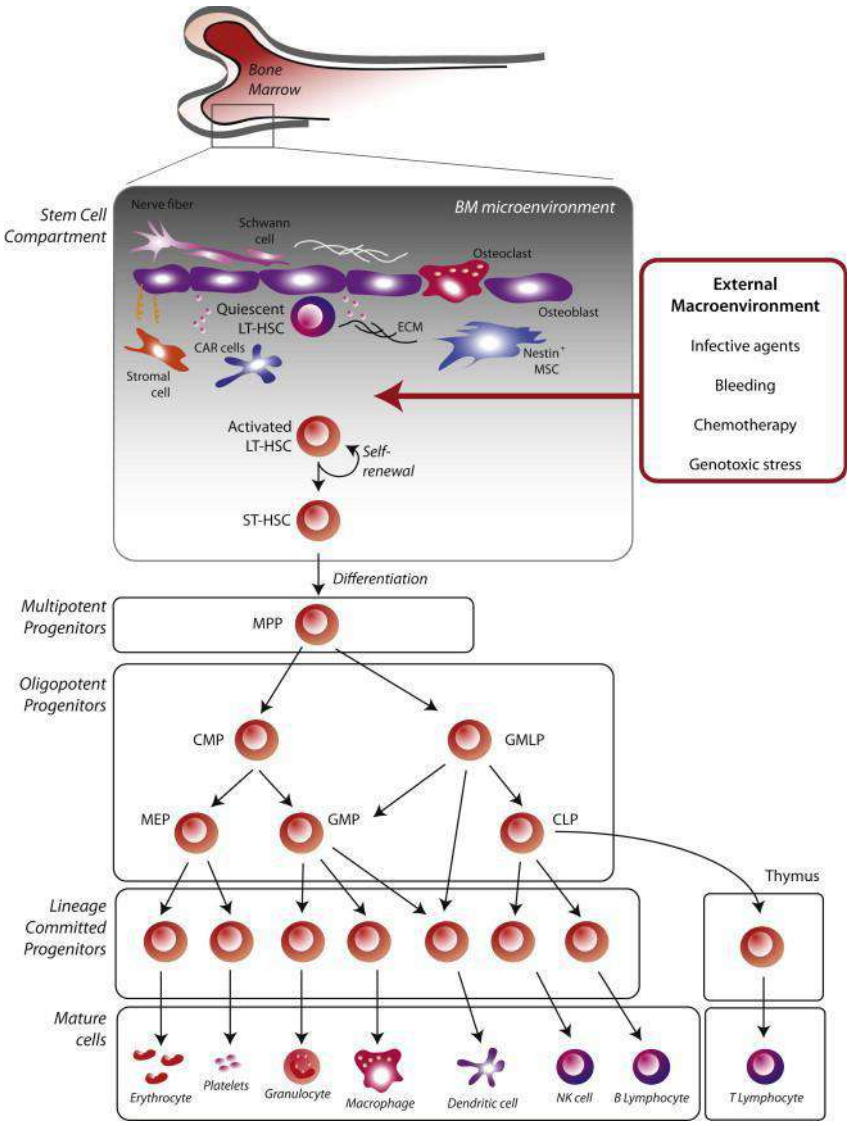



**Figure 1.1** LT-HSC that resides in the BM microenvironment ensures the formation of many different mature blood cells. The BM microenvironment is tightly regulated so hematopoiesis is maintained. Stimuli cause the LT-HSC to self-renew or become activated and give origin to ST-HSC that differentiate into MPP cells and eventually into committed progenitors that have high proliferation levels to produced vast amounts of mature blood cells (adapted from Rossi et al., 2012).

### 1.1.3 Phenotypical identification of Hematopoietic stem and progenitor cells (HSPC)

To study and to better understand HSCs biology, HSPCs identification and purification is required. Phenotypical identification and isolation can be accomplished using monoclonal antibodies and a technique called fluorescence-activated cell sorting (FACS). Briefly, a fluorescent molecule is conjugated to antibodies, which recognize characteristic antigens on the cell membrane. By using FACS analysis is possible to measure the cell fluorescence to identify and sort cells.

The first marker found as highly enriched in hematopoietic multipotent and oligopotent cells even if still heterogeneous, was the Cluster of differentiation 34 (CD34), a glycoprotein present at the cell surface where it functions as a ligand for L-selectin (Civin et al., 1984; Weissman and Shizuru, 2008). Early experiments showed that CD34+ cells were able to reconstitute haematopoiesis after lethal irradiation (Berenson et al., 1988; Weissman and Shizuru, 2008). Later Baum and colleagues identified a population of cells CD34+, CD90+ and Lin-, proposed as the first phenotypic signature for HSCs. Lineage (Lin) markers consist in different molecules typically expressed by specific lineage population and mature cells. CD90 (also known as Thy-1) is a surface glycoprotein probably implicated in cell-matrix interaction (its function is still not fully understood). This cell population contains multipotent progenitors able to differentiate into T-cells (Baum et al., 1992) and it can be derived from fetal and adult marrow, fetal liver, cord blood and mobilized peripheral blood. Furthermore, these cells are able to give long-term multilineage reconstitution (LTMR) *in vivo* (Weissman and Shizuru, 2008). It is possibile to use the surface marker CD38 in order to achieve further enrichement in CD34+ population. CD38 (or cyclic ADP ribose hydrolase) is progressively expressed during differentiation. Both *in vitro* and *in vivo* studies have demonstrated that CD34+ $CD38^{lo/-}$ cells are able to sustain long-term culture differentiation and to repopulate immunodeficient mice (Hao, Thiemann et al., 1996; Bhatia et al., 1997). Also CD133, a pentaspan transmembrane glycoprotein,

can be used to better characterize the most primitive population of HSCs (Yin et al., 1997). More recently, Notta and collegues proposed CD49f as a positive marker for HSC isolation. $CD49f^{+}$ cells are highly efficient in repopulating the hematopoietic compartement, whereas their loss leads to transient MPP engraftment (Notta et al., 2011). Latterly, Fares and collegues suggested Endothelial Protein C Recpetor (EPCR) as a novel marker for expanded human LT-HSC. EPCR is a constituent of endothelial barrier protection, thus being fundamental for its anti-inflammatory function. EPCR+ cells resulted as a subset of a triple positive population uniformly expressing CD34, CD90 and CD133 and showed both short-term and long-term reconstitution potential in mice (Fares et al., 2017).

Currently we classify HSPC as either primitive HSC (CD34+CD133+CD90+), early progenitors (CD34+CD133+CD90−), committed progenitors (CD34+CD133−) and most differentiated cells (CD34−) (Genovese et al., 2014). To complete the phenotypical identification of the lineages CLPs are CD34+CD38+Lin−CD10+CD45RA+ (Galy et al., 1995) CMPs are CD34+CD38+L3Ra+CD45RA-, GMPs CD34+CD38+L3Ra+CD45RA+ and MEPs are CD34+CD38+L3RalowCD45RA- (Manz et al., 2002).

In contrast to humans where CD34 was the first marker found to be enriched in Hematopoietic Stem/Progenitor Cells (HSPCs) (Civin et al., 1984), murine HSC are first enriched as lacking linage markers of mature cells (Lin-). The addition of an antibody against the murine Stem Cell Antigen (Sca-1) and against the tyrosine kinase receptor c-kit identified the Lin-c-Kit+Sca+ (LSK) population which is very heterogeneous, mostly composed of progenitors and where true HSCs represent less than 10% of this subset (Ikuta et al., 1992). Moreover, this phenotype is only appropriate for identifying HSPCs present in steady-state adult BM of certain mouse strains, e.g. C57Bl/6. Additionally, the LSK phenotype is not useful in other mouse strains, e.g. Balb/c, or if HSPCs are activated and proliferating (particularly

upon in vitro culture), or from earlier timepoints of mouse embryonic development (Randall and Weissman, 1998; Dooner et al. 2008; Kim I et al. 2006). Addition of the markers CD150 and CD48, represented a major improvement for further enrichment of the LSK population of mouse HSPCs. The frequency of true HSCs within the $CD150^{+}CD48^{-}$ LSK (LSK/SLAM) population is about 40% in normal adult mouse BM (Kiel MJ et al. 2005). This phenotype also enables improved resolution of HSCs in various experimental contexts and from embryonic sources. (Kiel MJ et al. 2005; Yilmaz OH et al. 2006).

### 1.1.4 Gene therapy exploiting viral vectors

The aim of gene therapy is to introduce, by a genetic engineering approach, a segment of genetic material into the cells of a patient either to replace a defective gene, or to provide a new function, in order to cure or slowdown the progression of disease (Verma and Weitzman, 2005). To achieve this goal, gene therapy requires technologies capable of efficient gene transfer into cells and which can guarantee a stable expression of the therapeutic gene at good levels in target cells, and its mainteinance in the progeny. Thus, the delivery of exogenous corrective DNA to the cells can be done through different platforms and is a crucial process for the successful clinical application of gene therapy. Delivery platforms can be divided in viral and non-viral methods. Non-viral platforms include naked-DNA transfection and electroporation among many others, but their main hurdle is low transfection efficiency (Ramamoorth and Narvekar, 2015). Viral vectors, on the contrary, can be considered as very efficient delivery vehicles for gene therapy and transgene expression, also in primary cells (Kay et al., 2001). However, viral vectors carry safety limitations that have to be carefully evaluated. Viral vectors can be integration competent or integration deficient. The genome of integration deficient viruses is present in the cells in an episomal form and is essentially diluted upon cells division. Instead, integration competent viruses stably integrate into the genome of the cells, thus remaining present in all progeny. Currently, adenoviral

(AdV) (Wold and Toth, 2013) and adeno- associated (AAV) (McCarty et al., 2004) vectors are the two main types of viral vectors mantained as episomal genomes in the cells, while the two main types of vectors that can integrate into recipient cells are retroviral (RV) (Maier et al., 2010) and lentiviral (LV) (Naldini, 2011) vectors. Because of their ability to stably integrate in the host genome, the integrating vectors are the best tool for gene transfer into actively proliferating cells as HSCs, potentially allowing a lifelong expression of the corrected gene product in the cells and their progeny. For this reason, ex vivo gene therapy of HSCs with RV and LV vectors followed by reinfusion in the patient is a common strategy that is currently applied in many clinical trials for various diseases that benefit from gene corrected HSC progeny (Naldini, 2015).

#### 1.1.4.1 Integrating Vectors gene therapy

RV and LV derive from two different species of *Retroviridae*, the Moloney Murine Leukaemia Virus (MLV) and the Human Immunodeficiency Virus 1 (HIV-1), which belong to *Gammaretrovirus* and *Lentivirus* genera, respectively. Viruses that belong to the family of *Retroviridae* are characterised by an envelope that surrounds a truncated cone-shaped capsid. Inside this protein structure there are two copies of the retroviral genome, which consists of a linear positively orientated single-stranded RNA (ssRNA). The dimension of the genome is variable depending on the genus and the species of the retrovirus, and it ranges from 7 to 10 kilobases (kb). The life-cycle is shared among *Retroviridae* family members. The binding between envelope glycoproteins and cellular receptors is fundamental for viral entry and, after the uncoating, the genetic information is completely reverse-transcribed from ssRNA to double-stranded DNA (dsDNA), which semi-randomly integrates into the host genome (Lewinski and Bushman, 2005). Finally, the infected cell releases new virions, which will spread in the host organism. The principle for recombinant viral vector construction is the physical segregation of *cis*- and *trans*-acting genetic elements in different constructs (plasmids) during vector production in packaging

cells. On one side, packaging constructs contain the *trans*-acting sequences of the original viral genome encoding for proteins involved in virion assembly and transfer of genetic material. On the other side, the engineered vector genome (transfer construct) contains the therapeutic transgene expression cassette together with the *cis*-acting elements from the original virus, that regulate genome reverse transcription, dimerization and packaging as well as pre- integration complex (PIC) nuclear translocation (in lentiviruses) and provirus integration. Thus, splitting cis- and trans-acting sequences allows the generation of replication-defective viral vector particles containing the transfer construct-derived viral genome which is the only one that can be encapsidated thanks to its packaging signal. Moreover, all the viral genes coding for pathogenic or dispensable proteins are deleted from the viral genome. Packaging constructs and vector genome transfer construct can be provided by transient plasmid DNA transfection or stably expressed in the producer cells (Kay, 2011).

To better understand this principle, in figure 1.2 are reported the different DNA constructs used for the LV production in packaging cells. LV have been derived from several different lentiviruses but the most widely used are derived from HIV-1. LV are hybrid particles, composed of HIV-1 enzymatic and structural capsid proteins, encoded by *gag* and *pol* genes, and the envelope proteins derived from a heterologous virus (pseudotype). The development of these constructs has followed three prototypic stages, referred to as generations. In the first generation (not represented), all the viral genes were still present (except the envelope gene, *env*), while the 5' and 3' LTR (long terminal repeat) regions were already substituted by the strong cytomegalovirus (CMV) promoter and the polyadenylation signal (polyA), respectively. The packaging signal was absent in order to avoid the encapsidation of the packaging construct in the viral vector (Naldini, Blomer et al., 1996). In the second generation of packaging construct (not represented), all the accessory genes (*vif*, *vpr*, *vpu*, *nef*) were deleted, however the regulatory genes *tat* and *rev* were still present. In the third generation of packaging construct, currently

used (A.1), *tat* is not present anymore and *rev* has been transferred into a different plasmid (A.2), under the control of rous sarcoma virus (RSV) promoter. More in depth, the genes *Gag*, *pro* and *pol* contained into the packaging construct encode for structural and enzymatic proteins; the Rev-responsive element (RRE) is bound by *rev* during RNA transcription in packaging cells to export in the cytoplasm full-length transcripts and reduce splicing. All the plasmids were created without sequence overlap or sequence homology, and this is necessary to eliminate the risk of DNA recombination between plasmids, which might result in replication competent retrovirus (RCR) production. Indeed, RVs or LVs produced in packaging cells must transduce target cells without inducing them to produce viral progeny. The envelope construct (B) encodes for envelope glycoproteins, which are often derived from the vesicular stomatitis virus (VSV) in order to broaden LV tropism. The transfer vector is the only construct whose RNA has to be encapsidated into the viral particle. This construct encodes for the transgene of interest, controlled by a custom promoter. Even if the wild type (wt) LTR transfer vector (C.1) contains intact LTR regions (included the U3 promoter/enhancer region), the expression of the transgene would not be possible in target cells without the presence of *tat* protein. Moreover, in all the types of transfer vectors, the packaging signal and the RRE are flanked by a couple of SD-SA sites: this configuration allows full length RNA transcripts encapsidation in packaging cells because of the expression of *rev* protein, whereas the probability of this dramatically decreases in target cells, deficient for *rev* protein.

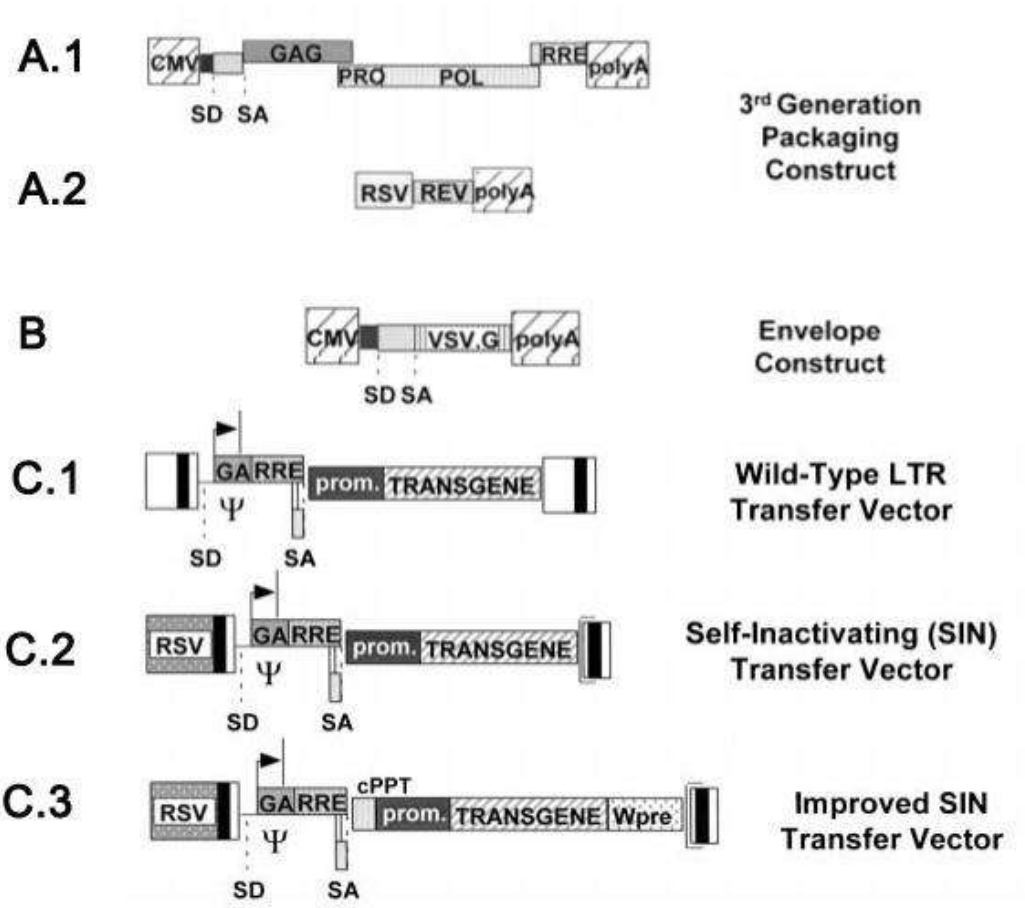



Figure 1.2 Represented above are the different DNA constructs that are used for LV production in packaging cells (adapted from Vigna and Naldini, 2000).

Furthermore, since the U3 LTR promoter activity might be one of the triggers of insertional oncogenesis process, the self-inactivating transfer vector (SIN) carries a large deletion in U3 of the 3' LTR region, and the U3 of the 5' LTR is substituted by a strong promoter for *Tat*-independent RNA transcription in packaging cells (C.2). Upon reverse transcription in target cells, the large deletion in the 3' LTR is copied at the 5' end, thus abolishing any HIV-derived promoter/enhancer activity. The improved version of SIN transfer vector includes also some additional elements, such as the central polypurine tract (cPPT) and the Woodchuck Hepatitis Virus (WHP) Post- transcriptional Regulatory Element (WPRE) to improve nuclear import performance and transgene expression, respectively (Vigna and Naldini, 2000).

RV vectors developed in the 1980s and early 1990s were the first to be shown to deliver genes into repopulating HSCs (Rivière et al, 1995; Williams et al,1984). However, despite its acceptable level of infectivity, this type of vector is not able to transduce non-dividing cells, thus constraining its employability in many contexts. Moreover, cases of RV silencing have been reported in different type of cells (Palmer et al., 1991; Challita 1994; Okita et al., 2007), whereas LVs do not seem to be susceptible to this epigenetic effect. Some of the elements that induce RV silencing in embryonic stem cells have been recently reported, such as the zinc finger protein Yin Yang 1 (YY1) and the Transcription intermediary factor 1-β (TIF1B) (Schlesinger et al., 2013). Furthermore, the sequence of the primer binding site (PBS) and the cellular protein binding sites in LTR regions seem stringently correlated with epigenetic switch-off through DNA methylation of RVs (Wolf and Goff, 2009). LV offer several advantages for gene therapy applications. Unlike RV, LV exploit active cellular mechanisms for the nuclear import of the viral genome, thus they can transduce a variety of cell types, including dividing and non-dividing cells, such as quiescent stem cells, hepatocytes and neurons. As for RV, integration of the LV into the host cell chromatin allows stable gene transfer to the target cell and its progeny. LV can carry larger and more complex gene cassettes than RV (LV can package up to 9 kb of genetic material) and transgene expression is unlikely to undergo transcriptional silencing. Importantly, since most of the human population is seronegative, LV can be injected systemically without the risk of pre-existing immunity against vector components (Blomer et al, 1997; May et al, 2000; Vigna & Naldini, 2000). However, LV technology has limitations as well. For instance, vector production still mostly relies on plasmid transient transfection because of the lack of widely used stable packaging cell lines and this represents a major hurdle for the industrial scaling-up of vector manufacturing. Although reassuring results have been reported in patients treated by LV-mediated HSPC gene therapy, the integrating nature of LV still imposes careful risk-benefit assessment for each therapeutic application. Moreover, concerning *in vivo* LV administration, the acute

toxicity profile, the risk of inducing anti-transgene immune responses and germ-line transmission need to be carefully evaluated.

Both RV and LV were used and are currently used in a large amount of HSC gene therapy clinical trials, writing a story of ups and downs. Reporting some examples, when patients with X-linked severe combined immunodeficiency disorder (SCID-X1) were infused with CD34+ cells treated with a RV that possessed intact long terminal repeats (LTRs) and expressed IL2RG, it resulted in initial success with SCID-X1 long-lasting correction and functional and polyclonal T cell recovery in 19/20 patients (Fisher et al., 2010). Unfortunately, in the long-term follow-up 5 patients developed leukaemia and one died as a result: insertional mutagenesis caused by semi-random integration of the vector was the cause of the leukaemia (Hacein- Bey-Abina, 2008). In fact, molecular analysis of malignant cells has revealed a vector- mediated transactivation of the oncogene LIM domain only-2 (*LMO-2*) or cyclin D2 (*CCND2*), due to the enhancer activity of U3 LTR region upon RV integration nearby their promoters (Hacein-Bey-Abina et al., 2003). Other clinical trials, despite their greater or lesser efficacy, showed the same unexpected hurdle. In the more recent clinical trials for CGD, based on the infusion of a RV transduced autologous CD34+ cells (Kang et al., 2010), long-term enfgraftment was difficult to achieve, probabily due to vector silencing, immune clearance of corrected cells or excessive production of ROS in stem and progenitor cells, and myelodysplasia with fatal outcome and clonal expansion were observed in 4 patients, caused by the occurence of insertional mutagenesis near oncogene promoters (Ott et al., 2006). Going further, ten Wiskott-Aldrich syndrome- patients received an infusion of autologous CD34+ cells transduced with a LTR-driven RV (Boztug et al., 2010; Braun et al., 2014), and despite stable engraftment of corrected cells and significant clinical benefit, molecular analyses highlighted that the corrective RV preferably integrated nearby proto-oncogenes (such as *LMO-2*), and 7 out of 10 patients developed leukemia as a serious adverse event associated with RV-gene therapy.

The occurrence of severe adverse events in HSC gene therapy clinical trials shows the necessity of further investigating on the major drawbacks of gene replacement approaches to fulfil the promise of safe and effective gene therapy. Given all these data, it is clear that currently one of the two major challenges in gene therapy is insertional mutagenesis. The second is represented by the non-physiological expression of the transgene, particularly relevant in the context of X-linked Hyper-IgM syndrome, as I will discuss later on.

#### 1.1.4.2 Insertional mutagenesis and genotoxicity

Insertional mutagenesis is an event where bases are added into the DNA, effectively mutating it and is considered a major hurdle in gene therapy with integrating viral vectors. These integrations don't necessary damage the genetic information causing mutations that can lead to cancer development: integration in an intron (not encoding for a mRNA) or in an untrascribed region is not genotoxic. However, as already mentioned, both RV and LV integrate their reverse transcribed genome in the host DNA in a non-specific semi-random manner (Bushman et al., 2005). Many groups identified some different preferred patterns of integration between RV and LV (Cattoglio et al., 2010; Cattoglio et al., 2010; Biasco et al., 2011). RV vectors have shown a tendency to integrate near genomic regions that play a role in the transcriptional regulation by RNA polymerase II, as promoters, DNase-I hypersensitive sites of transcribed genes, transcription factor binding sites, methylated CpG islands, nucleosome-wrapped DNA. On the contrary, LV vectors prefer to integrate in the body of the gene, after the transcription start site (TSS) (Bushman et al., 2005; Roth et al., 2011; Cavazza et al., 2013). In addition, epigenetic analyses suggest that RV integration sites are often in proximity of histone modifications, such as H3K4me1, H3K4me3, H3K27ac (Cavazza et al., 2013; Figure 1.3). The mechanism that is responsible of the different patterns of integration between RV and LV is not fully understood, but the viral integrase seems to play an important role (Lewinski et al., 2006; Felice et al., 2009).

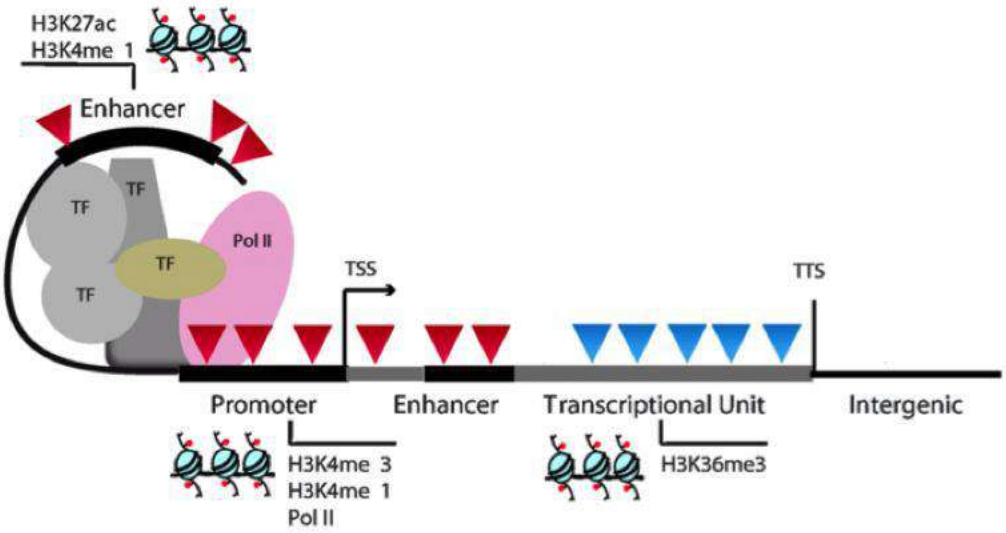



**Figure 1.3** Integration of RV and LV vectors into the genome. RV and LV vectors prefer different integration sites, MLV- derived RV vectors (red triangles) integrate near regulatory regions, while HIV-derived LV vectors (blue triangles) integrate in the transcribed gene away from regulatory elements. TSS = transcriptional start site, TTS = transcriptional termination site, TF = transcription factor and Pol II = RNA polymerase II (Adapted from Cavazza et al., 2013).

Given those observations, RV and LV do not have the same safety profile in terms of genotoxicity. In the latest years, many groups tried to make a comparison between RV and LV mediated oncogenicity (Montini et al., 2006; Zychlinski et al., 2008; Arumugam et al., 2009; Modlich et al., 2009; Montini et al., 2009). As an example, Montini's group demonstrated the crucial contribution of viral integration site selection (ISS) and vector design to oncogenesis. Exploiting a tumor-prone mouse model of HSC therapy they showed that insertions of retroviral vectors at oncogenes and cell-cycle genes were enriched in early-onset tumors, indicating cooperation in tumorigenesis. In contrast, tumorigenesis was unaffected by lentiviral vectors and did not enrich for specific integrants (Montini et al., 2006). In addition, they also demonstrated that transcriptionally active long terminal repeats (LTRs) are major determinants of genotoxicity even when reconstituted in LVs and that self-inactivating (SIN) LTRs enhance the safety of γRVs. Furthermore, by comparing the genotoxicity of vectors with matched active LTRs, they observed preferential targeting of cancer genes by γRVs (Montini et al., 2009).

In the same years, Baum and his group developed an *in vitro* immortalization assay (IVIM) to measure the transforming potential of SIN and non-SIN vectors in murine BM Lin- cells. With this assay SIN RVs were demonstrated to be significantly less genotoxic when compared with corresponding LTR-driven RVs (Modlich et al., 2006). Taken together, these data demonstrate that SIN LVs outperform SIN and non-SIN RVs in terms of safety.

Further investigations highlighted which mechanisms may be involved in triggering insertional mutagenesis genotoxicity (Figure 1.4 A). As already presented, in case of LTR- driven (non-SIN) vector integration, the enhancer activity of the LTR U3 regions may transactivate proto-oncogenes, whose promoters can be located near the integration site of the vector (Figure 1.6 B). Initially this situation was thought to be extremely rare, however a similar event of transactivation was probably the primary cause of adverse events in SCID-X1 and CGD clinical trials. In the first case, patients who developed acute lymphoblastic leukaemia after treatment showed a vector integration into LMO-2 proto- oncogene locus and an aberrant expression of the gene in the expanded monoclonal T cell population, caused by the enhancer-mediated effect of the LTR U3 region (Hacein-Bey- Abina et al., 2003). Similarly, the four cases of myelodysplasia with fatal outcome reported in the CGD clinical trial showed a non-SIN RV vector integration near different proto-oncogenes, such as *MDS-EVI1*, *PRDM16* and *SETBP1* (Ott et al., 2006). Using LV instead of RV and SIN vectors instead of non-SIN vectors may partially alleviate this type of events. However, also other mechanisms can trigger insertional mutagenesis genotoxicity. For example, it has been shown that the presence within the vector of strong enhancers/promoters can activate genes located near to the integration site (Modlich, Navarro et al., 2009). In addition, it would be advisable to avoid strong promoters/enhancers for transgene expression and to evaluate the insertion of an insulator into the LTR regions (Cesana et al., 2014). Another issue could be the presence, even if U3-enhancer sequences are deleted in SIN vectors, of both canonical and cryptic polyA sequences in the R unit of LTR regions. These

sites may induce the production of aberrant 3'- or 5'- truncated messenger RNAs (mRNAs) and, as a consequence, truncated proteins (Figure 1.4 B). 3'-truncated mRNAs are transcribed starting from an endogenous promoter close to the integration site and are prematurely terminated as soon as the RNA polymerase encounters an LTR polyA signal. 5'-truncated mRNAs start from the viral or transgene promoter and the readthrough goes on until the RNA polymerase reaches an endogenous polyA. Nonsense-mediated RNA decay or aberrant protein production may induce tumorigenesis (Uren et al., 2005; Almarza et al., 2011). Similarly, the presence of cryptic splicing sites or aberrant splicing events may lead to mutant proteins with enhanced or reduced activity, which can trigger the tumorigenesis process (Cesana et al., 2012; Moiani et al., 2012). Moreover, insertional mutagenesis might be triggered in case of vector- mediated disruption of some important genomic elements, as miRNA targets and promoters. The relevance of these mechanisms has been demonstrated after the recent results in LV-based–β-thalassemia gene therapy clinical trial (Cavazzana-Calvo et al., 2010). Integration site distribution analyses of the SIN LV showed dominance over time of integration in the intron 3 of *HMGA2* gene. Molecular analyses showed that the predominant form of *HMGA2* mRNA was aberrant: it was truncated by alternative splicing with a cryptic splicing acceptor located in the LTR region and it was polyadenylated within the adjacent R region. This process leads to the loss of let-7 microRNA (miRNA) target sites at the 3' end of the transcript and it resulted in an excess of *HMGA2* RNA. Further evaluation and long-term follow up are needed to assess if this clone might influence haematopoiesis or prelude to multistep leukaemogenesis (Cavazzana-Calvo et al., 2010).

Taken together, these data suggest that even if SIN LV has a safer genotoxic profile when compared with RV, it is important to take into consideration that many of the mechanisms that can trigger insertional mutagenesis are not limited to LTR-driven vectors. As a consequence, both RV and LV genotoxicity must remain strictly monitored during long- term follow-up in HSC gene therapy clinical trials. Notably,

the consequences of insertional mutagenesis and subsequent dysregulated gene expression do not have to be as detrimental as before mentioned. In fact, results can be different depending on cell type and inidivudal genetics. Dysregulation of genes in T-cells, as an example, resulted in a loss of T-cells instead of a leukemic clonal expansion that occurred in HSCs (Recchia et al., 2006).

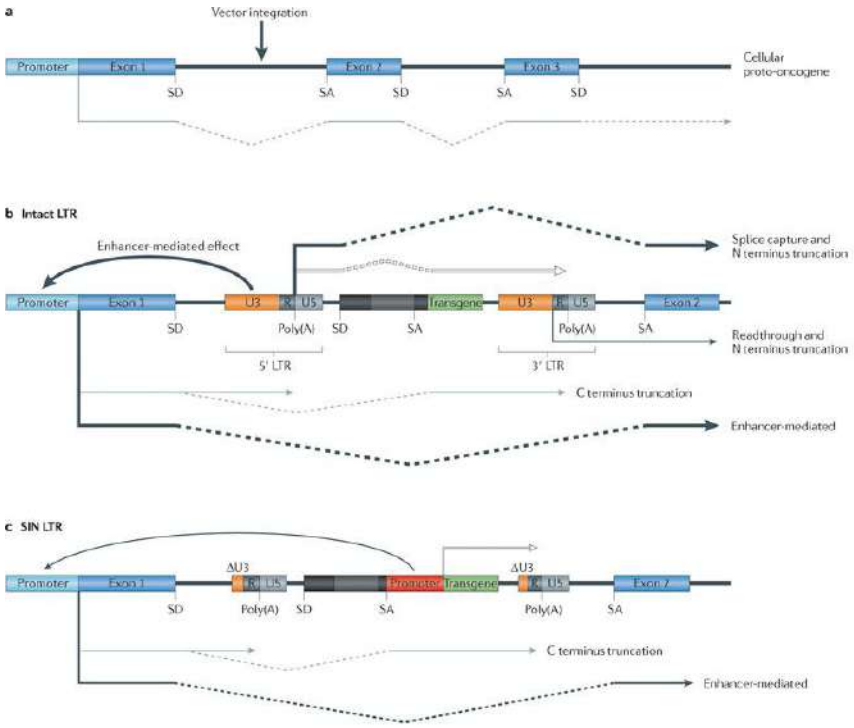



**Figure 1.4** Certain genotoxic events could lead to activation of the transforming potential of a cellular proto- oncogene after integration of a retroviral vector within or near the proto- oncogene. **A**) A vector integration site is represented in the first intron of a proto-oncogene. **B**) A conventional RV is integrated. The grey arrow below the gene indicates the normal transcript, while the broken segments indicate intronic sequences that are removed by splicing. The two LTRs contain strong enhancers and promoter elements in the U3 region; the R and U5 regions are also indicated. The white arrow indicates the vector transcript, which encodes the transgene. The integration of the vector, through enhancer-mediated effects on the proto-onogene promoter, may lead to an up-regulated transcription of this gene. Alternatively, splice capture from the promoter in the vector 5' LTR could give rise to a chimeric transcript encoding an N-terminally truncated form of the oncogene with constitutive activity and transforming potential. Another possible mechanism leading to an N- terminally truncated form of the oncogene is readthrough transcription originating from the vector 3' LTR. This event is less probable when the vector encodes a transgene, because of promoter interference (occlusion) between the two LTRs. A C-terminally truncated form of the oncogene may be produced by the termination of the trancription at the polyA sites contained in the LTR, thus possibly having constitutive activity and transforming potential. This event can occur both with and

without aberrant splicing between the cellular and the vector splice sites. **C**) All LTR-dependent events just presented are abrogated by the use of a SIN vector, thus deleting the U3 transcriptional control elements (ΔU3) from the LTRs and expressing the transgene from an internal promoter. A residual trouble could be long-range transcriptional activation of the oncogene caused by enhancer elements comprised into the internal promoter. However, this concern can be alleviated using an exogenous promoter with only moderate activity. (Naldini, 2011).

#### 1.1.4.3 Regulation of transgene expression

Expression of the incorporated transgene, in some cases, must be tightly regulated to achieve stable or physiological levels of expression. Factors that considerably influence transgene expression are vector type, design and integration site (Naldini, 2011). RV vectors, as already mentioned, are more prone to epigenetically silencing than LV vectors, which have more robust transgene expression (Ellis, 2005; Naldini, 2011). Moreover, the integration site significantly influences the expression of the transgene. If the vector integrates in a heterochromatin region, the expression may be switched off. On the contrary, euchromatin region should ensure a stable gene expression. However, change in the chromatin state during HSCs differentiation can compromise an efficient transgene expression and make its regulation difficult. Depending on tolerability of transgene expression and therapeutic needs, strong promoters such as viral LTR or PGK (promoter of an "housekeeping gene") can be used to regulate transgene expression to reach a stable and a quite strong expression. However, for some diseases a regulated expression of the transgene is a necessary. β-thalassemia is an example: high expression of β-globin must be uniquely limited to erythroid compartment due to β-globin toxicity (Bank et al., 2005). As a consequence, a strong promoter can't provide this kind of regulation, thus in β-thalassemia gene therapy clinical trial, HSPCs have been transduced with a SIN-LV encoding for the β-globin transgene under the control of the locus control region (LCR). LCR is a genomic element positioned 60 kb upstream of the human-β globin gene which allow to express sufficient levels of the transgene in the erythroid lineage (May et al., 2000).

Another approach that aims to restrict transgene expression to specific compartments is based on microRNA (miRNA) exploitation (Brown et al., 2006; Brown and Naldini, 2009). In some context, ectopic expression of the transgene in HSPCs is toxic or alters cellular behaviour, for example leading to a disregulation of cell growth. By adding the target sequence for a miRNA differentially expressed in HSPCs within the 3' UTR of the transgene, its expression could be suppressed specifically in the primitive cells that express that miRNA (Gentner et al., 2010). However, the introduction of elements that provide a semi-physiological expression of the transgene is not always possible, due to the complexity or the size of the regulatory region. Another example of a disease in which transgene expression is required to be physiological to avoid adverse events is the case of the hyper-immunoglobulin M immunodeficiency (HIGM). HIGM is a disease where, in most cases, a genetic mutation in the CD40 ligand gene (*CD40LG)* results in the inability of T-cells to activate B-cells. Pre- clinical studies in mice showed that a constitutive or unregulated expression of *CD40LG* resulted in lymphoproliferations that progressed into lymphomas (Brown et al., 1998; Sacco et al., 2000). To achieve physiological expression of the transgene, all its regulatory elements must be incorporated into a viral vector, which is impossible due to size constraints. Thus, creating a potential cure for HIGM with conventional gene therapy is highly unlikely.

Concluding, insertional mutagenesis by semi-randomly integrating vectors and non- physiological gene expression are the two major challenges in gene therapy. However, new strategies that involve artificial nucleases to integrate corrective genes into precise locations within the genome are rapidly finding ground and look promising in overcoming these two major hurdles in conventional gene therapy.

### 1.1.4.4 AAV-mediated gene therapy

AAV belongs to *Parvoviridae* family and it is reported the existence of different serotypes.

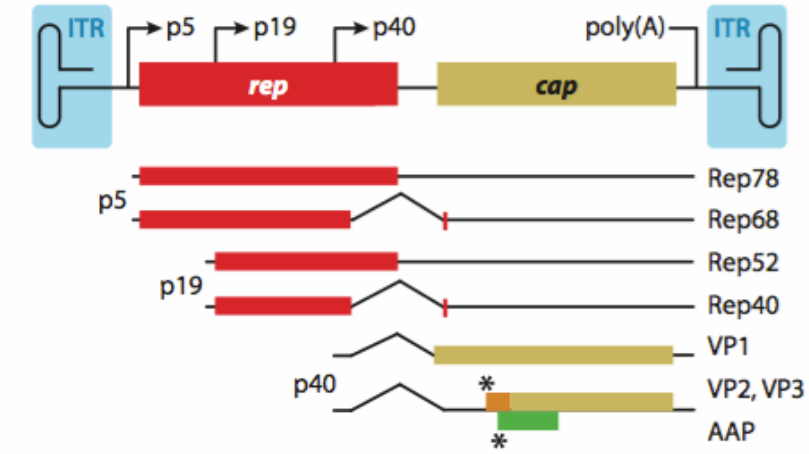



**Figure 1.5** AAV genetic map. AAV genome contains three open reading frames. The *rep* orf is reported in red and it codes for Rep78, Rep68, Rep52, and Rep40. This proteins are synthesized starting from the p5 and p19 promoters. The p40 promoter allows to produce three capsid proteins from the *cap* orf, reported in *yellow*. The ratio of VP1, VP2, and VP3 in the capsid is approximately 1:1:10. The spliced mRNA that codes for VP3 from a conventional AUG start codon also codes for the VP2 protein, which has the addition of N-terminal residues (*orange*), from a weak upstream ACG start codon, indicated with *. Moreover, VP2/VP3 mRNA codes also for an assembly-activating protein (AAP), reported in green, from a weak CTG start codon, indicated with *. AAP facilitates the assembly and the maturation of the capsid, and it is not present in the mature capsid. In the figure are also reported the ITRs, not in scale. (Samulski et al., 2014)

AAV is a small, non-enveloped virus that packages a single-stranded linear DNA genome, approximately 5 kb long (Berns et al., 2007; Srivastava et al., 1983), which could be both positively and negatively oriented. Infection could be initiated with particles containing either strand. AAV genome contains three open reading frames (orfs) that code for functional proteins. The *rep* orf codes for four Rep proteins (Rep78, Rep68, Rep52, and Rep40) that are synthesized from mRNAs initiated from the p5 and p19 promoters. The two larger proteins (Rep78 and Rep68) have site-specific, single-strand endonuclease, DNA helicase, and ATPase activities that are required for AAV DNA replication. The two smaller Rep proteins (Rep52 and

Rep40) are required for packaging DNA into capsids. The p40 promoter initiates an mRNA that is alternatively spliced to make three capsid proteins from the *cap* orf. The coding regions of AAV are flanked by inverted terminal repeats (ITRs) that are 145 bases long and have a complex T-shaped structure. These repeats are the origins for DNA replication and serve as the primary packaging signal. ITRs are the only cis-active sequences required for making AAV vectors and the only AAV-encoded sequences present in AAV vectors, while cap e rep sequences can be delivered in trans (Figure 1.5) (Samulski et al., 2014).

AAV DNA is replicated by the so-called rolling hairpin mechanism, and this process has been completely reconstituted *in vitro* with purified components (Berns et al., 2007; Nash et al., 2008). Moreover, to establish a productive viral infection, AAV requires a co- infection with a helper virus, and this feature surely helps preventing inappropriate spread of AAV following clinical application. Adenovirus, but also baculovirus and herpesvirus, can supply complete helper activity in cell culture. Briefly, the Adenovirus (AdV) helper functions have been identified as AdV E1a, E1b, E4 orf6, DBP (E2A), and VA (viral associated) RNA. Both E1a and DBP act as transcriptional activators that induce the AAV p5 promoter (Chang et al., 1990; Shi et al., 1991; Chang et al., 1989). E1a also induces S phase in host cells (Berk et al, 2007), which is needed to increase the level of the cellular DNA replication enzymes needed for DNA replication. Instead, E1b, E4 orf6, and VA RNA, perform various tasks including promoting second-strand synthesis of AAV; inhibiting p53- induced apoptosis; preventing entry into mitosis; shutting off host cell translation; promoting AAV mRNA transport to the cytoplasm; and inhibiting the interferon-induced double-stranded RNA–activated protein kinase R (DAI/PKR) (Samulski et al., 2014) (Figure 1.7).

Gene therapy vectors using AAV can infect both dividing and quiescent cells and persist in an extrachromosomal state without integrating into the genome of the host cell, although in the native virus some integration of virally carried genes into the host genome might occur.

As already mentioned for integrating vectors, the principle of turning these viruses into gene delivery systems is based on the spatial segregation of essential sequences and genes in different plasmids during vector production in packaging cells. The first production system of the current AAV vectors was based on a two-plasmids system, a pAAV containing the transgene and a plasmid containing the rep-cap genes, in which overlapping AAV sequences were totally eliminated, thus preventing the risk of wild type AAV generation in the recombinant virus batch. At that time, the exact set of AdV genes needed to provide complete auxiliary functions were not clearly identified. Therefore, the first method of AAV production was based on double transfection of target cells with the transgene plasmid and a *rep*/*cap* plasmid expressing *rep* and *cap*, followed by an infection step few hours later with wild type AdV at low Multiplicity of Infection, MOI. However, this method required extensive purification and heat inactivation, in order to eliminate the wild type AdV, which represented a serious problem of safety. The replacement of the wild type AdV with a replication defective AdV had partially solved the safety problem. The most important step was achieved in the late 1990s by replacing the infection step with the transfection of a plasmid encoding the complementary adenoviral helper functions E2, E4 and VA. This led to the establishment of a tri-transfection method, which is the standard approach for AAV-mediated gene transfer that is used today.

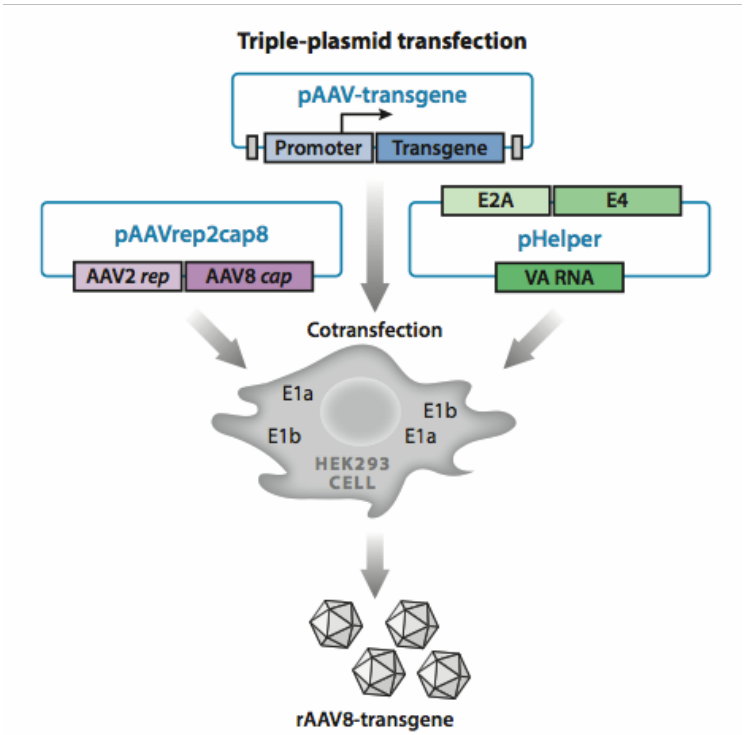



**Figure 1.6** AAV standard production method (Samulski et al., 2014)

HEK293 cells expressing adenovirus E1a and E1b are co-transfected with an adenovirus helper plasmid (pHelper), a *rep*/*cap* plasmid expressing *rep* and *cap* (of different serotypes of interest), and the transgene plasmid carrying the AAV-transgene cassette (pAAV- transgene), containing only the ITRs (Figure 1.6) (Samulski et al., 2014). This allowed the total elimination of adenoviral particles in the process of production (Merten et al., 2005).

In the last decade, AAV have become the vectors of choice for *in vivo* gene therapy. For example, in clinical trials for familial lipoprotein lipase (LPL) deficiency, an AAV1-based vector encoding a gain-of-function LPL variant resulted in persistent gene expression and protein activity, thus allowing a sustained decrease in the incidence of pancreatitis (Stroes et al., 2008). Based on the outcomes and the safety

profile, this product called Glybera, received marketing approval in Europe in 2012, and it represents the first approved gene therapy in Western nations. Other monogenic disorders in which AAV vectors demonstrated safety and efficacy include Leber's congenital amaurosis type 2 (Hauswirth et al., 2008; Bainbridge et al., 2008; Maguire et al., 2008; Maguire et al., 2009; Jacobson et al., 2012; Bennet et al., 2012), choroideremia (MacLaren et al., 2014) and haemophilia B (Nathwani et al., 2011). Currently the most convincing pre-clinical and clinical results in liver-directed gene therapy have been achieved using these vehicles (George et al, 2017; Mount et al, 2002; Nakai et al, 1998; Nathwani et al, 2011).

There are several advantages to using AAV vectors in gene therapy. First of all, they derive from non-pathogenic viruses and are weakly immunogenic (Cao et al, 2007; Dobrzynski et al, 2004; Mingozzi et al, 2003). They are "adeno-associated" so they need a co-infection with an adenovirus or a herpes virus for a productive replication, thus they do not express viral proteins and can efficiently transduce both dividing and quiescent cells. As for AdV, a number of viral serotypes have been isolated from different species, which show different tissue-tropism and transduction properties. The availability of different serotypes allows choosing the best one for each gene therapy strategy, but on the other hand the wide spread in the population of these viruses increases the likelihood of finding patients already immunized against the chosen serotype, or in the worst case against a conserved epitope of the virus (Zaiss & Muruve, 2008). Indeed, a large portion of the population presents neutralizing antibodies specific to capsids and this markedly limits gene delivery by many natural vectors (population with antibodies against AAV1 serotype: 67%, AAV2: 75%, AAV5: 40%, AAV6: 46%, AAV8: 38%, AAV9: 47%). Moreover, following cellular transduction, AAV capsid epitopes can become cross-presented on MHC class I molecules, which leads to the elimination of corrected cells by specific cytotoxic T cells and thus to the loss of transgene expression. Of note, the decline of coagulation factor IX expression reported in an early clinical trial for hemophlia B. Furthermore, many epitopes for AAV2 and

AAV8 have been identified, and they are among the most polymorphic ones, thus making difficult to engineer an AAV capsid that could evade recognition (Kotterman et al., 2014). Another important limitation, besides pre-existing immunity, is the small size of their genome. Vectors based on AAVs can package genomes up to 5 kb at near wild type titers and infectivity, beyond which packaging efficiency significantly decreases, and genomes with 5' truncation become encapsidated. Thus, this strategy can't be applied when the transgene of interest is longer than 5 kb (Kotterman et al., 2014).

## 1.2 Gene therapy in T cells

### 1.2.1 T cells biology

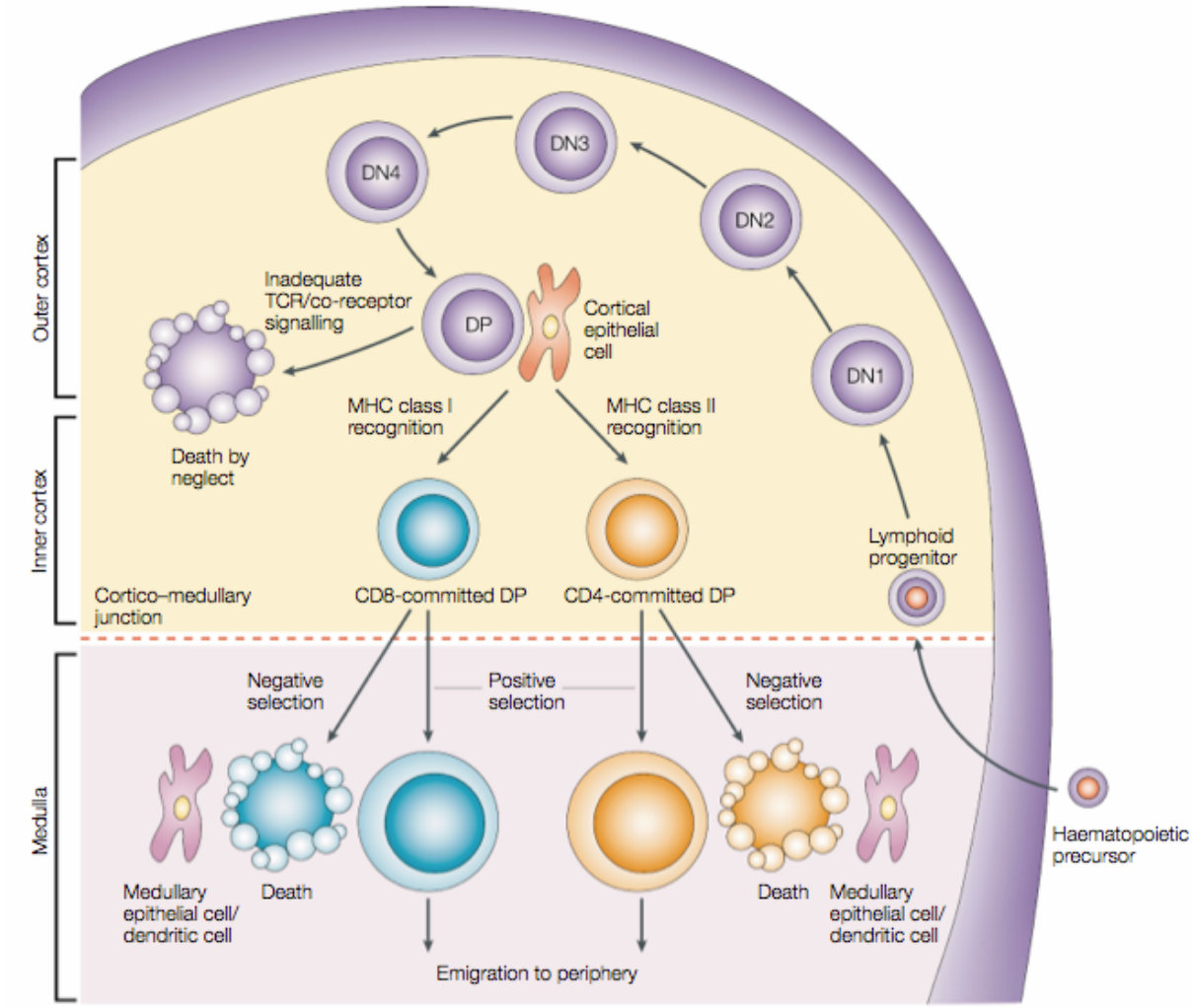



**Figure 1.7 T-cell development in the thymus. DN= double negative; DP= double positive (Germain et al., 2002).**

Committed lymphoid progenitors originate in the bone marrow from hematopoietic stem cells and migrate through the blood to get to the thymus. In the thymus, these cells lose the potential to develop B-cell or natural-killer-cell, thus resulting in a double- negative (DN; no CD4 or CD8) committed T-cell precursor. In mouse DN thymocytes can be subdivided further into four sequential stages of differentiation, based on the presence of two surface markers: DN1, $CD44^{+}CD25^{-}$; DN2,

$CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$; and DN4, $CD44^{-}CD25^{-}$. Then, double-negative T cells can form either γδ or αβ-expressing cells. For αβ TCR-expressing cell, DN3-stage cells have on their surface a pre- TCR-α, which is encoded by a non-rearranging locus. Pre-TCR-α pairs with the TCR β-chain, which is the product of some DNA rearrangements. The pre-TCR pair is associated with different proteins, which constitute the CD3/z complex involved in signal. After β-selection (late DN3 and DN4), the cells undergo 6–8 cell divisions, after which DNA recombination at the TCR-α locus produces the second chain composing the mature TCR. The expression of pre-TCR-α is lost during this stage, thus resulting in low levels of surface expression of αβTCR assembled with CD3/z complex. Meanwhile, the thymocytes begin to express co- receptor molecules: most often CD8 first, followed by CD4 forming a large population of double-positive (DP; CD4+CD8+) cells, which constitute 90% of the lymphoid compartment in the thymus of young individuals. Most (~90%) of these DP thymocytes express a TCR which do not interact well with the available self-peptide–MHC ligands on cortical epithelial cells, thus the intracellular signals needed to survive are not generated, and the cells undergo death by neglect. On the contrary, a small fraction of immature T cells (~5%) express a TCR which binds very well to self-ligands, thus likely being able to cause autoimmune pathology left the thymus. Then, a rapid apoptotic death is promoted (negative selection). Cells that express a TCR, which recognizes self-ligands and generates a signal, which is intermediate between the two resulting in neglect or negative selection, initiate a multi-step process known as positive selection, which eventually results in lineage-specific differentiation into either CD4+ or CD8+ mature T cells (Germain et al., 2002) (Figure 1.7). In the end, each T lymphocyte expresses a unique TCR on its surface as a result of maturation and selection in the thymus, forming the so-called naïve T cells. Naïve T cells circulate through blood and the lymphatic system, and inhabit in secondary lymphoid organs. These cells have not been activated yet by the encounter of a foreign antigen. Antigenic peptides can be presented to the naïve T cells in secondary lymphoid organs on MHC molecules on the cell surface of dendritic cells (DC), which can

also provide co-stimulatory signals for effective T cells activation. The variability of αβTCR ensures the presence of at least few naïve T cells with high-affinity for virtually any antigenic peptide. TCR-antigen interaction triggers a cascade of intracellular signalling events, thus activating the naïve T cells. The activated T cells undergo clonal expansion rapidly proliferating, then migrate through the tissues in order to reach the sites where the antigen is present, thus performing different effector functions. Cytotoxic CD8+ T cells are effective in the direct lysis of infected or malignant cells presenting the antigen, while CD4+ T helper cells produce cytokines that can be directly toxic to the target cells, can stimulate other T cell effector functions and can induce inflammatory mechanisms and antibody production (Priniciple of immunopharmacology, 2011, Springer).

Most of the effector T cells disappear after the elimination of the antigenic agent, however some remain forming memory T cells. While naïve T cells live for few months and effector cells disappear at the end of the immune response, memory T cells could survive for years in lymphoid organs and peripheral tissue, thus ensuring a secondary immune response if the same antigen appears again, either through an immediate effector functions in peripheral tissues or an activation and clonal expansion in lymphoid organs. This response is much faster than naïve T cells, so, in the case of infection, the elimination of the pathogens occurs at an early stage, preventing the disease spreading (Priniciple of immunopharmacology, 2011, Springer).

To summarize, in the primary lymphoid organs, the bone marrow and the thymus, T cells haematopoiesis and clonal selection occur. Then, the T-cell dependent immune response begins in the secondary lymphoid organs, which are spleen, lymph nodes, payer's patches, tonsils, bronchial, nasal and gut-associated lymphoid tissues. Here, specialized T cell-rich zones host a concentration of naïve T lymphocytes. Naïve T cells reside in the spleen for just few hours and in the lymph nodes for 24 hours before leaving and migrating in the bloodstream to enter new

lymphoid organs, repeating the cycle until they become activated (Priniciple of immunopharmacology, 2011, Springer).

### 1.2.2 T cells subsets and their phenotypical identification

αβTCR T cells can be subdivided into several groups based on lineage markers and related functional activities. CD4 and CD8 are the two major surface co-receptor molecules, defining two separate T cell lineages with different functions. CD4+ cells recognize the antigen in the context of MHC class II molecules, which are expressed only on the surface of professional antigen presenting cells, such as DC, B cells, and macrophages, and they are called T helper cells. Two further major functional subpopulations of T helper cells can be distinguished on the basis of their cytokine profile. Th1 cells produce mainly IFN-γ, but also IL-2, TNF-α and lymphotoxin and they enhance pro-inflammatory cell-mediated immunity. Th2 cells, instead, produce primarily IL-4, -5, -6, -10 and -13 and promote non-inflammatory immediate immune responses. It has been demonstrated that they are fundamental in B cell production of IgG, IgA, and IgE. Th1 and Th2 polarizations seem to be mutually antagonistic: the nature of the antigen and the surrounding cytokine microenvironment drive CD4+ cells polarization. As an example, IFN- γ and IL-12 has been shown to support Th1 cells, while IL-4 and IL-10 assist Th2 development. However, several recent studies have highlighted more complex patterns of cytokine interaction in different models of immune response. For example, another separate subset of CD4+ cells is the Th17. It was found that IL-23 induces the production of T cells which secrete mainly IL-17 A, a pro-inflammatory cytokine. These cells seem to be the major effectors in some diseases, such as rheumatoid arthritis, psoriasis and Chron's disease. Moreover, they are the first subset generated during infection (Priniciple of immunopharmacology, 2011, Springer).

CD8+ lymphocytes are activated by antigenic peptides presented by MHC class I molecules, which are expressed on all nucleated cells, and form effector cytotoxic T lymphocytes (CTL). CTLs proliferate in the presence of IL-2, expanding their number many thousand-fold at the peak of a primary immune response. Their ability is to destroy more than one target cell containing the antigen. This destruction requires the establishment of cell contact with the target cell and antigen recognition, but not co-stimulatory signals. Two major pathways of cytotoxicity via TCR signaling have been described: i) $Ca^{2+}$-dependent perforin/granzyme-mediated apoptosis, and ii) $Ca^{2+}$-independent Fas ligand/Fas-mediated apoptosis. i) Lytic granules, which consist of secretory lysosomes containing granzymes, perforin and proteoglycan serglycin, are able to be transported in the target cells, inducing caspase-dependent and –independent apoptosis. ii) The presence of Fas ligand on CTL mediates apoptosis in the target cell. Fas molecule is a member of the TNF receptor superfamily, with an intracellular death domain initiating caspase-dependent apoptosis upon Fas-ligand binding (Priniciple of immunopharmacology, 2011, Springer).

Another independent subset of T cells consists of regulatory T cells or Treg, which appears mostly within the CD4+ T cell set, but it is also reported among CD8+ T cells. Treg are critical in the maintenance of peripheral tolerance, in the down-modulation of the immune response and in the prevention of autoimmune diseases. They directly affect Th1, Th2, Th17, CTL and B cell reactions against both self antigens and foreign antigens: immunosuppressive cytokines such as TGF-β, IL-10, IL-35 play an important role in their function (Priniciple of immunopharmacology, 2011, Springer).

T cells can also be subdivided on the basis of their functional status, thus distinguishing naive, effector and memory cells, which express specific cell surface markers. (Priniciple of immunopharmacology, 2011, Springer).

Naïve T cells circulating in the blood, express L-selectin (CD62L), CC chemokine receptor 7 (CCR7) and leukocyte function antigen-1 LFA-1. These molecules are needed for the processes of rolling, adhesion and extravasation of the cells through endothelial venules found in lymphoid tissues in peripheral lymph nodes and mucosal lymphoid organs. Another expressed molecule is CD45RA, an isoform protein member of the protein tyrosine phosphatase (PTP) family (CD45), expressed on all hematopoietic cells. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation (Priniciple of immunopharmacology, 2011, Springer).

Activated effector T cells express CD69, which is a human transmembrane C-Type lectin protein and a very early activation antigen, and CD25 (IL-2RA; the interleukin 2 (IL2) receptor α (IL2RA) and β (IL2RB) chains, together with the common γ chain (IL2RG) constitute the high-affinity IL2 receptor). Other important surface receptors of activated T cells are: CD40 ligand, which interacts with B cells (see below) and with APC through binding to CD40, leading to the up-regulation of CD80 (B7-1) and CD86 (B7-2) on APC; and CD28, which binds to CD80 and CD86 and propagates a co-stimulatory signal, enhancing T cells activation. Tumor necrosis factor (TNF) receptor family molecules OX- 40, CD27, and 4-1BB, also can be found on primary activated T cells, sustaining T cell proliferation and survival upon their binding with the APC (Priniciple of immunopharmacology, 2011, Springer).

The model proposed for the memory includes different cellular subsets. Effector memory T cells (TEM) migrate to inflamed peripheral tissues and display immediate effector function, whereas central memory T cells (TCM) home to T cell areas of secondary lymphoid organs, have little or no effector function, but rapidely proliferate and differentiate to effector cells in response to antigenic stimulation (Sallusto et al., 2004). Recently, a third subset of memory T cells has been reported. *In vitro* and *in vivo* studies led to the identification of a subset of T cells that showed

multiple stem cell-like properties, so-called stem cell memory T (TSCM) cells (Gattinoni et al., 2011). TSCM cells precede TCM cells in differentiation. As a matter of fact, while TSCM cells display greater multipotency, as they are capable of generating all memory subsets, including TCM cells, no other memory subset has been shown to regenerate TSCM cells (Mahkne et al., 2013).

Several markers can be used to distinguish these subpopulations. CD44 is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. It is highly expressed in the central and effector memory subsets. CD45RO and CD45RA are two isoforms of the CD45 family already presented. CD45RA is mainly located on naive T cells and, surprisingly, on TSCM, while CD45RO is located on all memory T cells. CD127 is the interleukin-7 receptor-α subunit, and it is expressed by all the subsets of memory T cells. CD27 and CD28 are two co-stimulatory molecules mainly expressed by naive T cells and some memory T cells, including central memory T cells and TSCM. The same pattern of expression is followed by CD62L, which, as already mentioned, is a L-selectin responsible of different migration processes. CD95 is a surface receptor that has the capacity to mediate apoptosis induction, and it's overexpressed in the TSCM subpopulations. Also the CCR7, a chemokine receptor that interacts with CCL19 and CCL21, is highly expressed on this T cell subset. In summary, TSCM is a relatively rare memory population CD27hi, CD28hi, CD45ROlow, CD45RAhigh, CD95hi, CD122high and CD127high. Central memory T cells are CD44high, CD45ROhigh, CD45RAlow, CD127high, CD62L high, while effector memory T cells show the same profile for CD44 and CD127 expression, but a lower expression of CD62L and CD45RA, showing instead high levels of CD45RO (Rosenblum et al., 2015) (Table 1).

| Memory T cell subset | | Human phenotype |
|---|---|---|
| Conventional T cells | Central memory | $CD44^{hi}$, $CD45RO^{hi}$, $CD45RA^{low}$, $CD127^{hi}$ and express high levels of IL-2 and intermediate levels of IFNγ and TNF |
| | Effector memory | $CD44^{hi}$, $CD45RO^{hi}$, $CD45RA^{low}$, $CD127^{hi}$, L-$selectin^{low}$, express high levels of IFNγ and TNF and express low levels of IL-2 |
| | Stem cell memory | $CD27^{hi}$, $CD28^{hi}$, $CD45RO^{low}$, $CD45RA^{hi}$, $CD95^{hi}$, $CD122^{hi}$, $CD127^{hi}$, L-$selectin^{hi}$, $CCR7^{hi}$ and express low levels of IFNγ and intermediate levels of IL-2 |

**Table 1** representation of T cells surface markers expressed by different memory T cells subpopulation (Rosenblum et al., 2015).

As published by Cieri and collegues, to simplify the phenotypical recognition of these T cell subsets, TSCM cells can be identified as CD62L+CD45RA+, central memory T cells as CD62L+CD45RA-, effector memory T cells as CD62L-CD45RA-, and effector memory RA cells as CD62L-CD45RA+ (Cieri et al., 2013) (Figure 1.8). Following this order, we move from the most primitive cells to the most differentiated cells.

In mice, CD4 and CD8 T cells can be categorized into memory and naïve phenotypes based on CD62L (L-selectin) and CD44 expression with the $CD44^{low}CD62L+$ population considered naïve ($T_N$), $CD44^{high}CD62L+$ population considered central memory ($T_{CM}$), the $CD44^{high}CD62L^{neg}$ population considered effector and/or effector memory ($T_{E/EM}$) and the subset of $CD44^{low}CD62L^{low}$ cells, which has been referred to as an activated effector phenotype (Wiede et al., 2009). It is known that CD4 and CD8 T cells differ in their distribution of these subsets in lymphoid and peripheral organs. While naïve frequencies within CD4 and CD8 populations remain relatively similar, the $CD44^{high}$ population is more central memory skewed in CD8 T cells and effector memory skewed in CD4 T cells in a resting organism (Dutton et al., 1998; Sallusto et al., 2004) [12, 13]. However, in the peripheral organs, tissue resident T cells within both the CD4 and CD8 T cell subsets are predominantly of the effector memory phenotype (Shin et al., 20013) [14].

### 1.2.3 *In vitro* T cells expansion

To fully exploit T cells for a treatment, it is important that the accumulation of effector lymphocytes, able to eliminate antigen-bearing cells, and memory lymphocytes, able to patrol the host for residual or recurrent disease, virtually life-long, remain preserved during the process of genetic manipulation, thus ensuring two complementary and critical functions of T lymphocytes.

*In vitro* expansion of T cells, by performing polyclonal activation with anti-CD3 monoclonal antibody (mAb) followed by culture with high-dose IL-2, induces progressive CD8+ T-cell differentiation towards a late effector state, resulting in phenotypic and functional changes

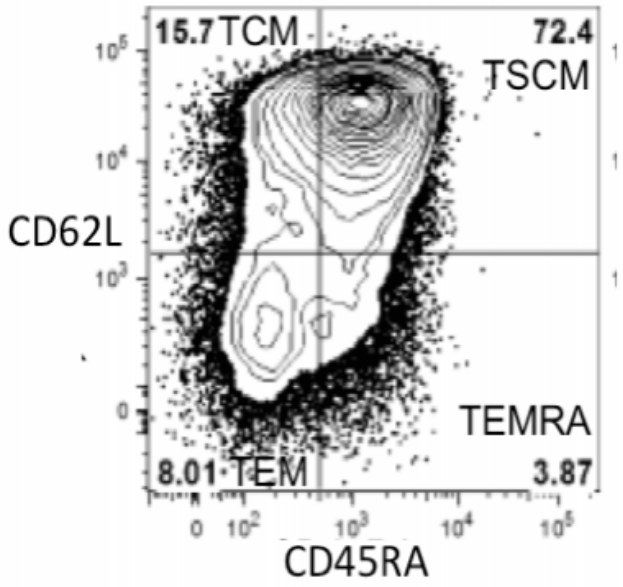



**Figure 1.8** Illustrative example of a flow-cytometry analysis plot, showing the four main memory T cells subpopulations, marked for CD62L and CD45RA, our reference surface markers (Adapted from Cieri, Camisa et al., 2013)

that make T cells less able to benefit from the activating cues present in the host. Therefore, it is necessary to generate large numbers of less-differentiated, TCM-

like cells. Based on the different need for co-stimulation of naïve versus memory and effector cells, co-stimulation associated to TCR triggering could induce naïve cells (TN) to proliferate, rendering these cells susceptible to retroviral transduction. The addition of anti-CD28 mAb generates higher numbers of gene-modified lymphocytes with maintained TCR hypervariable region and homing receptors repertoires (Berger et al., 2003; Coito et al., 2004) that resist apoptosis (Brentjens et al., 2003). Monoclonal antibodies conjugated to cell-sized beads deliver a stronger signal than soluble or plated-bound monoclonal antibodies (Skov et al., 2000), probably because by mimicking APC, they facilitate polarized interaction that are essential to the formation of the immunological synapse, recruiting a wider repertoire of lymphocytes, in particular TN cells. Bondanza and colleagues showed that CD28 co-stimulation through cell-sized beads fosters the generation of gene-modified TCM, whereas stimulation with anti-CD3 alone ends up mainly with TEM (Bondanza et al., 2006). *In vivo*, the maintenance of the pool of TCM lymphocytes depends on the disposal of homeostatic γ-chain cytokines promoting TCM survival and proliferation, even in the absence of antigen (Surh et al., 2006). Among these, IL-7 is involved mainly in TCM survival (Kaech et al., 2003; Kondrack et al., 2003; Schluns et al., 2000) whereas IL-15 induces lymphocyte proliferation (Prlic et al., 2002; Schluns et al., 2002). It was demonstrated that IL-7 and IL-15 are required for the expansion of human alloreactive gene-modified TCM cells able to self-renew, resist activation-induced cell death *in vitro*, and cause GvHD in a fully humanized mouse model. Of interest, gene- modified TCM cells cultured with IL-7 and IL-15 proved as potent as unmanipulated lymphocytes in mediating alloreactivity (Kaneko et al., 2009).

As already presented, more recently it has been described in humans that a self-renewing, stem cell-like memory T-cell subset, TSCM, (Gattinoni et al., 2011), is able to persist long term *in vivo* and is capable of differentiating into effectors upon antigen re-encounter. Cieri and colleagues identified a protocol to differentiate *in vitro*, expand and gene modify in clinically compliant conditions human TSCM

able to expand and differentiate into effectors upon serial transplantation in immunodeficient mice. The phenotype can be induced starting from naïve precursors in response to CD3 and CD28 engagement in the presence of IL-7 and IL-15. While CD28-costimulation and IL-15 have a role in the homeostasis and proliferation of T cells, thus being critical for an optimal expansion of these cells, IL-7 seems uniquely capable of instructing lymphocytes towards the stem-like phenotype. Preservation of a TSCM functional phenotype is critical for the generation of cellular products able to persist and mediate functions in treated patients (Cieri et al., 2013).

### 1.2.4 Adoptive T cell therapy for cancer

Besides to HSPC, that have been one of the earliest target cell type of gene therapy, also genetically engineered T lymphocytes are suitable candidates for the treatment of malignancies, particularly of the hematopoietic system, and for otherwise intractable viral diseases. In fact, T lymphocytes can be easily collected from peripheral blood, expanded and/or activated, engineered *in vitro* and then reinfused back into the patient. In the past few decades, the potency of the immune system in the development and treatment of cancer has been a major focal point of research (Davis et al., 2013). One of the earliest clinical trials of T-cell gene therapy for cancer immunotherapy has been reported in 1995 by Bonini *et al*. (Bordignon et al, 1995). The authors transduced allogeneic T lymphocytes with a viral vector carrying the thymidine kinase gene of the Herpes Simplex virus, namely TK and infused them into patients with relapsing leukemia. This procedure resulted in an improvement in immune-mediated leukemia killing (graft *versus* leukemia), while at the same time allowed controlling GVHD by the administration of Ganciclovir, which led to the death of TK-expressing donor T cells. Although targeted therapy and immunotherapy with immune checkpoint blockade have greatly improved the survival of, amongst others, melanoma and non-small cell lung cancer patients, a

large proportion of patients still develop disease progression upon these therapies (Luke et al., 2017; Mayor et al., 2016). Adoptive cell therapy (ACT) may provide an additional treatment option for these patients. Currently, ACT can be classified into three different types with each their own mechanism of action:

i. ACT with the naturally occurring tumor-infiltrating lymphocytes (TIL), which consists of ex vivo expansion of TIL from resected tumor material and adoptive transfer into the patient following a lymphodepleting regimen and subsequent support of interleukin-2 (IL-2). With this regimen, tumor responses of around 50% have been achieved in patients with metastatic melanoma in several phase I/II clinical trials (Rosenberg et al., 2011; Andersen et al., 2016; Besser et al., 2013).

ii. ACT using T cell receptor (TCR) gene therapy, in which peripheral blood T cells are isolated and genetically modified in vitro to express TCRs that target specific tumor antigens. Trials for melanoma and sarcoma patients pointed out significant and prolonged tumor regression, using genetically modified TCRs specific for MART1, melanoma-associated antigen 3 (MAGE-A3), glycoprotein 100 (gp100) and cancer testes antigen (NYESO-1) (Johnson et al., 2009; Robbins et al., 2011; Morgan et al., 2013; Morrison, 2014).

iii. ACT with chimeric antigen receptor (CAR) modified T cells (June et al., 2015) (Figure 1.9), which holds the capacity of the same effector function as TCR-modified T cells, but independently of MHC expression (Gross et al., 1989). For the recognition by the modified TCR indeed, antigen presentation via the major histocompatibility complex (MHC) is required. However, it is well known that many cancer types can escape T cell-mediated immune responses by downregulation or loss of their MHC expression (Garrido et al., 2017).

Fig. 1.10 shows an overview of the process for adoptive cell therapy with TIL, TCR gene therapy and CAR-modified T cells.

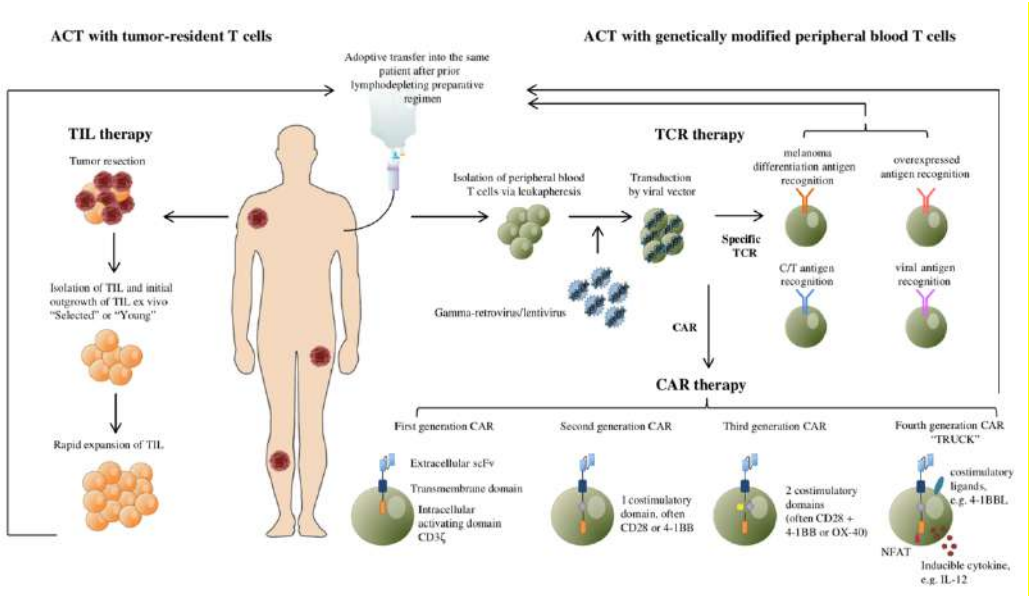



Fig. 1.9 Schematic overview of the processes for adoptive cell therapy (ACT) of tumor-infiltrating lymphocytes (TIL), ACT with T cell receptor (TCR) gene therapy and ACT with chimeric antigen receptor (CAR)- modified T cells. In ACT with TIL, tumor-resident T cells are isolated and expanded ex vivo after surgical resection of the tumor. Thereafter, the TILs are further expanded in a rapid expansion protocol (REP). Before intravenous adoptive transfer into the patient, the patient is treated with a lymphodepleting conditioning regimen. In ACT with genetically modi- fied peripheral blood T cells, TCR gene therapy and CAR gene therapy can be distinguished. For both treatment modalities, peripheral blood T cells are isolated via leukapheresis. These T cells are then transduced by viral vectors to either express a specific TCR or CAR, respectively (Maartje et al., 2018).

#### 1.2.4.1 CAR T cell therapy

CARs combine the affinity of antibody-like recognition with the T-cell-activating function (Maher, 2012). A CAR is a synthetic construct that, when expressed in T cells, mimics T cell receptor activation and redirects specificity and effector function toward a specified antigen. For cancer therapy, this is accomplished by linking an extracellular single-chain variable fragment of a monoclonal antibody

specific for a tumor cell surface antigen to the intracellular signaling module of a TCR (thus maintaining the downstream signaling) that activates T cells upon antigen binding. The earliest "first-generation" CARs contained only a CD3ζ or Fc receptor gamma signaling domain (Eshar et al 1993), and the addition of one (second generation) or more (third generation) costimulatory domains such as CD28, 4-1BB, or OX40 induced more cytokine production and T cell proliferation (Maher et al., 2002; Kershaw et al., 2005; Imai et al., 2004) (Figure 1.10). The signaling modules in a CAR are usually selected based on analysis of tumor recognition *in vitro* and in preclinical *in vivo* models (Srivastava et al., 2015; Kawalekar et al., 2016; Zhao et al., 2015). Because of their ability to recognize native cell- surface antigens independently of antigen processing or MHC-restricted presentation, CARs do not have to be matched to the patient HLA and can recognize tumors that have down-regulated HLA expression.

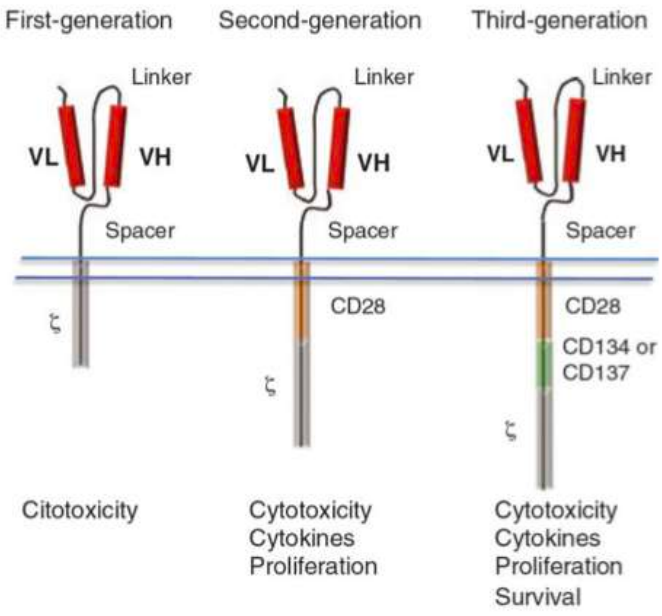



**Figure 1.10** A CAR is constructed by fusing intracellular domains of the TCR complex with the variable regions of the heavy and light chains (respectively VH and VL) of a monoclonal antibody, which confers antigen specificity. First-generation CARs only contain the T-cell signalling domain that transmits the activation signal, that is, the CD3 ζ chain (left). Second-generation CARs incorporate also a single costimulatory domain, like CD28 or 41BB, which increases T-cell expansion and release of cytokines (middle). Third- generation CARs incorporate at least two

costimulatory domains, like CD28 and 41BB, which further improve T-cell survival and *in vivo* persistence (right) (Casucci et al., 2012).

B cell malignancies are an attractive target for CAR T cells because they express B cell lineage-specific molecules such as CD19, CD20, and CD22 that are not expressed on other tissues. To prepare CAR T cell products for treatment of patients, T cells are obtained from the blood, activated *in vitro* and modified to express the CAR by viral or non-viral gene delivery. CAR T cells are then re-infused into the patient, often after the administration of lymphodepleting chemotherapy to promote engraftment and proliferation of transferred cells. Remarkable antitumor effects of CD19-specific CAR-T cells have been demonstrated in patients with chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL) and non-Hodgkin lymphoma (NHL) (Turtle et al., 2017; Gardner et al., 2017; Lee et al., 2015; Brentjens et al., 2013; Kalos et al., 2011; Kochenderfer et al., 2012). CAR T-cell therapies have also been developed to target solid tumors and first signs of efficacy have been reported in the context of neuroblastoma treatment (Louis et al., 2011). A major challenge for CAR T cell therapy in solid tumors is identifying target antigens expressed homogeneously throughout the tumor and not on normal vital tissues.

Despite these early and promising results (some of them are summarized in table 1.1), a number of factors that can affect efficacy and safety of this strategy have to be taken into account. Conditioning regimens (fludarabine and/or cyclophosphamide) may be necessary to reduce the number of circulating T cells and to promote *in vivo* expansion of transferred cells by limiting the competition for cytokines (Gattinoni et al., 2005; Paulos et al., 2007), thus increasing engraftment and persistence (Klebanoff et al., 2005; Dudley et al., 2008; Uttenthal et al., 2012). Another major controversy in the field is defining the optimal cell product for infusion. That is, the input dose of cells and whether to purify selected subsets of cells for culture and subsequent genetic engineering or to use bulk cell products that contain mixtures of $CD4^+$ helper, $CD8^+$ cytotoxic, naive, central

memory, effector memory cells. For example, cell culture conditions can be optimized to promote the expansion of T-central memory cells using anti-CD3 and anti-CD28 coated beads with IL-7 and IL-15 (Kaneko et al, 2009). Furthermore, preclinical studies have shown that the increase of the total number of adoptively transferred cells leads to a progressive improvement in tumor regression (Klebanoff et al., 2011), even if complete cancer remission has been achieved in patients with a specific range of administered anti-CD19 CAR T-cell dose (Kalos et al., 2011; Porter et al., 2011). Typically, T cells are isolated from peripheral blood apheresis, cultured *in vitro* and re-infused as a defined number of cells per kilogram of body weight. However, the effective cell dose varies among patients, because *in vivo* T cell replication and expansion after transplantation are patient-specific. Other factors that can influence the long-term maintenance of efficacy are the downregulation or the loss of expression of the targeted antigens as well as the tumor microenvironment.

Indeed, few targets with homogeneous expression on epithelial cancers have been identified, and outgrowth of antigen-null tumor cells after CAR T cell therapy is an anticipated resistance mechanism. The success of CAR-T cells in B cell malignancies targeting CD19 for example, is tempered by outgrowth of $CD19^-$ tumor cells in some ALL patients (Turtle et al., 2016; Gardner et al., 2016; Sotillo et al., 2015). A strategy to circumvent tumor escape is to target multiple antigens simultaneously, so that only tumor cells that lack expression of all target molecules could escape an anti-tumor immune response (Grada et al., 2013). In terms of potential safety risks associated with these therapies, the most critical side effects are related to on-target off-tumor activity, off-target reactivity and cytokine-release syndromes (Casucci et al., 2015). In the first case, modified T cells could trigger a potent cellular immune response against the body tissues that express the target antigen, even at low levels (Johnson et al., 2009). In the second case, T cells could react against peptides in proteins other than the targeted ones, thus inducing the release of several inflammatory cytokines (such as IFN-γ and IL-6) and

macrophage activation in the patient that could experience the symptoms of a cytokine storm (high fevers, rigors, nausea and diarrhea) (Maude et al., 2014).

| Target antigen | Target disease | T-cell therapy | No. patients | Responses | References |
|---|---|---|---|---|---|
| GP100 | Melanoma | TCR | 16 | 1 CR and 2 PR | Johnson et al., 2009 |
| MAGEA3 | Melanoma, oesophageal and synovial sarcoma | TCR | 9 | 1 CR and 4 PR | Morgan et al., 2013 |
| NYESO-1 | Melanoma and sarcoma | TCR | 17 | 2 CR and 7 PR | Robbins et al., 2011 |
| CD19 | ALL | CAR | 21 | 18/21 CR (86%) within 28 days. 16 of these patients were also MRD-negative | Maus et al., 2014a |
| | ALL | CAR | 20 | 14/20 CR (70%) within 28 days. 12 of these patients were also MRD-negative | Lee et al., 2014 |
| | ALL | CAR | 30 | 27/30 CR (90%) at day 28. 22 of these patients were also MRD-negative | Maude et al., 2014 |
| GD2 | Neuroblastoma | CAR | 19 | 3 CR within 6 weeks, 2 CRs sustained >21 months | Louis et al., 2011 |

ALL, acute lymphoblastic leukaemia; CR, complete response; MRD, mixed residual disease; PR, partial response. Please note that only positive responses are shown here.

Table 1.1 Some published reports about positive clinical responses to T-cell therapies, both exploiting genetically modified TCR therapies and CAR therapies.

To date, two CAR T therapies have been granted FDA approval for the treatment of patients with hematologic malignancies. The first approval granted in 2017 was awarded to 4-1BB-based CD19 CAR T cell therapy tisagenlecleucel (CTL019, Kymriah, Novartis, Basel, Switzerland) for the treatment of patients with acute lymphoblastic leukemia (ALL) that is refractory or in second or later relapse. In particular, all patients who were not leukopenic received lymphodepletion regimen before receiving a median dose of $3.1 \times 10^6$ cells/kg CAR T cells. Also in 2017, the FDA granted a second CAR T therapy approval to the CD28-based CD19 CAR T cell product axicabtagene ciloleucel (Axi-cel, Yescarta, Kite Pharma/Gilead, Los Angeles, CA) for the treatment of patients with diffuse large B cell lymphoma (DLBCL) who have not responded or have relapsed after two prior treatment regimens. In the phase I/II ZUMA-1 trial (NCT02348216) patients received $2 \times 10^6$ cells/kg autologous CAR T cells after fludarabine and cyclophosphamide lymphodepletion (Neelapu et al., 2017). In 2018, tisagenlecleucel gained a second FDA approval, this time for the treatment of adult patients with relapsed/refractory DLBCL.

## 1.3 Targeted genome engineering

Genome engineering refers to the strategies and techniques developed for the targeted modification of the genome of living organisms. Recent advances in the development of these technologies based on programmable nucleases have substantially improved our ability to introduce precise changes at specific genomic loci of eukaryotic cells and therefore hold great promise for revolutionizing the gene therapy arena. One particularly promising technology is known as gene targeting and is based on the cellular homologous recombination (HR) pathway, which has evolved mainly to promote genetic recombination during meiosis and the repair of DNA double-strand breaks (DSBs) before mitosis. Following the discovery that induction of a DSB increases the frequency of homology-directed repair (HDR) by several orders of magnitude, targeted nucleases have emerged as the method of choice for improving the efficiency of HDR-mediated genetic alterations. HDR allows researchers to use an exogenous DNA template to specify the outcome of the DSB repair. Upon introduction of a targeted DSB, HDR machinery may use exogenously provided DNA templates with sequence similarity to the break site to repair the lesion, incorporating any changes encoded in the template DNA. In the absence of a repair template the lesion may be repaired by directly rejoining the two DSB ends in an error-prone process called non-homologous end joining (NHEJ). Although NHEJ- mediated DSB repair can be accurate, this type of repair eventually results in the formation of small insertion or deletion mutations (Indels). Indels introduced into the coding sequence of a gene can cause frameshift mutations that lead to mRNA degradation by nonsense-mediated decay or result in the production of nonfunctional truncated proteins. Thus, NHEJ may be used to suppress gene function similarly to RNAi, but it may lead to permanent inactivation by introducing loss-of-function mutations into the gene in targeted cells. To date, four major classes of nucleases have been developed to enable site-specific genome editing: meganucleases, zinc finger nucleases (ZFNs), transcription activator–like effector nucleases (TALENs) and CRISPR-associated nuclease Cas9 (Table 1.2). These nuclease systems can be classified into

two categories based on their mode of DNA recognition: ZFNs, TALENs and meganucleases achieve specific DNA binding via protein- DNA interactions, whereas Cas9 is targeted to specific DNA sequences by a short RNA guide molecule that base-pairs directly with the target DNA and by protein-DNA interactions. Meganucleases are endonucleases with large (>14-bp) recognition sites, the DNA binding domains of which are also responsible for cleavage of target sequences. ZFNs and TALENs are chimeric enzymes consisting of a DNA binding domain fused to the nuclease domain of the restriction enzyme FokI. In this configuration, the DNA-binding domain directs the non-specific FokI cleavage domain to a specific DNA target site (Cathomen et al., 2008). Re-targeting of ZFNs and meganucleases for a new DNA sequence requires protein engineering, whereas re-targeting of TALENs requires complex molecular cloning for the construction of two new TALEN genes. In contrast, the Cas9 protein is invariant and can be easily re-targeted to new DNA sequences by changing a small portion of the sequence of an accompanying RNA guide that base-pairs directly with target DNA. Another potential advantage of Cas9 is its ability to introduce multiple DSBs in the same cell (also referred to as multiplexing) via expression of distinct guide RNAs. All four types of nucleases have been shown to achieve efficient genome editing in a wide range of model organisms and mammalian cells, and efforts are now underway to develop these tools as therapeutics (Turitz Cox et al., 2015).

| | Zinc finger nuclease | TALEN | Cas9 | Meganuclease |
|---|---|---|---|---|
| Recognition site | Typically 9–18 bp per ZFN monomer, 18–36 bp per ZFN pair | Typically 14–20 bp per TALEN monomer, 28–40 bp per TALEN pair | 22 bp (20-bp guide sequence + 2-bp protospacer adjacent motif (PAM) for *Streptococcus pyogenes* Cas9); up to 44 bp for double nicking | Between 14 and 40 bp |
| Specificity | Small number of positional mismatches tolerated | Small number of positional mismatches tolerated | Positional and multiple consecutive mismatches tolerated | Small number of positional mismatches tolerated |
| Targeting constraints | Difficult to target non-G-rich sequences | 5′ targeted base must be a T for each TALEN monomer | Targeted sequence must precede a PAM | Targeting novel sequences often results in low efficiency |
| Ease of engineering | Difficult; may require substantial protein engineering | Moderate; requires complex molecular cloning methods | Easily re-targeted using standard cloning procedures and oligo synthesis | Difficult; may require substantial protein engineering |
| Immunogenicity | Likely low, as zinc fingers are based on human protein scaffold; FokI is derived from bacteria and may be immunogenic | Unknown; protein derived from *Xanthamonas* sp. | Unknown; protein derived from various bacterial species | Unknown; meganucleases may be derived from many organisms, including eukaryotes |
| Ease of *ex vivo* delivery | Relatively easy through methods such as electroporation and viral transduction | Relatively easy through methods such as electroporation and viral transduction | Relatively easy through methods such as electroporation and viral transduction | Relatively easy through methods such as electroporation and viral transduction |
| Ease of *in vivo* delivery | Relatively easy as small size of ZFN expression cassettes allows use in a variety of viral vectors | Difficult due to the large size of each TALEN and repetitive nature of DNA encoding TALENs, leading to unwanted recombination events when packaged into lentiviral vectors | Moderate: the commonly used Cas9 from *S. pyogenes* is large and may impose packaging problems for viral vectors such as AAV, but smaller orthologs exist | Relatively easy as small size of meganucleases allows use in a variety of viral vectors |
| Ease of multiplexing | Low | Low | High | Low |

Table 1.2 Comparison of different programmable nuclease platforms (Turitz Cox et al., 2015).

### 1.3.1 Rna-guided CRISPR nucleases

Distinct from the site-specific nucleases described above, the CRISPR/Cas system has recently emerged as a potentially facile alternative to ZFNs and TALENs for inducing targeted genetic alterations, representing a system that is markedly easier to design, highly specific, efficient and well-suited for high-throughput and multiplexed gene editing for a variety of cell types and organisms.

#### 1.3.1.1 Discovery of the CRISPR arrays in prokaryotic genomes

The clustered regularly interspaced short palindromic repeats (CRISPR) were first identified in 1987 by Ishino and colleagues who detected five 29 bp identical direct repeats with 32 bp variable spacers downstream of the isozyme-converting alkaline phosphatase (iap) gene on the chromosome of Escherichia coli (Ishino et al., 1987). Although the biological role served by these repetitive motifs remained obscure, several organisms were found to possess this feature. Similar repeats were found soon in other prokaryotic species such as Mycobacterium tuberculosis (Groenen et al., 1993), Haloferax volcanii and Haloferax mediterranei (Mojica et al., 1995) and

Archaeoglobus fulgidus (Klenk et al., 1997; Jansen, 2002); however, they were not detected in eukaryotic or virus sequences (Jansen et al., 2002). Mojica and co-workers performed a comparative in silico study of those repetitive elements to determine structure and sequence similarity, as well as their phylogenetic distribution (Mojica et al., 2000) showing that CRISPRs display a high degree of homology between phylogenetically distant species and a wide distribution in bacteria and archaea. To date CRISPR/Cas systems have been found in almost 50% of bacterial and 85% of archaeal genome sequences available (Grissa et al., 2007). A critical discovery came in 2005 with three independent studies showing that spacers matched sequences of extrachromosomal origin, including phages, prophages and plasmids, suggesting they were possibly the memory of a novel immune system (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Furthermore, there was a positive correlation between the presence of spacers matching a particular phage and phage resistance (Bolotin et al., 2005; Pourcel et al., 2005). The first direct evidence that CRISPR/Cas could protect bacteria from phages or plasmids was provided by two key studies. Firstly, Barrangou obtained phage resistant mutants after challenging wild-type S. thermophilus with phages. Analysis of the CRISPR I locus from the generated phage insensitive mutants showed that each immune mutant expanded its CRISPR array by insertion of one or several new spacers. The newly acquired spacers interestingly matched genomic regions from the phage used in the challenge. The artificial introduction of these naturally acquired spacers in the original host resulted in de novo immunity against that phage, while removal of spacers matching to the phages resulted in phage sensitivity (Barrangou et al., 2007). Whereas typically only one spacer is added upon a phage challenge, increased resistance is obtained by the addition of more spacers against a certain phage (Barrangou et al., 2007; Brouns et al., 2008; Deveau et al., 2008). Subsequently, Marraffini and Sontheimer showed that CRISPR/Cas systems can also prevent both conjugation and transformation of plasmids in Staphylococcus epidermidis (Marraffini et al., 2008). All these findings made by different groups contributed to the consensus that the CRISPR/Cas system is an

active prokaryotic immune system against propagation of viruses and conjugative elements (He and Deem, 2010).

#### 1.3.1.2 CRISPR/Cas9 a potent technology for gene editing: mechanism and improvements

The CRISPR/Cas9 system can be broadly described as a RNA guided endonuclease (RGEN) and since the first use of CRISPR/Cas9 in mammalian cells many improvements have been made to make gene editing more specific and tolerable. Multiple types of CRISPR/Cas9 systems exist throughout bacteria and archaea, most targeting DNA and some RNA (Rath et al., 2015). Cas9 is exclusively associated with the type II CRISPR. Based on the variety of Cas genes, type II CRISPR loci are subdivided into three subtypes (IIA–IIC) (Makarova et al., 2011; Chylinski et al., 2013). Type II CRISPR loci mostly consist of the cas9, cas1, and cas2 genes, as well as a CRISPR array and tracrRNA. Type IIC CRISPR systems contain only this minimal set of genes, while types IIA and IIB present some other additional genes (Chylinski et al., 2013). However, Cas9 proteins exhibit sequence homology and length variability which do not reflect the subtype classification of their parental CRISPR locus. Of >1,000 Cas9 nucleases identified from sequence databases (UniProt) based on homology, protein length range from 900 to 1600 aminoacids. Despite this diversity, all Cas9 proteins share similar domain architecture (Makarova et al., 2011; Chylinski et al., 2013, 2014; Fonfara et al., 2014), consisting of the RuvC and HNH nuclease domains and the REC domain. It's mainly the conservation of the REC domain that makes length difference among Cas9 proteins (Jinek et al., 2014). The most widely described and used system for gene editing is the type II CRISPR system from S. pyogenes because SpCas9 protein is well distributed within the nuclease as opposed to Cas9 protein from S.thermophilus (Lander, 2016), hence we will focus on this system (Figure 1.11). In bacteria proto-spacer acquisition is driven by Cas1, Cas2, but also Csn2 and Cas9 (Heler et al., 2015; Wright et al., 2016) and upon infection with viral DNA the CRISPR locus is transcribed by RNA polymerase into precursor CRISPR RNA

(pre-crRNA), which is processed into different CRISPR RNAs (crRNA). CrRNA, that harbours the target sequence, is fused with tracrRNA and together recruits Cas9 protein to form a Cas9:crRNA:tracrRNA complex that cleaves DNA at the target site. Cleavage will occur 3 basepairs upstream of the PAM sequence (here a 5'NGG) and, consequently, if there is no PAM sequence cleavage will not occur. Genome editing has been made easier by fusing crRNA and tracrRNA into a chimeric small guide RNA (sgRNA), resulting in higher gene editing efficiencies (Jinek et al., 2012). SgRNA is ~100 bp long and specificity is provided in the first 20 nucleotides. These nucleotides can be easily altered to target any genomic locus followed by a PAM sequence. Moreover, two conserved domains in the Cas9 protein cut each in a different DNA strand. The histidine-asparagine-histidine (HNH) domain cuts the DNA strand complementary to the sgRNA, the RuvC domain cleaves the non-complementary stand. HNH is a single nuclease domain, while RuvC domain consists of three subdomains across the linear protein sequence, with RuvC I near the N-terminal region of Cas9 and RuvC II/III flanking the HNH domain near the middle of the protein. Recently, two structural studies highlighted the structural mechanism of RNA-guided DNA cleavage by Cas9. First, single-particle EM reconstructions of the SpCas9 revealed a large structural alteration between Cas9 unbound to nucleic acid and Cas9 in complex with crRNA and tracrRNA, thus forming a central channel to accommodate the RNA-DNA heteroduplex (Jinek et al., 2014). Second, a high-resolution structure of SpCas9 in complex with sgRNA and the DNA complementary strand further unveiled the domain organization. It consists of an α-helical recognition (REC) lobe and a nuclease

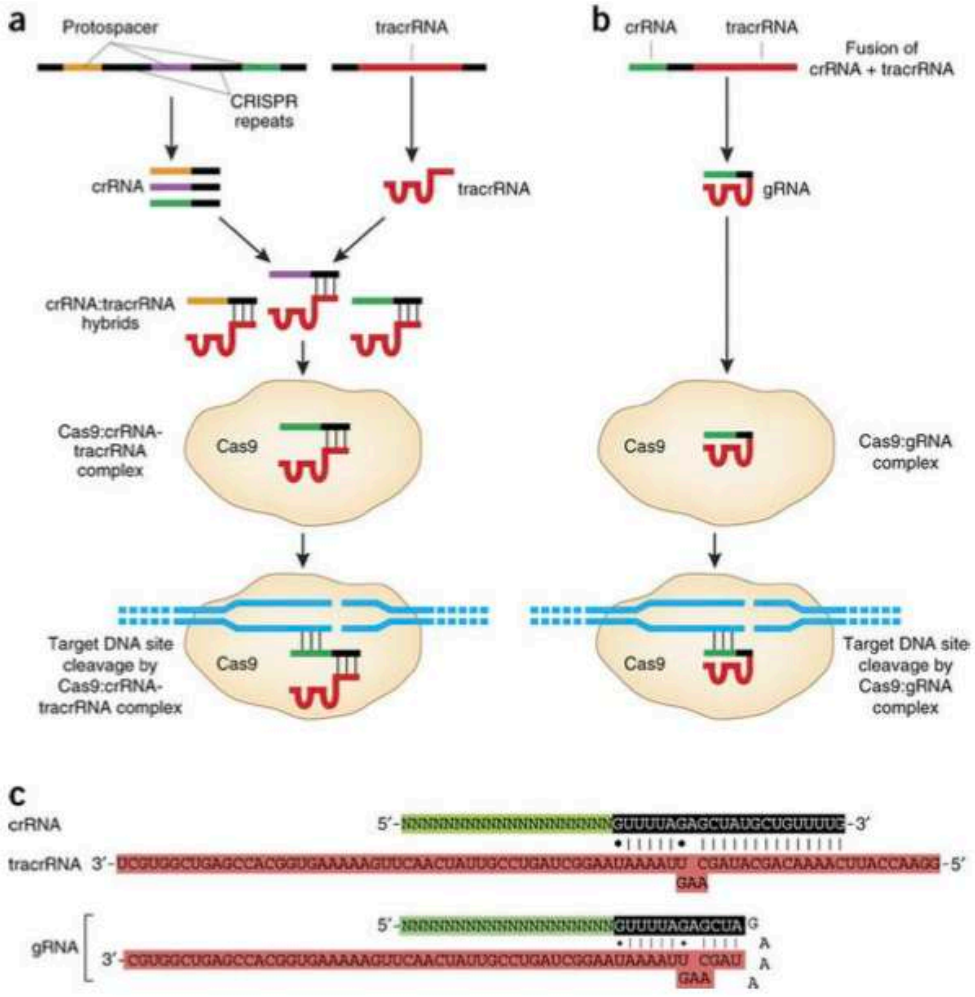



**Figure 1.11** A) Mechanism of the naturally occurring Type II CRISPR/Cas9 system. B) An engineered CRISPR/Cas9 system used for targeted genome editing. C) Differences between the naturally occurring Cas9:crRNA-tracrRNA complex and engineered Cas9:sgRNA complex. (Adapted from Sander and Joung, 2014)

and a PAM- interacting (PI) C-terminal region (Nishimasu et al., 2014). Together, these two studies suggest that SpCas9 unbound to any nucleic acid exhibits an autoinhibited conformation, in which the HNH domain active site is blocked by the RuvC domain and is positioned away from the REC lobe (Jinek et al., 2014). Thus, unbound Cas9 cannot bind or cleave target DNA. Only the guide RNA serves as a scaffold for Cas9 folding and organization (Nishimasu et al., 2014). The crystal structure of SpCas9 complexed with RNA-DNA heteroduplex also revealed that an arginine-rich bridge helix (BH) within the REC lobe is responsible

for contacting the 3′ 8–12 nt of the RNA-DNA heteroduplex (Nishimasu et al., 2014), which correspond with the seed sequence, crucial for DNA target recognition, identified through guide sequence mutation experiments, thus facilitating target binding (Jinek et al., 2012; Cong et al., 2013; Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013; Mali et al., 2013).

#### 1.3.1.3 Targeting Specificity

Because genome editing leads to permanent modifications within the genome, the targeting specificity of Cas9 nucleases is of particular concern, especially for gene therapy clinical applications. Streptococcus pyogenes Cas9 specificity has been extensively characterized by multiple groups using mismatched guide RNA libraries, in vitro selection, and reporter assays (Fu et al., 2013; Hsu et al., 2013; Mali et al., 2013; Pattanayak et al., 2013). The CRISPR/Cas9 is known to target many off-targets sites in the genome because mismatches in the targeting sequence are tolerated to a certain extent and are better tolerated when Cas9 is present at high concentrations (Hsu et al., 2013; Pattanayak et al., 2013), leading to higher off-target activity. When Cas9 concentration is decreased, significant improvement in the on- to off-target ratio are reported (Hsu et al., 2013). In addition, Cas9 requires extensive homology between the gRNA and target DNA in order to cleave, but it can remain bound with only a short region of complementary sequence, thus suggesting that Cas9 has many off-target binding sites but cleaves only a small fraction of them (Wu et al., 2014). Taken together, these evidences can result in thousands of potential off-target sites that can have severe implications and for this reason have to be carefully evaluated with experimental off-target screening methods and improved with better CRISPR/Cas9 design. Tipically, potential off-target sites can be computationally determined by searching for high similarity sequences to the desired target locus. However, wholegenome sequencing or other unbiased ways of labeling DNA DSBs genome-wide may be essential to find off-

target sites that are not computationally predictable by sequence comparison. Unbiased genome-wide characterizations have been previously applied to characterize ZFN off- targets (Gabriel et al., 2011), thus it could be easily adapted for Cas9 nuclease activity. One of the most notable improvements for the CRISPR/Cas9 system is the use of truncated guide RNAs (tru-gRNA) that instead of a 20 bp complementary region only have 17-18 bp and show similar on-target activity and a >5000 fold decrease of undesired mutagenesis at some off-target loci (Fu et al., 2014). Researchers found that lengthening the 5' end actually decreased on-target activity (Cho et al., 2014; Hwang et al., 2013). Possibly these nucleotides at the 5' end are historically present to compensate for mismatches, because the CRISPR system is an immune system against mutating viral invaders. Hence a shorter complementary region would be more specific, because less mismatches would be allowed in the sgRNA (Fu et al., 2014). This adjustment of the sgRNA design can significantly improve specificity and can easily be combined with other improvements to further reduce off target activity, such as the addition of a two guanine (GG) 5' end extension of the sgRNA (Cho et al., 2014). The mechanism behind the off-target reduction is still not clear, but the hypotesis is the disruption of the interaction with a stabilizing protein with the 5′ end of the gRNA. Another way to enhance CRISPR/Cas9 specificity is the chemical substitution of the 5' and 3' terminal three nucleotides with modified ribonucleotide bases, like 2'-O-methyl-3'phosphorothiorate (MS) and 2'-O-methyl-3'thioPACE (MSP). This holds particularly true when they are used in a RNA-only or Ribo-Nucleo-Protein (RNP) CRISPR/Cas9 system. MS and MSP modified sgRNA demonstrate better on-target/off-target ratios than unmodified sgRNA and lower cytotoxicity in primary cells (Hendel et al., 2015), thus indicating that cytotoxicity is at least partially triggered by sgRNA. Other strategies to improve the specificity of CRISPR/Cas9 system involve the Cas9 protein itself. For example, SpCas9 can be converted into a DNA "nickase", that creates a single-stranded break (SSB) by catalytically inactivating the RuvC or HNH nuclease domains via point mutations (Gasiunas et al., 2012; Jinek et al., 2012; Sapranauskas et al., 2011). In this case, the cleavage

results in overhang-ends. To improve on-target DSB specificity, a paired-Cas9 nickase approach can be used to increase the total number of bases that are specifically recognized in the target site. The double Cas9 nickase strategy requires two paired nickases which can target by two gRNAs the site of interest, inducing a DSB with overhang-ends, basically using the same hetero-dimerization strategy that ZFNs and TALENs are using to be highly specific. This strategy reduces off-target mutations up to 1500-fold (Ran et al., 2013). However, off- target mutations induced by the second sgRNA can reduce the efficiency of this method (Tsai and Joung, 2016), thus implicating the necessity to consider the off-target effects of each of the gRNAs in the pair. Another strategy is the use of an RNA-guided FokI nuclease based on fusion between catalytically inactive Cas9 and the FokI nuclease domain, can also (Fu et al., 2014; Guilinger et al., 2014; Tsai et al., 2014). Other groups improved Cas9 protein through rational protein engineering creating mutant forms of monomeric Cas9 that show increased specificity. One group hypothesized that neutralisation of positively charged residues within a specific Cas9 groove weakens binding efficiency and would result in a need for Cas9 to acquire more Watson-Crick base pairing to function (Slaymaker et al., 2016). Through structure-guided mutagenesis, "enhanced specificity" SpCas9 (eSpCas9 1.1) was assembled that contained three substitutions of positively charged residues with alanine, thus reducing the interaction with the non-target DNA strand. eSpCas9 mutants retained on-target efficiency and showed reduced off-target activity (Slaymaker et al., 2016). Another group made a high- fidelity variant of SpCas9 (SpCas9-HF1) designed to have fewer non-specific DNA contacts. They hypothesised that disruption of hydrogen bonds, that facilitate binding between sgRNA and the target DNA strand, would allow for only high affinity, on-target, DNA binding to occur and subsequently cleave DNA (Kleinstiver et al., 2016). Four amino acids were substituted with alanine to nullify hydrogen bond formation and minimal disruption was found when testing the mutant Cas9 with mismatched sgRNA. They reported that SpCas9-HF1, which contains N497A, R661A, Q695A, and Q926A substitutions, reduces nearly all off- target activity to undetectable levels (measured

by GUIDE-seq), while retaining >70% on-target activity comparable to wt-Cas9 with >85% of sgRNA tested on human cells (Kleinstiver et al., 2016). They also noted that substitution of basic DNA contacting residues sites could further reduce off-target activity with SpCas9- HF1. Moreover, some argue even more promisingly, RGEN system has been discovered and is not based on Cas9 but on Cpf1. Notable advantages of this system over Cas9 are i) the smaller size of Cpf1 enzyme because it contains only a single identified nuclease domain (in contrast to the two present in Cas9), making it easier to deliver into cells, ii) the fact that Cfp1 leaves overhang-ends after cleavage that could benefit HDR in contrast to Cas9 that leaves blunt-ends, iii) that cleavage occurs far from the recognition site allowing for multiple opportunities for gene correction even when the cut site is mutated, and iv) new flexibility in choosing target sites, because Cfp1 employs different PAM sequences, v) the fact that Cpf1 don't need a tacrRNA to process crRNA arrays and both crRNA and tracrRNA to mediate interference (Deltcheva et al., 2011). In fact, Cpf1 processes crRNA arrays independent of tracrRNA, and Cpf1-crRNA complexes alone cleave target DNA molecules, without the requirement for any additional RNA species, thus simplyfing the design and delivery of genome-editing tools and being advantageus because shorter RNA oligos are easier and cheaper to synthetize (Zetsche et al., 2015).

### 1.3.2 Targeted genome editing in human HSCs

The clinical goal of gene therapy is the genetic manipulation of human HSCs and targeted genome editing could undoubtedly lead to an improvement of the strategies and a reduction of side effects. However, efficient gene targeting in hematopoietic progenitors and, more specifically, HSCs has not been reported for years and the identification of the factors that affect gene editing in HSC has been a fundamental step to overcome this hurdle. In this subchapter, I will briefly summarize these factors and the possible solutions to improve gene targeting efficiency.

#### 1.3.2.1 Delivery vehicles for editing machinery and gene targeting efficiency

To achieve suitable levels of gene targeting, efficient systems for the delivery of the editing machinery are required. However, a big difference in gene targeting efficiency has been reported between cell lines and primary cells, like T cells or HSPCs, even using the same reagents. The main causes of this difference are likely the lower permissiveness to viral vector of primary cells (Lombardo et al., 2007) and an insufficient intracellular concentration of both the nucleases and the donor vectors. Genovese and colleagues overcame these problems by combining a physical and a viral delivery method. In particular, they used mRNAs electroporation to transiently express ZFNs directed against the exon 5 of *IL2RG* gene or the intron 1 of *PPP1R12C* gene (*AAVS1* locus) and IDLV infection to provide the DNA donor template for HDR. In this way, ZFN persists short-term in the cells, thus avoiding toxicity. Moreover, they have demonstrated that IDLV is less toxic compared to plasmid in CD34+ cells and permits to achieve significantly better results in term of gene targeting efficiency. Finally, it has been shown that the timing of transduction and nucleofection is extremely relevant. Indeed, the better results in term of gene targeting efficiency and NHEJ levels have been obtained by combining IDLV 24 hours before ZFN mRNA electroporation, thus reducing the competition between the two delivery platforms and giving the time to viral vector to complete the reverse transcription and to be imported in the nucleus (Genovese et al., 2014). The following year Wang and colleagues proposed the combination of ZFN mRNA and AVV6 viral vector to achieve HDR-mediated genome editing. They demonstrated that this combination allowed high levels of gene editing efficiency also in the most primitive compartement in CCR5 and *AAVS1* locus of mobilized blood HSPCs, without adversely affecting the growth and the differentiation potential of the cells. Moreover, HSPCs resulted capable of engrafting in mice and differentiating into multiple lineages stabily edited. Finally, AAV6 showed a relatively flexibility in the delivery timing, which resulted optimal between 24 h pre- and 1 h post-electroporation (Wang et al., 2015). Subsequently,

Dever and collaborators demonstrated that AAV6 transduction can be coupled with CRISPR/Cas9 platform to successfully and efficiently edit HSPCs (Dever et al., 2016). Recently, also the co-delivery of donor template by single-stranded phosphorothioate-modified oligodeoxynucleotides (ssODNs) in combination with the nucleofection of CRISPR/Cas9 platform showed more than 20% gene correction of CGD patient-derived HSPCs, with stable engraftment of edited cells upon NSG xenotransplantation (De Ravin et al., 2016). Another issue that can impact on gene targeting efficiency may be the delivery of the nucleases and their stability into the cells. For example, Hendel and collegues demonstrated that modifications at the three terminal nucleotides at 5' and 3' ends of the gRNAs comprising 2′-*O*-methyl (M), 2′-*O*-methyl 3′phosphorothioate (MS), or 2′-*O*-methyl 3′thioPACE (MSP), enhanced intracellular stability, thus increasing the efficiency of gene editing (measured as indel frequencies) both when co-delivered with Cas9 mRNA and when electroporated as ribonucleoprotein complex (RNP) in cell line. Moreover, by nucleofecting the ribonucleoprotein complex, a partial protection of the gRNA from degradation has been observed, thus improving the stability of the platform. Even when tested in primary cells, chemically modified gRNAs facilitate high frequencies of gene disruption in human T cells and HSPCs and show low cytotoxicity (Hendel et al., 2015).

#### 1.3.2.2 Toxicity of gene editing procedure

Another factor which impacts on HSPCs gene targeting outcome is toxicity. In fact, genome editing procedure is *per se* more toxic in primary cells than in cell lines (Urnov et al., 2005). Recently, Genovese and colleagues have shown that HSPCs are highly sensitive to gene editing (Genovese et al., 2014). In particular, while IDLV or AVV alone mildly affects HSPC viability, innate cellular response dependent on pattern-recognition- receptors (PRRs) could be triggered (Sadler and Williams, 2008) by contaminants, often present particularly in laboratory grade IDLV preparation, which can enter into the cells through the electroporation procedure. Moreover, the presence of exogenous RNAs, such as nuclease-encoding

mRNAs, in the cytoplasm of the cells can also activate the innate cellular response in gene targeted HSPCs. The activation of these reponses based on Interferon-1 (IFN-1) correlates with HSC fate reprogramming and with the exit from G0 phase, proliferation, differentiation and exhaustion (Essers et al., 2009; Passegue and Ernst, 2009). More recently, Liu and colleagues have also demonstrated that strong PRR recognition can trigger apoptosis in human CD34+ cells (Liu et al., 2012). The electroporation of chemically modified nuclease-encoding mRNAs may reduce the activation of these responses and the toxicity (Warren et al., 2010; Hendel et al., 2015). Thus, better vector purification and the electroporation of modified nuclease-encoding mRNAs might reduce the toxicity. Another cause of toxicity in HSPCs could results from DNA DSB. As a matter of fact, HSPCs have been already reported to be highly sensitive to genotoxic insults (Milyavsky et al., 2010). After DSB induction DDR is activated in HSCs, and it could lead to apoptosis, differentiation or senescence. Thus, specificity of artificial nucleases in HSC gene targeting is fundamental, because higher off-target activity means multiple DSBs in the genome and in turn higher toxicity (Pattanayak et al., 2011). For this reason, an accurate optimisation of reagents is required in order to achieve higher efficiency of HSC gene targeting.

#### 1.3.2.3 Primitive HSCs low permissiveness to HDR

The low permissiveness to HDR of primitive HSCs has been hypotezised as another hurdle to overcome in order to obtain high gene targeting efficiency. Genovese and colleagues have recently observed a lower permissiveness to HDR of ZFN-induced DSBs (Genovese et al., 2014). In fact, the HDR/NHEJ ratio is lower in more primitive HSPCs both *in vivo* and *in vitro* after gene targeting procedure (Genovese et al., 2014). This observation is supported by the fact that HDR is preferred in S/G2 phases to repair DSBs, while NHEJ is more widely used in G1 and, maybe, G0. This means that more committed HSPCs tend to repair DSBs exploiting HR, whereas primitive HSCs preferably recruit the NHEJ pathway (Mohrin et al., 2010). In fact, primitive HSPCs rarely cycle, thus infrequently having the possibility to

repair DSBs using the HDR pathway. A significant augmentation of gene targeting efficiency has been achieved by doubling the pre- stimulation time of HSPCs (48 h instead of 24 h). Indeed, a prolonged time of stimulation favours the transit through the S/G2 phases, when HDR preferably occurs (Genovese et al., 2014). Other applicable solutions, showing an improvement in HDR efficiency but not yet evaluated in HSPCs, could be i) increasing the percentage of similarity between donor DNA template and substrate (Orlando et al., 2010; Beumer et al., 2013); ii) abolishing of alternative repair pathway(s) (such as NHEJ) through the addition into the medium of compounds (such as the NHEJ-inhibitor Scr7) (Beumer et al., 2013; Maruyama et al., 2015).

#### 1.3.2.4 In vitro HSC maintenance and expansion

The current strategy for HSC gene therapy is *ex vivo* genetic manipulation of autologous HSCs before reinfusing the HSPCs back in the patient for clinical benefit. This method benefits from high gene editing rates and affords control over the dosage of delivered molecules (Cox et al., 2015). But prior to the genetic manipulation, HSCs have to be maintained *in vitro* and they must be not allowed to differentiate. Moreover, the HSCs must re-engraft into the BM, which is an inefficient process that might require perilous ablative conditioning regimens to deplete the patient's BM from non-functional HSCs (Bunn et al., 2011; Cox et al., 2015). *In vitro* expansion of HSCs before genetic manipulation could clearly benefit their application as clinical experience has shown that transplantation of a higher dose of non-edited HSCs results in better patient survivability (Wagner et al., 2002).

HSCs can divide in 3 different way: 1) symmetrical self-renewal cell division, where division of the parent HSC results in two identical HSCs that maintain all stem cell properties, 2) Asymmetrical self- renewal division, where the parent HSC divides in an identical daughter cell and a cell committed to differentiation, 3) Symmetrical HSC division, where the parent HSC divides in two identical daughter cells that are committed to differentiation (Figure 1.12). Unfortunately, it is still not

possible to sustain symmetrical self-renewal division *in vitro* (Walesek et al., 2012). Due to restricted gene targeting efficiency in the most primitive HSC (Genovese et al., 2014) and differentiation of HSCs in culture (Walasek et al., 2012), the clinical use of modified HSC is limited to disease where gene edited cells have a selective advantage over non-edited cells. In this case engrafted HSCs can expand *in vivo* and abolish the disease phenotype. Diseases where a small concentration of therapeutic proteins is sufficient for correction of the phenotype might also benefit from gene therapy when the cell fitness of gene edited cell is equal (Cox et al., 2015).

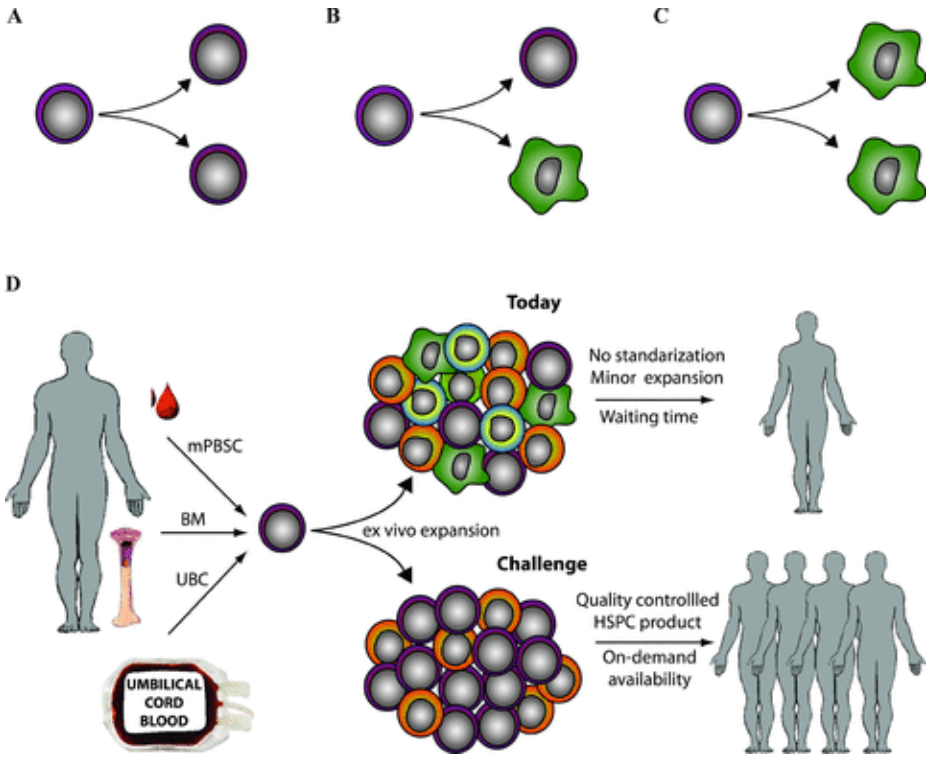



**Figure 1.12** Three different types of HSC division. A) Symmetrical self-renewal cell division. B) Asymmetrical self-renewal division. C) Symmetrical HSC division. D) Currently, ex vivo expansion results in differentiation, where severely reducing *in vitro* differentiation has clinical benefits (Adapted from Walesek et al., 2012).

Understanding regulatory intrinsic HSC pathways and extrinsic regulators that determined HSC fate in the HSC microenvironment is important when developing ideal HSC culture conditions that promote symmetrical self-renewal. Hematopoietic cytokines, like SCF, TPO, IL-3, IL-6, IL-11, granulocyte macrophage colony stimulating factor (GM-CSF), and FMS-related tyrosine kinase 3 ligand (FLT-3L) have positive effect on self-renewal and proliferation *in vitro* (Sauvageau et al., 2004; Walesek et al., 2012). Cytokine mixtures that contain at least SCF, FLT-3L and TPO became the standard for HSPC survival, proliferation and maintenance *in vitro* (Walesek et al., 2012).

In order to improve gene targeting efficiency and better maintain HSCs, Genovese and colleagues have tried to add at the medium in the 48h pre- stimulation protocol the small molecules StemRegenin1 (SR1) and 16,16-dimethyl-PGE2 (dmPGE2) (Genovese et al., 2014). In the last years, high-throughput screenings have permitted to find molecules potentially useful for HSC expansion and SR1, an aryl hydrocarbon receptor antagonist, has been proposed as a potential candidate for this purpose (Boitano et al., 2010). SR1 has been proved to increase both the total amount of cord blood (CB)-derived CD34+ cells and the long-term repopulation yield after transplantation in mice. In parallel, similar high- throughput studies on zebrafish haematopoiesis have found that the lipid molecule PGE2 can influence HSC numbers in embryos (Hoggatt et al., 2009; Goessling et al., 2011). Even if the precise mechanism by which PGE2 induces HSC expansion and preserves stemness is currently only a hypothesis (upregulation of survivin and downregulation of activated caspase-3), two independent studies have observed an increased frequency of repopulating cells and a prolonged *in vivo* competitive advantage (Hoggatt et al., 2009; Goessling et al., 2011). Genovese and colleagues have shown that dmPGE2 increases the percentage of gene targeted HSPCs, while SR1 slightly reduces it. In addition, SR1 significantly boosts human cell engraftment. It is likely that dmPGE2 increases HSPC proliferation and activation facilitating IDLV and homology-mediated DSB repair, while SR1 help primitive HSPC maintenance thus

augmenting the targeting efficiency in immunodeficient mice. Overall, the tailored protocol results in a substantial improvement in gene targeting efficiencies with 2-fold more gene targeted cells within the most primitive HSPC population (CD34+CD133+CD90+) (Genovese et al., 2014). This barrier was partially rescued, by the same group and other, by extending culture condition, in order to drive also the most primitive cells into replication, while preserving their engraftment capacity, and by optimizing editing reagents and protocol for human HSPCs to avoid triggering innate immune cellular responses (Schiroli et al., 2017, Wang et al., 2015, De Ravin et al., 2016). Despite all these improvements and the efficacy of a 10% of WT cells to rescue physiological immune-reconstitution in SCIDX-1 mice, the % of HDR-edited long-term repopulating cells in xeno-transplant recipients remains low ($\leq 20\%$) and this might be a limit in other application, where more robust correction is needed, particularly of long-term repopulating HSC. For this reason, ongoing studies on HSC gene editing aims to further improve the efficiency of HDR in the most primitive cells by favoring HDR versus NHEJ, or to select *in vitro* edited HSC in order to obtain a pure population that might be further expanded before transplantation. Other factors that play a role in the regulation of self-renewal and differentiation of HSPC are micro RNA (miRNAs) of the miR-125 family, expression of which results in promotion of self-renewal in HSCs (Guo et al., 2010; Kim et al., 2012). Other approaches aim to ameliorate gene editing by acting at the basal levels (Rees et al., 2018) or by silencing genes or regulatory sequences by acting at the epigenetic level of the targeted locus (Amabile et al., 2016).

Another important aspect is associated with the induction of DBS in HSC that might induce adverse effects on their key functional proprieties, such as long-term hematopoietic reconstitution, by triggering both DDR pathways: HDR and NHEJ. It has been shown that the induction of DSB triggers DDR with p53 pathway activation, p21 induction, and delayed cell proliferation. It was also showed that the response correlates with the specificity of nucleases, lesser specificity, which

cleaved DNA at the target and few other off-target sites, caused a more robust DDR induction, thus higher p53 response (Schiroli et al., 2019). For this reason, the inhibition of p53 response would be another possible approach to increase the yield of edited cells.

Overall, these findings mark the birth of a new era in cell and gene therapy, in which the use of genome editing results to be feasible on human HSPC and, by improving its features even further, would be ready for clinical testing.

### 1.3.3 Therapeutic genome editing strategies

Genome editing based therapy can be achieved through a number of approaches including correction or inactivation of deleterious mutations, introduction of protective mutations, addition of therapeutic transgenes and disruption of viral DNA. Pathogenic mutations can be classified as causing either gain or loss of function in a gene product. A gain-of-function mutation, such as those found in the HTT gene in Huntington disease and in FGFR3 in achondroplasia, results in the expression of a pathogenic gene product and may be treated by using NHEJ-mediated mutations to specifically inactivate the mutant allele while leaving the wild-type allele intact on the homologous chromosome (Fig. 1.13 a). Nucleotide expansion disorders, such as Huntington disease, could instead be treated by NHEJ-based deletion of the pathogenic insertion via the creation of two DSBs on both sides of the expansion (Figure 1.13 b). However, some gain-of-function mutations, such as the SOD1 G93A mutation found in some individuals with amyotrophic lateral sclerosis (ALS), are point mutations, which may not be sufficiently different from the wild type allele on the homologous chromosome to be distinguished by the current generation of programmable nucleases, potentially leading to an undesirable complete loss of protein function if the mutation is targeted using NHEJ. In such cases HDR could instead be used to change the gain-of-function allele to the wild-type sequence, restoring gene function while preserving physiological levels of gene expression (Fig. 1.13 c).

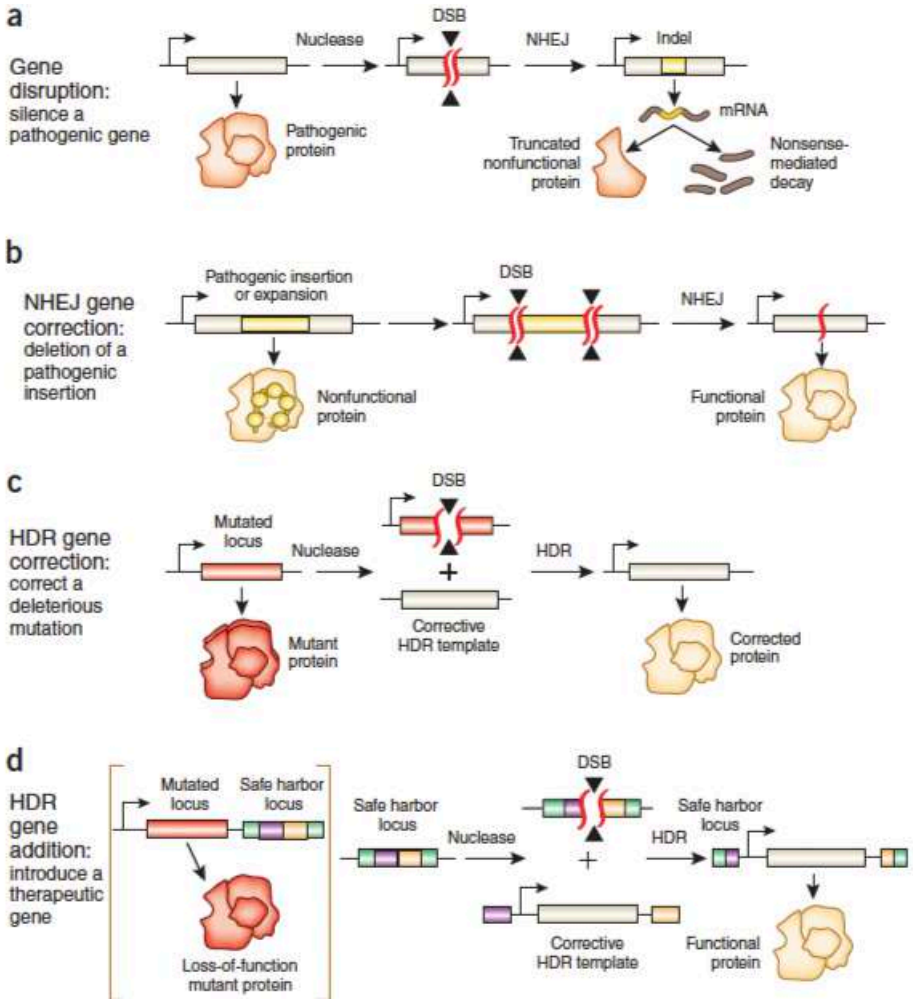



Fig. 1.13 Types of therapeutic genome modifications. The specific type of genome editing therapy depends on the nature of the mutation causing disease. (a) In gene disruption, the pathogenic function of a protein is silenced by targeting the locus with NHEJ. Formation of Indels in the gene of interest often results in frameshift mutations that create premature stop codons resulting in a nonfunctional protein product or nonsense-mediate decay of transcripts, suppressing gene function. Gene disruption may also be used to introduce protective loss of-function mutations into wild-type genes to generate a therapeutic effect. (**b**) In NHEJ gene correction, two DSBs targeted to both sides of a pathogenic expansion or insertion may be resolved by NHEJ, causing a therapeutic deletion of the intervening sequences. This form of treatment would require multiplexed targeting of disease-causing mutations. (**c**) HDR gene correction can be used to correct a deleterious mutation. A DSB is induced near the mutation site in the presence of an exogenously provided, corrective HDR template. HDR repair of the break site with the exogenous template corrects the mutation, restoring gene function. (**d**) An alternative to gene correction is HDR gene addition, which introduces a therapeutic transgene into a predetermined locus. This may be the native locus, a safe harbor locus or a non-native locus. A DSB is induced at the desired locus, and an HDR template containing sequence similarity to the break site, a promoter, a transgene and a polyadenylation sequence is introduced to the nucleus. HDR repair restores gene function in the target locus, albeit without true physiological control over gene expression (Turitz Cox et al., 2015).

Similarly, also loss-of-function mutations, such as those found in Tay-Sachs disease, would necessitate precise sequence changes by HDR gene correction. Genome editing also provides the possibility of introducing protective mutations into affected individuals to reverse illness. These known protective mutations may be loss of function, as in the case of CCR5 mutations in HIV, which can be introduced via NHEJ-mediated gene disruption or change of function as for APP in Alzheimer disease and therefore require correction by HDR. For deleterious loss-of-function mutations and protective gain-of- function mutations, a therapeutic effect may also be achieved by introducing a copy of the wild-type gene or gain-of-function mutant, respectively (Fig. 1.13 d). The therapeutic transgene may be inserted into a new locus, including identified 'safe harbor' loci (regions of the genome whose disruption does not lead to discernible phenotypic effects) to restore missing gene function. As previously described, Gene insertion may also be used to stably confer novel functions to specific cell types, as with the insertion of chimeric-antigen receptors (CAR) into T cells. Programmable nucleases may also be targeted to foreign DNA, such as viral genomes that are either integrated as proviruses or maintained extrachromosomally. Targeting of extrachromosomal DNA may lead to cleavage and subsequent destruction of viral genomes, while mutagenesis of the provirus genome at important coding sequences or regulatory regions may inactivate viral replication rendering latent viruses incapable of propagating infection. Additionally, multiplexed nucleases like Cas9 could be used to excise proviruses from the genomes of infected cells, leading to their degradation by cellular nucleases. Efforts to develop genome editing nucleases for antiviral therapy have focused primarily on HIV. One study demonstrated the possibility of targeting Cas9 to cleave LTR sequences of HIV, significantly reducing the expression of HIV genes in T cells (Hu et al., 2014). Similar strategies have shown promise with human papillomavirus (HPV) (Kennedy et al., 2014) and hepatitis B virus (HBV) (Lin et al., 2013; Bloom et al., 2014) but most infectious diseases face the same problem as HIV: currently, there are no therapeutic platforms capable of delivering genome editing nucleases to the majority of infected cells thus achieving

complete removal of the virus. Genome editing has been successfully applied to a number of diseases at the preclinical and clinical level (Table 1.3). An example of successful ex vivo editing therapeutic strategy, is that one utilized in the HIV treatment. In particular, in the clinical trials based on the use of the ZNF technology for the disruption of chemokine receptor type 5 (CCR5) (NCT00842634 and NCT01044654). In 1996, Alkhatib and colleagues proved that human chemokine C-C receptor 5 (CCR5) operates as co-receptor to enforce the interaction between HIV-1 gp160 and CD4+ T cells and to lead to membranes fusion (Alkhatib et al., 1996). In the same year, an independent group showed that people who carry in homozygosis a particular mutation in *CCR5* gene (the so-called *CCR5*-Δ32 allele) are perfectly healthy and largely protected from HIV-1 infection (Samson et al., 1996). Moreover, Thimoty Ray Brown (a.k.a. "The Berlin Patient"), a HIV seropositive adult living in Berlin, was completely cured from HIV after the developement of acute myeloid leukaemia (AML) following the treatment with allogeneic hematopoietic stem cell transplantation from a donor homozygous for *CCR5*-Δ32 mutation (Hutter et al., 2009). Thus, all these data gave rise to a strong rationale for the development of genome editing approaches in CD4+ T cells that aim to abolish the infectivity of R5-tropic strain exploiting ZFN technology through NHEJ-mediated site-specific gene disruption (Hoxie and June 2012). However, as expected, this strategy cannot lead to protection from HIV strains that can use C-X-C chemokine receptor type 4 (CXCR4) as co-receptor, but it restricts progression towards acquired immunodeficiency syndrome (AIDS) (Liu et al., 1996). For this reason, two different papers report the feasibility of an analogous strategy to disrupt *CXCR4* gene in place of or in addition to *CCR5* gene in CD4+ T cells (Wilen et al., 2011; Didigu et al., 2014). ZFNs provided, for the first time, an efficient and relatively simple platform for inducing site- specific mutations or modifications of genomes and are considered the most mature nuclease technology in the gene therapy field (Carlson et al., 2012). Early results from this trial suggest that genome editing through ZFNs of the CCR5 locus is safe, although the follow-up time has been too short to provide a full understanding of the risks and efficacy of treatment.

Another example of ex vivo editing therapy have also been successfully demonstrated in the previously mentioned study in which a mutated IL2RG gene was targeted for correction with ZFNs in hematopoietic stem cells (HSCs) obtained from a patient suffering from SCID-X1 (Genovese et al., 2014). First, HSCs were transduced using an integration-deficient lentivirus containing an HDR template encoding a therapeutic cDNA for IL2RG. Following transduction, cells were electroporated with mRNA encoding ZFNs targeting a mutational hotspot in IL2RG to stimulate HDR-based gene correction. This strategy resulted in gene- corrected HSCs from the SCID-X1 patient being obtained in culture at therapeutically relevant rates.

On the other hand, the first example of successful in vivo editing therapy was demonstrated in a mouse model of hemophilia B (Li et al., 2011). Restoring factor IX activity to above 1% of normal levels in severely affected individuals can transform the disease into a milder form, as infusion of recombinant factor IX into such individuals prophylactically from a young age to achieve such levels largely ameliorates the most severe bleeding complications (Löfqvist et al., 1997). In addition, factor IX is synthesized and secreted by the liver, an organ that can be transduced efficiently by viral vectors encoding editing systems. Using hepatotropic adeno-associated viral (AAV) serotypes encoding ZFNs and a corrective HDR template, up to 7% of mutated, humanized factor IX alleles could be genetically corrected in murine liver tissue (Li et al., 2011). This resulted in improvement of clot formation kinetics, a measure of the function of the clotting cascade, demonstrating for the first time that in vivo editing therapy is not only feasible but also efficacious in treating this condition. Building on this study, other groups have recently used in vivo genome editing of the liver with Cas9 to successfully treat a mouse model of hereditary tyrosinemia and to create mutations that provide protection against cardiovascular disease (Yin et al., 2014; Ding et al., 2014).

| Disease type | Nuclease platform | Therapeutic strategy |
|---|---|---|
| Hemophilia B | ZFN | HDR-mediated insertion of correct gene sequence |
| HIV | ZFN and CRISPR | NHEJ-mediated inactivation of CCR5 |
| Duchenne muscular dystrophy (DMD) | CRISPR and TALEN | NHEJ-mediated removal of stop codon, and HDR-mediated gene correction |
| Hepatitis B virus (HBV) | TALEN and CRISPR | NHEJ-mediated depletion of viral DNA |
| SCID | ZFN | HDR-mediated insertion of correct gene sequence |
| Cataracts | CRISPR | HDR-mediated correction of mutation in mouse zygote |
| Cystic fibrosis | CRISPR | HDR-mediated correction of CFTR in intestinal stem cell organoid |
| Hereditary tyrosinemia | CRISPR | HDR-mediated correction of mutation in liver |

Table 1.3 Examples of applications of genome editing to therapeutic models (Turitz Cox et al., 2015).

Besides therapeutic applications, genome engineering is progressively becoming a powerful and versatile tool for the tailored site-specific modifications of cells to address biological questions and/or develop improved biotechnology reagents. The availability of new technologies based on the "easy-to-use" CRISPR/Cas9 nuclease system, recently led to the application of genetic inactivation of the CCR5 locus in human embryos as a strategy to prevent HIV infection, which is endemic in China. As of November 2018, it has been announced that two "gene-edited" chinese twins are born. A detailed experimental report of this study is not yet available, however it has been claimed that one of the two twins is chimeric, while the other is homozygous for CCR5 gene disruption. With regard to this announcement, the scientific community has expressed serious scientific and ethical concerns, since no genetic manipulation system is spared from potential genotoxicity and the rationale for this study may not sustain the potential risks at which the born babies are exposed to, such as long-term genotoxicity, which can be even transmitted to the following generations and increased susceptibility to other infections and autoimmunity (Ajuebor et al, 2006; Bonfa et al, 2014). The debate around germ-line editing remains heated and international guidelines have been issued (2017).

## 1.4 X-linked hyper-immunoglobulin M immunodeficiency

The Hyper-immunoglobulin M immunodeficiencies (HIGM) are a group of primary immunodeficiency disorders characterized by defects that affect B-cell class switching. These patients have normal B- and T- cell development and normal or high serum levels of IgM, but make very limited antibody responses against antigens that require T-cell help. Thus, immunoglobulin isotypes other than IGM and IgD are produced only in trace amounts. Defects have been found in both T-cell helper function and in the B cells themselves. The most common form of hyper-IgM syndrome is X-linked hyper-IgM syndrome, or CD40 ligand deficiency (70% of HIGM1 patients) which is caused by mutations in the gene encoding CD40 ligand (CD154) (Korthauer et al., 1993). CD40 ligand is normally expressed on activated T cells, enabling them to engage the CD40 protein on antigen-presenting cells, including B cells, dendritic cells and macrophages. In males with CD40l deficiency, B cells are normal, but in the absence of engagement of CD40, their B cells do not undergo isotype switching or initiate the formation of germinal centers. These patients therefore have severe reductions of circulating levels of all antibody isotypes except IgM and are highly susceptible to infections by pyogenic extracellular bacteria. Because CD40 signaling is also required for the activation of dendritic cells and macrophages for optimal production of IL-12, which is important for the production of IFN-γ by Th1 cells and NK cells, patients with this deficiency also have defects in type 1 immunity and thus manifest a form of combined immunodeficiency. Inadequate cross-talk between T cells and dendritic cells via CD40L-CD40 interaction can lead to lower levels of co-stimulatory molecules on dendritic cells, thus impairing their ability to stimulate naïve T cells. These patients are therefore susceptible to infections by extracellular pathogens that require class-switched antibodies, such as pyogenic bacteria, but also have defects in the clearance of intracellular pathogens, such as mycobacteria (Hayashi et al., 1999), and are particularly prone to opportunistic infections by *Pneumocystis*

*jirovecii* (Levy et al., 1997; Winkelstein et al., 2003), which is normally killed by activated macrophages and *Cryptosporidium* (Hayward et al., 1997).

The CD40l gene is located on chromosome Xq26 and encodes for a type II transmembrane glycoprotein, with molecular weight that varies between 32 and 39 kDa because of post-translation modifications (Kooten et al., 2000). CD40LG is a member of the TNF superfamily and is characterized by a sandwich extracellular structure composed of a β-sheet, α-helix loop, and a β-sheet (Fig. 1.14). It is expressed in a trimeric form on the cell membrane and comprises a CD40 binding domain on the cell surface, a short transmembrane domain and a cytoplasmic tail. A soluble form of CD40LG with functions similar to those mediated by the transmembrane form, has been reported by different groups (Graf et al., 1995; Mazzei et al., 1995). *CD40LG* expression is very tightly regulated and it is primarily expressed by activated CD4+ T cells, as well as activated B cells and platelets; under inflammatory conditions it is also induced on monocytic cells, NK cells, mast cells and basophils. CD40L is also expressed on a number of additional immune-related cells, including γδ T, NK, NKT, and dendritic cells (DC) and macrophages, as well as nonimmune cells.

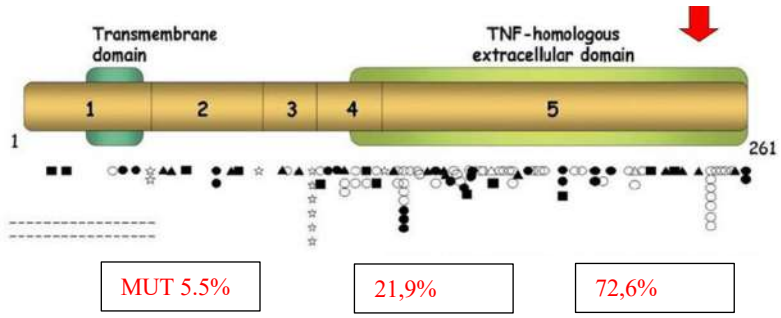



**Figure 1.14** Illustration of *CD40LG* gene. The protein domains are indicated in green, while numbers in black represent the exons that constitute the gene. The percentages written in red refer to the percentage of mutations reported in literature occurring in exon 1, exon 2/3/4 and exon 5.

### 1.4.1 CD40L-CD40 signaling

CD40LG is the ligand of CD40, a co-stimulatory receptor included in TNFR superfamily. CD40 is a 48 kDa type I transmembrane protein (van Kooten et al., 2000) and it is mainly expressed by B cells, but also by dendritic cells (DCs), monocytes, platelets, and macrophages as well as by non-hematopoietic cells such as myofibroblasts, fibroblasts, epithelial, and endothelial cells (Banchereau et al., 1995; Bourgeois et al., 2002; van Kooten et al., 1997). CD40-CD40LG receptor-ligand pair principally mediates signaling depending on adaptor proteins known as TRAFs (TNF receptor-associated factors), which are recruited to the CD40 cytoplasmic domain (Figure1.15). In addition to serving as simple adaptors that promote the assembly of multiprotein complexes, five of the six known TRAFs also function as E3 ubiquitin ligases. This activity contributes to activate two distinct NF-κB pathways resulting in the activation of canonical (NF-κB1) and non-canonical (NF-κB2) NF-κB, transcriptional regulators involved in B cell survival, maturation, proliferation and activation (Bishop et al., 2013). CD40L stimulation of CD40 also activates PI 3 kinase, which leads to the activation of Akt by PDK1 (IMMunology).

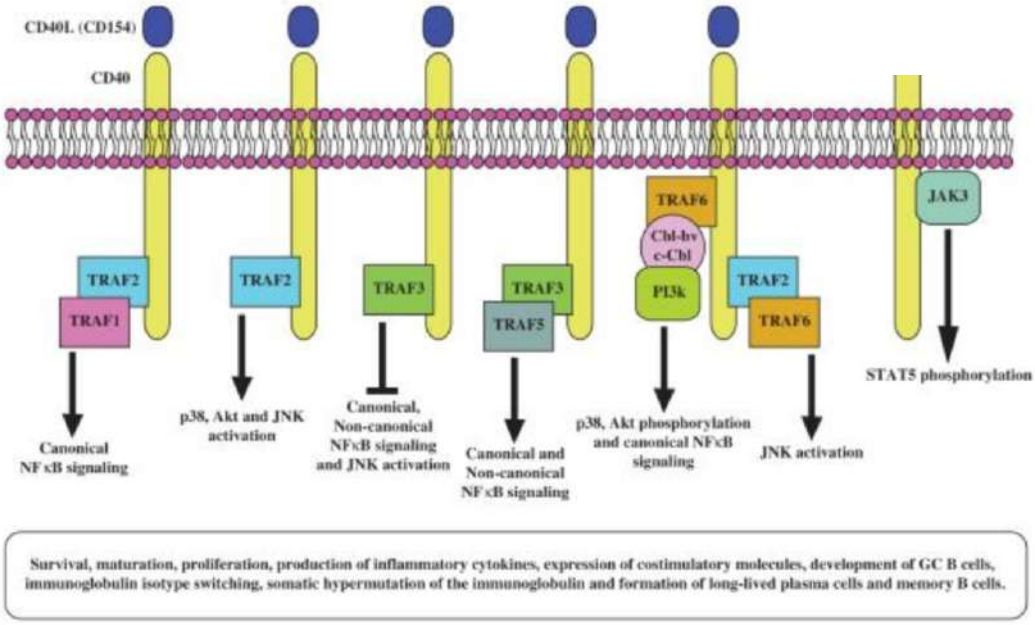


**Figure 1.15** CD40/CD40L interaction molecular signaling After CD40 activation, TRAFs 1, 2, 3, 5, and 6 are recruited to CD40 cytoplasmic tail, thus activating different signaling pathways. Also Janus family kinase 3 can bind the cytoplasmic tail (Elgueta et al., 2009).

### 1.4.2 CD40/CD40LG and its role in humoral immunity

In a thymus-dependent (TD) humoral immune response, B cells require CD40 signaling for the generation of high titers of isotype-switched, high affinity antibodies and for development of humoral immune memory. The physical interaction between antigen-activated B cells presenting the antigen in the context of MHC II and cognate CD4+ T- helper cells specific for the antigen presented results in an immunological synapse, in which the T cell provides to the B cell contact-dependent and –independent stimuli (Lanzavecchia et al., 1985; Rock et al., 1984). The binding between CD40/CD40LG is essential for the initiation and the progression of a TD humoral immune response. CD40 engagement triggers B cells proliferation, expansion, differentiation and antibody isotype switching *in vitro* (Banchereau et al., 1994; Barrett et al., 1991; Clark et al., 1986; Jabara et al., 1990). *In vivo*, CD40 engagement is required for GC formation and progression, as well as antibody isotype-switching and affinity maturation. All these processes are also essential for the generation of long-lived plasma cells and memory B cells (Foy et al., 1994; Foy et al., 1993). The blockade of CD40/CD40LG interactions or genetic knock-out of CD40 or CD40LG causes a complete loss of TD humoral immunity, while the thymus independent humoral immunity remains unaffected (Banchereau et al., 1994).

During infection with a pathogen, it enters the lymph and the proximal draining lymphoid organs or, if the infection is systemic, the spleen through the blood. The direct encounter of a cognate antigen by naïve follicular (NF) B cells, which resided in B-cells follicles, can occur, otherwise the antigen can be delivered to NF B-cells through tissue-resident DCs, that after antigen uptake in the periphery can migrate to secondary lymphoid organs (Bergtold et al., 2005; Pape et al., 2007; Wykes et

al., 1998). Lymphoid-resident DCs can also present processed antigen to Th cells through their MHC II in the T-cell zones of the lymphoid organs, leading to the clonal-activation of antigen-specific T cells. CD40/CD40LG interactions between DCs and T helper cells are necessary for DCs maturation and survival. Then, maturation and survival of DCs lead to expansion and differentiation of specific T-helper cells. The humoral response to a TD antigen has been well defined as a sequence of events that are precisely ordered in time. First of all, NF B cells are activated and an induction of different surface molecules can be observed hours after TD activation. After that, the cells begin to proliferate and to differentiate into plasmablasts secreting IgM and then IgG, located outside of the B-cell follicles (day 2-12 post-immunization). Activated B cells can also seed secondary follicles, rapidly proliferate and again interact with T helper cells forming germinal centers, in which isotype switching and the somatic hypermutation occur (day 9- 20). Germinal center B cells further differentiate into long-lived plasma cells and memory B cells containing high-affinity BCRs of the switched isotypes (day 20+) (Jacob et al., 1992; Jacob et al., 1991; MacLennan et al., 1994).

CD40LG plays an essential role in the events just presented: when DCs activate T helper cells in the T cell zone, the T cells transiently express *CD40LG* (Van den Eertwegh, 1993). Then, they position themselves at the border of B-cell follicles and T-cell zone where they interact with cognate B cells through CD40/CD40LG (Garside et al., 1998) (Figure 1.31). The helper T cells expressing *CD40LG* activate NF B cells to differentiate into plasmablasts. The blockade of CD40LG/CD40 interactions result in a complete ablation of plasmablast response (Foy et al., 1993; Figure 1.31). In parallel to this response, a subset of oligoclonal antigen-responding activated NF B cells colonizes the B-cell follicles where they form GC structures (Jacob et al., 1991; MacLennan et al., 1994; Figure 1.31). Whether a B cell will differentiate into a plasmablast or whether it will seed a GC is related to some B-cell intrinsic and extrinsic factors. The first include the affinity of the B cell for the antigen: a high initial affinity BCR results in a drive-force to B cells differentiation

in plasmablasts, whereas moderate affinity drives the B cells to seed the GC (Benson et al., 2007; O' Connor et al., 2006; Paus et al., 2006; Phan et al., 2006). CD40/CD40LG interaction is an extrinsic factor that impacts on B-cell fate. There is a range of CD40 signaling that is "acceptable" in inducing a GC response. In the absence of CD40 stimulation, no GC response is observed, but when CD40LG signaling is provided, a GC response initiates and matures. However, in the presence of enhanced CD40 stimuli during a primary immune response, B cells are selectively triggered to differentiate into plasmablast, and no GC is formed (Erickson et al., 2002). Then, CD40/CD40LG signaling is essential both for plasmablast differentiation and for GC initiation.

Once committed to the GC lineage, B cells encounter another specialized subset of activated T cells, called T follicular helper cells (FH), which provide cytokines and cell- contact signals, including CD40LG, necessary to sustain and propagate the GC reaction (Breitfeld et al., 2000; King et al., 2008; Schaerli et al., 2000). After the T/B cell encounter at the borders of the B-cell follicles, activated T helper cells can further differentiate into T FH cells, thus entering the B-cell follicles thanks to the heightened expression of CXCR5 chemokine (King et al., 2008; Ansel et al., 1999). T FH cells are characterized by CD40LG, ICOS and CXCR5 expression and by the secretion of IL-21 cytokine, which stimulate B- cell proliferation, isotype switching, and differentiation into plasma cells (King et al., 2008). CD40LG has been shown as critical for T FH cells functions. As the GC follicles mature, the structure becomes polarized into light and dark zones. The dark zone shows the presence of high density and rapidly dividing B cells, called GC centroblasts, and it is the place in which somatic hypermutation occurs (Rogerson et al., 1991). CXCL12 is the chemokine secreted by dark zone resident stromal cells and allows to the GC centroblast, which express CXCR4, to home into this zone (Allen et al., 2004; Allen et al., 2007). The GC light zone is composed by follicular dendritic cells (FDCs) and stromal cells which secrete CXCL13, the ligand for CXCR5, thus it is here that T FH cells are found, exploiting CXC13 gradient. The B cells into the

light zone are called centrocytes and are characterized by the expression of CXCR5. Within this zone, the interactions between B cells, T helper cells and complexed antigens occur and result in the centrocytes selection, based on the highest affinities for antigen: they receive survival and differentiation signals through their BCR and CD40 engagement (Allen et al., 2004; Allen et al., 2007). In fact, the engagement of CD40 by centrocytes protects the cell from Fas-mediated apoptosis by inducing Bcl and c-FLIP, two anti-apoptotic proteins, however other signals may exist (Liu et al., 1989; Hennino et al., 2001; Tuscano et al., 1996). These germinal centre B cells exit the germinal centre, either as memory B cells, or as long-lived plasma cells that contribute to serological memory (King et al., 2008; Figure 1.16). Concluding, it is clear that CD40 signaling is fundamental for GC response: blockade of this signaling or genetic ablation of the genes prevents the clonal proliferation of B cells as well as the formation of GC structures. Consequently, antibody isotype switching and affinity maturation result impaired, leading to a loss of the generation of long-lived plasma cells and memory B cells. From a molecular point of view, as discussed previously in this manuscript, after CD40 activation different TRAFs protein could be recruited on the CD40 cytoplasmic tale. The contribution of these factors in regulating humoral immunity has been studied *in vivo* through transgenic mice containing CD40 receptor with mutation in the cytoplasmic binding sites for TRAF proteins. Through disruption of TRAF6-binding site, an ablation of affinity maturation and generation of plasma cells occur. Mutagenesis of TRAF6, TRAF2- TRAF3 binding sites arrest GC formation. In contrast, CD40-induced B cell proliferation and early Ig production occurred even when all TRAF sites where ablated, thus suggesting the existence of other functional sites in the CD40 cytoplasmic tail (Elgueta et al., 2009).

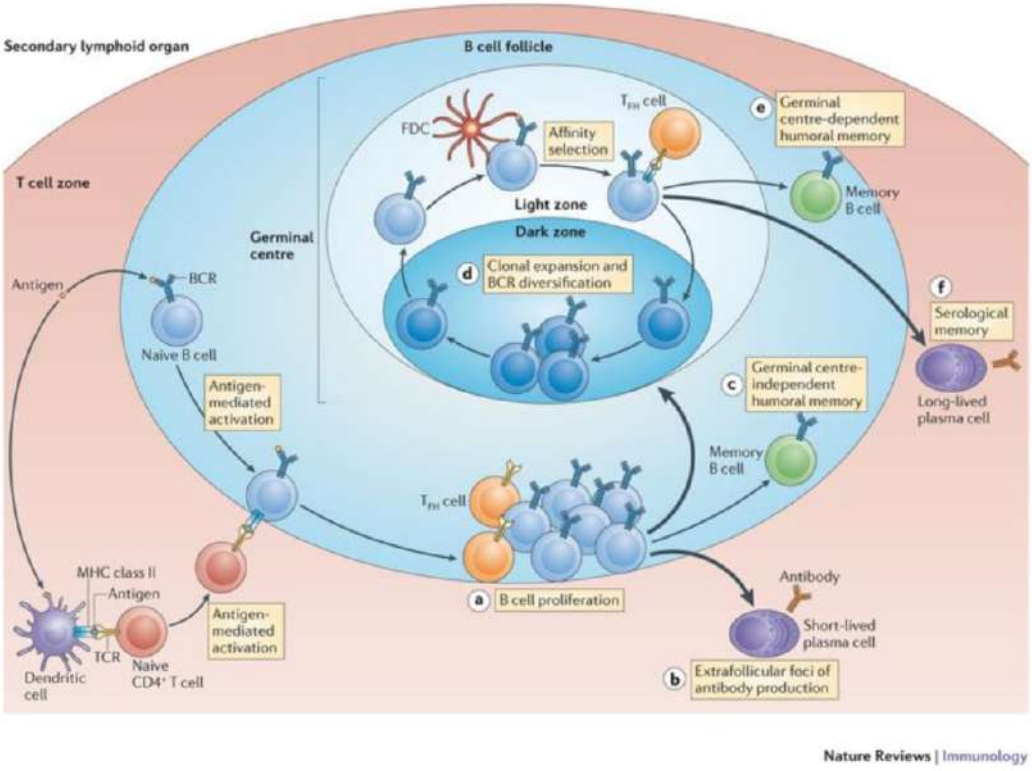



**Figure 1.16** Antigen-activated B cells migrate at the border of the B cells follicles, whereas T cells migrate at the border of the T cell zones in secondary lymphoid organs, thus leading the two players to establish stable interactions. B cells receive helper signals from cognate $CD4^+$ T cells. Activated B cells and T cells then move to the outer follicles. Here, B cells proliferate (part a). Some of these proliferating B cells differentiate into short- lived plasma cells (part b), which give rise to the extrafollicular foci, and some other become memory B cells (part c; germinal centre-independent memory B cells). Alternatively, the activated B cells can return to the follicle and can rapidly proliferate, thus forming the germinal centre (part d), which is composed by a dark and a light zone. In the dark zone, antigen-specific B cells undergo clonal expansion and B cell receptor (BCR) diversification through somatic hypermutation. When released from cell cycle, B cells move to the light zone, where affinity selection takes place, through interaction with immune complex-coated follicular dendritic cells (FDCs) and antigen-specific T follicular helper cells (TFH cells). At this point, the affinity-matured B cells can either re-enter the germinal centre cycle or exit the germinal centre, as memory B cells (part e; germinal centre-dependent memory B cells) or as long-lived plasma cells (part f). These two cell types contribute to serological memory. B cells can receive different signals, which possibly determine their fate: stronger signals (indicated by bold arrows) favour development into plasma cells or germinal centre B cells, whereas weaker signals (indicated by narrow arrows) determine memory B cell differentiation. TCR=T cell receptor. (Kurosaki et al., 2015)

### 1.4.3 CD40/CD40LG and its role in cell-mediated immunity

While the role of CD40/CD40LG signaling in the TD humoral immunity is well known, its function in cell-mediated immunity (CMI) is less clear. There is unquestionable evidence that the development of some CMI responses requires this signaling. This is mostly evident in the role of this receptor-ligand pair in the development of cytotoxic T cells to tumors, virus and in the development of some T-cell-dependent autoimmune diseases. To give some examples, it has been reported in 2013 that *CD40LG* expression and other "helper T-cells characteristics" are a feature of not only CD4+ helper T cells, but also of a subset of MHC I restricted CD8+ T cells, mainly of a distinct subset of memory CD8+ T cells. The fact that *CD40LG* expression is a feature of resting memory CD8+ T cells supports the notion that these cells may exert their helper functions not only in the first effector phase of immune response, but also during a secondary challenge. One of the functions of *CD40LG* during this secondary pathogen encounter could be to protect APCs from cytotoxic T-cells mediated elimination, as reported for CD4+ T cells. Moreover, these cells show a cytokine profile very similar to CD4+ helper T cells. In fact, the majority of CD8+ T cells that express *CD40LG* do not produce IFN-g, however in human virus specific responses IFN-g expressing CD8+ cells has been reported. Thus, it suggests the existence of different *CD40LG*-expressing CD8+ cells subset, in analogy to CD4+ T cells subsets (Frentsch et al., 2013).

Concerning the role of CD40LG/CD40 interaction in tumor, the first report of importance of this pathway was published in 1997. It was shown that anti-CD40LG monoclonal antibody treatment inhibited the generation of protective immune response and prevented the therapeutic value of potent tumor vaccine. These results were also confirmed in CD40LG- deficient mice: they were unable to generate a protective anti-tumor immune response following a protective vaccination regime (Mackey et al., 1997). Based on this data, early studies showed that CD40 agonistic antibody generated CTL responses able to eradicate the tumor in a lymphoma system, and able to increase anti-tumor vaccine efficacy (French et al., 1999; Diehl

et al., 1999; Sotomayor et al., 1999). Going on, in 2014, it has been found that direct T cells activation via CD40LG generates high avidity CD8+ T cells against endogenous tumor antigens. After repeated CD40LG co-stimulation in mice, low avidity CD8+ T cells improved functional avidity, tumor killing, proliferation and they demonstrated higher TCR expression on their surface. Furthermore, CD8+ T cells increase anti-apoptotic signals, thus decreasing pro-apoptotic ones. Moreover, memory T cells demonstrated higher stem-cell like phenotypes. As a result, a robust immune response breaking immunological tolerance was generated, thus controlling tumors in two different mouse models (p53 and gp100 as tumor associated antigens; Soong et al., 2014). However, the efficacy of a single agent was questioned. It is clear that robust development of adaptive immunity relies on the interplay of both innate and adaptive immunity, thus the contribution of TLR agonists to CD40 stimulation was evaluated. It was reported that TLR agonists used in combination with CD40 agonists provided a therapeutic efficacy in different tumor models (Ahonen et al., 2008).

### 1.4.4 CD40LG deficiency: clinical features

Since *CD40LG* is a gene located on chromosome X, the disease predominantly manifests in male subjects, even if occasional symptomatic female carriers with skewed lyonization (skewed X chromosome inactivation occurs when the inactivation of one X chromosome is favored over the other, leading to an uneven number of cells with each chromosome inactivated) have been reported. Patients usually present in early childhood (with a median age of <12 months) with recurrent respiratory tract infections and Pneumocystis jirovecii pneumonia (PCP).[12,13] PCP is a presenting feature of this syndrome in around 40% of cases. In the presence of normal T lymphocyte counts and a negative human immunodeficiency virus test, this will be the most likely underlying diagnosis in male infants presenting with PCP. Chronic cryptosporidial infection is another common infection in these patients: symptomatic chryptosporidiosis may occur, leading to failure to thrive and

weight loss with persistent diarrhea. Molecular studies for cryptosporidium infection, based on PCR of parasite DNA in patients with CD40LG deficiency suggest that subclinical infection is common and in many cases the organism is not detectable by stool microscopy, but only by PCR (McLauchlin et al., 2003). Diseases of the bile ducts (cholangiopathy), with the organism found in biliary tree, are a common complication of both clinical and subclinical infections. The results could be the development of cholangitis potentially leading to cirrhosis with risk of cholangiocarcinoma (Hayward et al., 1997; Rodrigues et al., 2004) and disturbed liver function, mainly tested with raised γ glutamyl transferase levels. In early series not treated with bone marrow transplantation, chronic liver disease was a feature in 50% of patients and was responsible for early death in many cases (Levy et al., 1997). Liver transplantation has been attempted, but with very poor results and recurrence of the disease in the transplanted liver. A single success of combined bone marrow and liver transplantation has been reported in 2000 (Hadzic et al., 2000). CD40/CD40LG interaction has been described to be important also in the handling and clearance of mycobacteria. However, tuberculosis is uncommon in patients (Levy et al., 1997; Winkelstein et al., 2003). An increased frequency of central nervous system infections (enteroviral meningoencephalitis[15] and JC virus progressive multifocal leukoencephalopathy),[16] often resulting in neurodegeneration,[12,17] has been reported. Other reported infections are cerebral toxoplasmosis, cryptococcosis, and *Cytomegalovirus*. Handling of CMV infection can be problematic in these patients: disseminated infection can be seen as an initial presenting illness. CMV has also been implicated in some cases of chronic sclerosing cholangitis (Levy et al., 1997; Winkelstein et al., 2003; Hayward et al., 1997). Lastly, *Parvovirus* infection was described in three cases with a partial *CD40LG* expression, thus leading to a late presentation of the disorder. In all cases anaemia was reported, which resolved upon commencement of immunoglobulin therapy (Seyama et al., 1998). Another common complication in CD40LG deficiency is neutropenia. It occurs at some stage in 50% of cases (Levy et al.,

1997). The clinical course of neutropenia may be transient or it may be prolonged and persistent. The cause of the neutropenia is not well understood. No anti-neutrophil antibodies are detected. However, since early myeloid progenitors express CD40 and ligation has been shown to stimulate myelopoiesis, lack of CD40LG and thus lack of CD40-mediated stimulation of precursors may play a role. Early reports reported that treatment with high doses of immunoglobulin helped resolve the neutropenia, but in the wider European experience, it was useful only in around half the cases (Banatvala et al., 1994; Levy et al., 1997). Also autoimmune complications are common in CD40LG deficiency-patients. Patients' mature naïve B-cells were shown to express auto-reactive antibodies suggesting a role for CD40/CD40LG interaction in mediating peripheral B-cell tolerance (Herve et al., 2007). Some cases of autoimmunity have been reported in different studies. In the Levy study seronegative arthritis affected 11% of patients and inflammatory bowel disease affected 6% of cases. Three patients showed thrombocytopaenia, and one autoimmune haemolytic anaemia. Other patients presented a variety of autoantibodies, however they were not associated with any disease at the time of the analysis (Levy et al., 1997). Schuster and collegues have reported other occasional cases of autoimmune disease in CD40LG deficiency (Schuster et al., 2005), while in the North American series 15% of patients had anaemia, even if insufficient detail was reported to be sure about the autoimmune nature of the disease (Winkelstein et al., 2003). Finally, patients with CD40LG deficiency suffer an excess risk of developing malignant diseases, affecting predominantly the biliary tree and the intestine (Hayward et al., 1997; Levy et al., 1997).

#### 1.4.4.1 Host immune response to Pneumocystis

*Pneumocystis* can infect a variety of mammalian species. Each species is infected by a genetically distinct member of the genus: *P. jirovecii* infects humans, *Pneumocystis carinii* and *Pneumocystis wakefieldiae* infect rats, and *Pneumocystis murina* infects mice. Key features of *P. murina* infection have rendered it experimentally useful for studying immunity to *Pneumocystis*, in part

as a result of the availability of mice with defined immune defects. Following exposure to the organism, immunocompromised murine hosts, such as those deficient in CD40 ligand (CD40L), develop a progressive pulmonary disease, which histologically and clinically resembles human *Pneumocystis* pneumonia (PcP) (Hernandez-Novoa et al., 2008). Among the genetic defects that have been studied to date in mouse models that do not lead to global loss of cell populations (e.g., Rag-deficient or *scid* mice), only the CD40-CD40L interaction has been shown to be absolutely required for ultimate control of *Pneumocystis* infection, since KO mice deficient in either CD40 or CD40L are highly susceptible to severe infection (Hernandez-Novoa et al., 2008, Bishop et al., 2005, Furuta et al., 2001). Increasing evidence suggests that the life stage form of Pneumocystis influences the host immune response (Evans et al., 2016; Evans et al., 2017). The Pneumocystis lifecycle has an infective ascus form and an asexual trophic form. The ascus form has up to eight ascospores and a cell wall consisting of the major surface glycoproteins (MSG), β-1,3 glucan and β-1,6 glucan. β-1,3 glucan is the most abundant and is the primary pro-inflammatory factor. Like the ascus form, the asexual trophic form expresses MSG but not β-glucans (Kottom et al., 2015). Asci β-glucan serves as a pathogen-associated molecular pattern (PAMP) that is recognized by pattern recognition receptors (PRRs) on phagocytic cells. This recognition is essential in inducing the host immune response by stimulating interferon gamma (IFN-γ) production by CD4+ T cells to drive Pneumocystis clearance, as shown in Figure 1.17. In contrast, the trophic form hinders dendritic cell proinflammatory cytokine production elicited by β-1,3-glucan, thereby suppressing the immune response. Evans et al. proposed that suppression could indeed be beneficial for immunocompromised hosts in that the lung pathology associated with PCP is reduced (Evans et al., 2016; Evans et al., 2017). Furthermore, T cells primed in the presence of the trophic form can effectively mediate the clearance of both trophic and ascus forms.

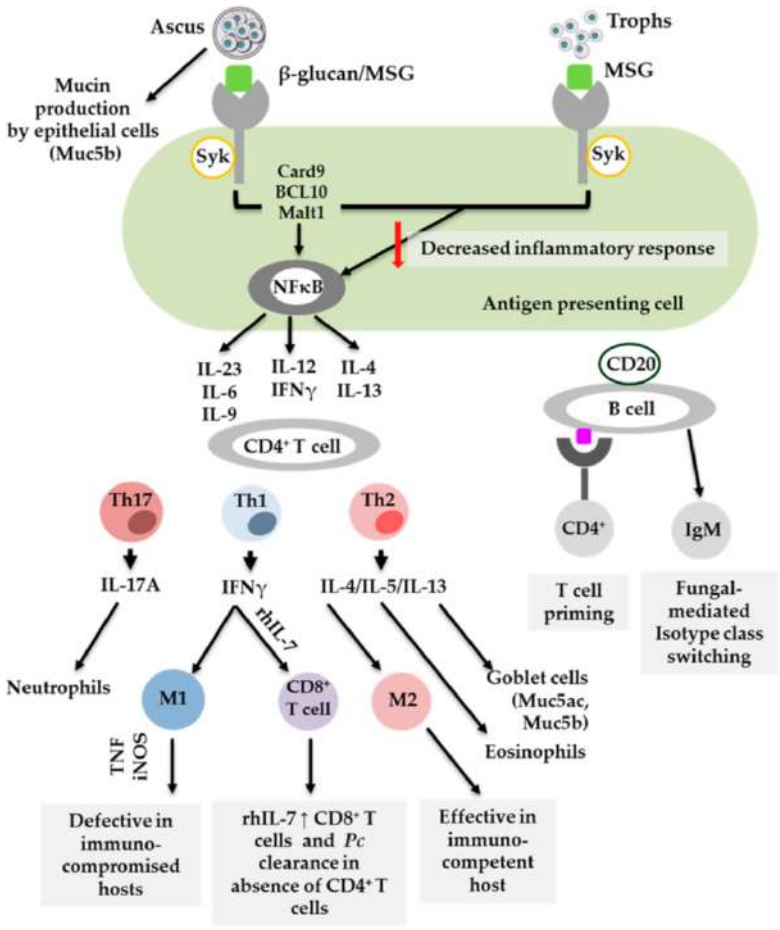



**Figure 1.** Cells which contribute to the host immune response to *Pneumocystis*.

Fig. 1.17 Cells which contribute to the host immune response to Pneumocystis (Otieno-Odhiambo et al, 2019).

Multiple types of cells have been shown to contribute to the host immune response to Pneumocystis. Carmona et al. found that human DCs stimulated by *Pneumocystis*-derived β-glucans interact with lymphocytes to produce IL-17 (Carmona et al., 2012). In contrast, Sassi et al. demonstrated that MSG seems not to be involved in DC activation thus suggesting that the trophic form may actually hinder effective DC activation, resulting in impaired $CD4^+$ T cell responses. Alveolar macrophages (AMs) are key resident effector cells of alveolar spaces and critical in the clearance of lung pathogens, including *Pneumocystis*. Upon activation by host cytokines, such as IFN-γ, TNFα and granulocyte macrophage colony

stimulating factor (GM-CSF), AMs destroy *Pneumocystis* through the production of reactive oxygen and nitrogen species. Recent studies investigating the response of macrophages to *Pneumocystis* infection suggested that the type of macrophage activation could also be significant and could affect the level of clearance. In particular, a study dissecting the role of M1/M2 macrophages in *Pneumocystis* infection showed that immunocompetent rats, exhibiting M2 macrophages, cleared the *Pneumocystis* infection effectively, while immunosuppressed rats exhibited a predominantly M1 phenotype, which resulted in defective *Pneumocystis* clearance (Nandakumar et al., 2017). $CD4^+$ T cells are crucial for *Pneumocystis* clearance because of their ability to recruit and activate effector cells, like macrophages (Cushion et al., 1988, Evans et al., 2016). However, the contribution of specific $CD4^+$ T cell subsets needs further investigation. T helper cells, namely, Th1, Th2 and Th17 seem to be activated by Pneumocystis in infected mice. In particular, Th17 has been shown to be involved in the recruitment of $CD4^+$ T cells producing interleukin 17A (IL-17A) in the lungs of infected animals. In addition, neutralization of IL-17A results in a significant increase in *Pneumocystis* lung burden at later stages of infection and mice lacking nuclear factor (NF)-κβ signalling within the lung epithelium exhibit impaired fungal clearance that is associated with reduced lung IL-$17^+$ $CD4^+$ T cells (Perez-Nazario et al., 2013; Limper et al, 1997; Ripamonti et al, 2017). $CD8^+$ T cells are thought to work in conjunction with $CD4^+$ T cells to elicit an effective immune response against *Pneumocystis*. The role of $CD8^+$ T cells in the absence of $CD4^+$ T cells remains controversial, with reports of both protective and detrimental responses. De la Rua and colleagues suggested that secondary immune responses to *Pneumocystis* involve $CD8^+$ T cells and alveolar macrophages (De la Rua et al., 2016). Finally, susceptibility to *Pneumocystis* is also influenced by B cells, which play a dual role in antibody production and antigen presentation (Kolls et al., 2017). In particular,

the role of antibodies in protection against acquisition of infection has been demonstrated by passive transfer of immune sera in mice (Gigliotti et al., 2002) and by vaccination in immunosuppressed non-human primates (Kling and Norris, 2016). Additionally, the suggestion that the IgM isotype has a predominant role in recognition of Pneumocystis infection (as well as in B cell isotype class-switching and Th2 and Th17 promotion of differentiation) both in mice (Rapaka et al., 2010) and in humans (Djawe et al., 2010; Tomás et al., 2016), further supports the role of antibodies in disease protection. Finally, two other studies demonstrated that B–T cell interactions are important for the generation of both effector and memory CD4+ T cells in Pneumocystis infection and that these B cells play an important role in early priming of CD4+ T cells (Lund et al., 2006; Opata et al., 2015).

### 1.4.5 CD40LG deficiency: therapeutic options

Up to date, the therapeutic options that are available for patients with CD40LG deficiency are mainly immunoglobulin replacement therapy and bone marrow transplantation. Immunoglobulin replacement therapy should be initiated on diagnosis, and it permits to largely correct the clinical consequences of humoral immunodeficiency. However, historically, long-term survival with conservative therapy has been poor, with 20% to 50% of patients surviving to the third decade. (Rezaei and Notarangelo, 2013) Hepatic disease and severe infections represent the major causes of death, (Levy et al., 1997) and many patients have chronic comorbidities. (Rezaei and Notarangelo, 2013) An alternative approach to treat the X-linked HIGM disease could theoretically be the administration of recombinant soluble CD40LG, however the potential clinical problems associated with unregulated ligation of CD40 strongly mitigate against this strategy (Mazzei et al., 1995).

Currently, the only curative treatment is hematopoietic stem cell transplantation (HSCT). Numerous published case reports and single-center experiences report

encouraging results, especially with an HLA-matched sibling donor (MSD). However, there is a risk of complications, and overall survival (OS) is not optimal. In the European retrospective analysis of 38 patients with CD40L deficiency receiving HSCT, OS was 68%, but 10% had autologous reconstitution. In the end, one of these patients achieved full engraftment after a second procedure. Moreover, one patient had extremely poor immunological reconstitution despite reaching a full donor engraftment. 32% of patients died from infection-related complications, particularly severe cryptosporidiosis, but no correlation has been found between donor type or conditioning regimen and the occurrence of infection. Transplantation was curative in 58% of patients. The cure rate was better in patients without liver disease (72%), even though the presence of a liver disease was not a predictor of survival. On the contrary, pre-existing lung disease and the use of a mismatched unrelated donor correlated with a decrease in survival (Gennery et al., 2004).

In another study on 130 patients who underwent HSCT for CD40L deficiency between 1993 and 2015, overall survival (OS), event-free survival (EFS), and disease-free survival (DFS) were 78.2%, 58.1%, and 72.3% 5 years after HSCT. Significantly better survival was observed in transplantations performed in 2000 or later, when improvement in diagnostic tools and clinical management allowed transplantation at a younger age, with a shorter time interval after diagnosis and lower organ damage burden. Indeed, a significant outcome improvement was observed in the past years in children less than 10 years old at the time of HSCT, in those undergoing transplantation within 2 years of the diagnosis of CD40L deficiency and in those without organ damage. In patients treated before 2000, pre-existing organ damage (mainly chronic lung disease, liver dysfunction, or both) before HSCT negatively influenced survival. Liver disease, especially sclerosing cholangitis, was the most important adverse risk factor, followed by protracted diarrhea and gastrointestinal infection by Cryptosporidium species. These clinical features were confirmed to negatively influence outcome

also in patients undergoing most recent transplantations, even if less profoundly. The presence of chronic lung disease, previously a significant risk factor (Gennery et al., 2004), did not significantly influence survival in recent transplantations. Type of CD40L gene mutation, previous clinical history of respiratory tract infections, including Pneumocystis jirovecii pneumonia, requirement of ventilation before transplantation, neutropenia, oral ulcers, failure to thrive, and absent Cryptosporidium species prophylaxis before HSCT had no significant influence on OS. Use of myeloablative regimens and HSCT at 2 years or less from diagnosis associated with higher OS and DFS. EFS was best with matched sibling donors as compared with unrelated or mismatched donors, as well as with myeloablative conditioning (MAC), and with bone marrow–derived stem cells as compared with peripheral-blood or cord-blood derived stem cells. Most rejections occurred after reduced-intensity (RIC) or nonmyeloablative conditioning, which associated with poor donor cell engraftment. Twenty-six deaths were reported, most occurred within 6 months (n=20), mainly due to infections, graft rejection or progression of pre-existing neurologic disease. Among survivors who ceased immunoglobulin replacement, T-lymphocyte chimerism was complete or predominantly donor but unfortunately, a minimum T-cell donor percentage reliably associated with immunoglobulin independence could not be retrieved based on available data. Interestingly, a higher percentage of complete donor chimerism (63.2%) was observed in transplantations in which patients did not experience viral infection after HSCT. Moreover, a decrease in T-lymphocyte chimerism was detected in patients in which viral infection occurred after HSCT, likely favoring the expansion of autologous T lymphocytes to replenish the niche (Ferrua et al., 2019).

### 1.4.6 Gene therapy approach for the treatment of HIGM1

Gene therapy approaches for CD40LG deficiency were tempted. In 1998 Brown and colleagues published a work in which murine bone marrow or thymic cells were transduced with retroviral vector containing the cDNA for the murine *CD40LG* and then transplanted in CD40LG-deficient mice. Three groups of mice were treated, either following lethal irradiation or not. Even low-levels of constitutive expression of the transgene stimulated humoral and cellular immune functions in these mice, upon infection with an attenuated strain of influenza A virus or upon a vaccination against a thymus- dependent antigen. Unfortunately, 63% of the mice developed T-lymphoblastic lymphomas involving thymus, spleen and other organs, at 6-9 months after transplantation. Since a group didn't receive irradiation but similarly developed lymphoblastic lymphomas of thymic origin, tumor induction was not associated with prior irradiation. Moreover, several other analyses support the conclusion that thymocytes carrying *CD40LG* transgene were responsible for neoplastic transformation in the thymus, but insertional mutagenesis was an unlikely contributor to lymphomagenesis because of only a minor proportion of tumor cells contained pro-viral DNA (Brown et al., 1998).
In 2000, Sacco and collaborators demonstrated that when the *CD40LG* gene was placed under the control of a T cell restricted promoter, its deregulated expression perturbs the homeostasis of the lymphoid subsets of lymph nodes, and generated a hyperplastic B cell expansion associated with a high risk of lymphoma progression. By performing an immunological phenotype of the lesion, the pattern was consistent with expanded mature B-cell population that has underwent CSR, thus suggesting that the neoplastic transformation occurred in the expanded B-cell population. In fact, the unregulated *CD40LG* expression caused the slow B-cell expansion and this finding was not unexpected, because it is known that *CD40LG* signaling is critical both in GC formation and GC maintenance. Since CD40-CD40LG interaction is known to be an anti-apoptotic stimulus, failure of the B cells to undergo apoptosis is suggested by the authors as a possible mechanism for abnormal B cells proliferation (Sacco et al., 2000).

In 2004, Tahara and co-workers achieved promising results by correcting *CD40LG* gene using a trans-splicing approach: bone marrow from mice lacking CD40LG was transduced with a lentivirus trans-splicer encoding the normal *CD40LG* exons from 2 to 5 and was transplanted into lethally irradiated syngenic CD40LG knock-out mice. Trans-splicing was determined to be 1.2% mean. Treated mice immunized with a thymus-dependent antigen showed production of specific IgG1 and a partial restoration of humoral immunity *in vivo*, suggesting that a low number of corrected T cells allows expansion of functional B cells. Moreover, treated mice infected with P. Carinii induced attenuation of the inflammation and of the pathogen infection in lungs. Finally, by 12 months after transplantation none of the mice had developed lymphoproliferative diseases, confirming that a regulated expression of *CD40LG* is required to develop a safe therapeutic approach and to avoid B or T lymphoproliferative diseases that can progress to frank lymphomas (Tahara et al., 2004). However, higher efficiencies of trans-splicing correction are necessary to confirm the safety of this approach and possibly to consider it for a clinical application.

Taken together, all these published results are highly relevant to the field of human gene therapy, because they highlighted that the tight control of the expression of highly regulated genes, as *CD40LG*, is essential. Thus, employing not only of the structural gene but also of the elements for its regulating expression is needed (Brown et al., 1998). Moreover, gene-editing strategies that can reconstitute physiologic expression control of the corrected gene might represent a more promising approach for the correction of diseases caused by tightly regulated genes.

### 1.4.7 CD40LG deficiency: why a gene editing approach?

As described in the previous chapter, *CD40LG* gene therapy approach showed evidence of efficacy. In Brown's and Tahara's work, cell-mediated and humoral responses against pathogens were fully or partially restored, in addition partial or

full thymus-dependent immunoglobulin reconstitution has been achieved, even starting from few *ex vivo* corrected cells and without conditioning in the first case, thus recapitulating that a low number of corrected CD4+ T cells should be sufficient to allow the expansion of functional B cells (Brown et al., 1998; Tahara et al., 2004). However, a physiological expression of the gene is fundamental to avoid the development of B or T lymphoproliferative diseases that can progress to frank lymphomas (Brown et al., 1998; Sacco et al., 2000). Taking into consideration all the pre-clinical results achieved with different gene therapy approaches, all the critical safety issues involved and the necessity of a tightly regulated gene expression (Brown et al., 1998), a gene editing approach could be promising for the correction of CD40LG deficiency. In fact, gene editing by exploiting nuclease mediated HDR to *in situ* correct *CD40LG* mutations, should allow to restore both physiological function and expression of the gene, which could be controlled by its own endogenous promoter. Since in HIGM1 the genetic defect is not lethal to T cells, this disease offers the great opportunity to develop not only a therapy approach based on gene-corrected autologous HSPCs, but also an adoptive T cells therapy approach with autologous corrected T cells. The capacity of T-cells to expand in large number with well-established clinical grade procedures and their resistance to oncogenic transformation after genetic manipulation (Newrzela et al., 2008), make these cells suitable for a first-in-human testing and validation of a gene correction strategy based on engineered endonucleases. Moreover, since even a low number of corrected CD4+ T cells could allow the expansion of functional B cells (Brown et al., 1998), they may be sufficient to restore the formation of oligoclonal but functional germinal centers in lymph nodes and spleen. Thus, this approach may induce the generation of protective immunity in recipient HIGM1 patients and possibly also the production of some long living memory B cells. However, multiple administrations of edited T-cells may be required to reconstitute a long-lasting and broad T cell repertoire that can mediate efficient T cell help in response to a wide range of pathogens. Additionally, even if CD40LG has a major critical function on CD4+ T cells, this molecule is also expressed on several other

hematopoietic cell types, such as activated B cells, platelets, NK cells, monocytes, basophils and eosinophils (Schonbeck and Libby, 2001). Thus, an adoptive T cell therapy approach may be an effective "bridge therapy" that prepares the patient for the definitive cure by an HSC transplant. As a matter of fact, it will be important to expand the gene editing strategy from the correction of T-cells to the correction of autologous HSPC, which can provide a much broader and prolonged therapeutic benefit.

### 1.4.8 CD40LG deficiency: published gene-editing strategy

In 2016, Hubbard and colleagues published a first attempt of gene editing strategy for the treatment of X-linked HIGM syndrome. A TALEN pair targeting the 5'UTR in the first exon of *CD40LG* (Figure 1.18, top) and a donor template within AAV packaging constructs were designed. TALEN-binding sites were deleted from donor template to avoid cleavage of the donor DNA as well as later cleavage of modified genomic DNA, and a GFP cassette was introduced downstream of the first ATG of *CD40LG* with a 2A-linked *CD40LG* cDNA directly downstream of GFP, followed by endogenous 3'UTR and polyA putative sequences (Figure 1.18, bottom). These templates were expected to reconstitute a controlled physiological *CD40LG* expression and GFP expression in corrected cells, conserving upstream promoter and intronic regulatory elements downstream of the inserted cDNA sequence. Moreover, *CD40LG* cDNA sequence was sequence diverged with synonymous mutations to avoid the possibility of unpredictable HDR between the gene-editing template and gDNA. T cells obtained from X-HIGM patients, who have *CD40LG* mutations resulting in decreased/absent *CD40LG* expression, were edited using the described TALEN and donor template, reaching a 14.3% editing rates mean. The surface expression of CD40LG on edited T cells was monitored over a 54-hour time course following a 2-hour PMA/Ionomycin activation: *CD40LG* expression in both non- edited healthy and edited cells peaked between 3 and 8 hours post-activation and return to baseline by 24 to 54 hours. Both the averaged MFI and the percentage of cells expressing CD40LG were comparable

throughout the time course for all 3 X-HIGM donors. However, edited cells showed lower levels of *CD40LG* expression both in terms of MFI and percentage compared to the unedited healthy cells, thus not reaching physiological expression levels. The authors also performed an *in vitro* class-switching assay to test the function of edited T cells in inducing naïve B-cell CSR: activated X-HIGM CD4+ T cells co-cultured with allogenic naïve B cells failed to induce B cells to undergo IgG class switch *in vitro*, while the function was restored following gene editing, at rates similar to those seen with activated healthy donor T cells. Thus, these findings demonstrated restoration of functional CD40LG in edited cells, despite the lower levels of *CD40LG* expression. To demonstrate the survival and the stability of edited compared with non-edited T cells, the authors performed an adoptive transfer *in vivo* of treated and non-treated T cells into NOD- scid IL2RG-/- (NSG) recipient mice. Editing percentage (GFP percentage) of transferred cells remains stable and edited CD4+ T cells recovered from spleens retained the ability to express surface CD40LG upon PMA/Ionomycin stimulation, thus demonstrating functional and genetic stability. Finally, a TCRVβ spectratyping was performed on non-edited and edited cells, showing no differences in overall complexity, which remained at high levels (Hubbard et al., 2016). Taken together, these data support the potential of this methodology and the hypothesis that the transfer of edited T cells could provide protective immunity in X-HIGM patients.

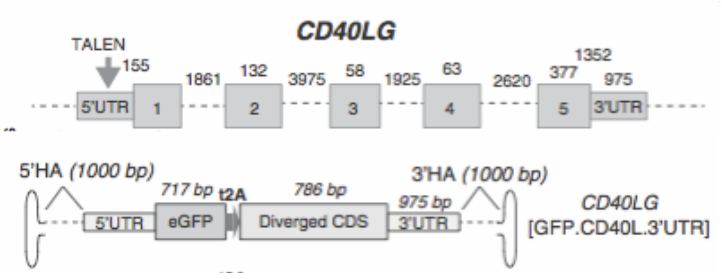



**Figure 1.18** Top: arrow indicates the position of TALEN target site into the 5' UTR of human *CD40LG* gene.
Bottom: Editing template contains two 1000 base pair 5' and 3' homology arms and a eGFP reporter cassette followed by a 2A sequence upstream to the *CD40LG* cDNA (which includes the endogenous 3'UTR) (Adapted from Hubbard et al., 2016).

A second attempt to provide the foundation for a gene editing approach for the treatment of the XHIM syndrome has been done by Kuo et al. in 2018, but in contrast to the previous work made by Hubbard and colleagues, this group focused on HSC-based genome-editing strategies that could provide permanent and robust immune reconstitution to patients. In this study both, TALEN and CRISPR/Cas9 platforms combined with a corrective cDNA donor were used to target the 5' UTR of the CD40L gene (Figure 1.19) in both primary T cells and HSCs in short-term cultures followed by assessment of HSCs long-term *in vivo* in NSG mice. The codon-optimized/divergent cDNA donor packaged as recombinant AAV6 vector was assembled to contain the 3' UTR of the CD40L gene, as stability of the mRNA transcript is dependent on binding of a polypyrimidine- tract-binding (PTB) protein to the 3' UTR (Anderson, 2010; Sai-fuddin et al., 2013). Additionally, PTB binding to the 3' UTR plays an important role in activated T cell viability and proliferation (Covey et al., 2015), which must be maintained for long-lasting immune correction *in vivo* (Vavassori and Covey, 2009).

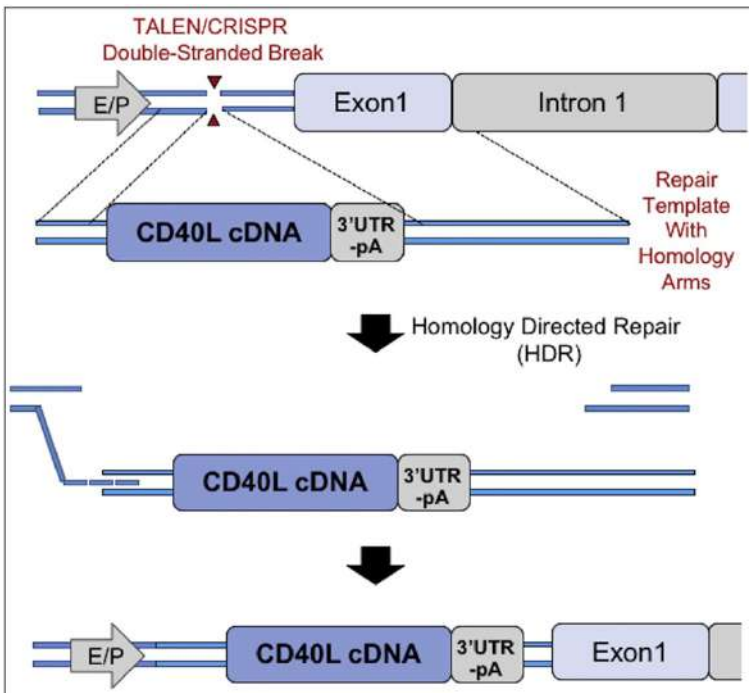


Fig. 1.19 Site-Specific gene integration of corrective cDNA cassette at the 5'UTR of CD40 Ligand overriding all known disease-causing mutations (Kuo et al, 2018).

Patient derived $CD4^+$ T cells transduced with AAV6 vector following TALEN mRNA electroporation, showed an upregulation to >20% CD40L expression upon anti- hCD3/anti-hCD28 immune stimulation, while 13% and 14% CD40L expression were achieved when CRISPR reagents were delivered as gRNA pre-complexed to Cas9 protein as ribonucleoproteins (RNPs) or as two RNA components (gRNA and Cas9 mRNA) respectively. Editing rates of <15% were achieved using both platforms.

Gene modification rates in TALEN-treated Primary Human $CD34^+$ Peripheral Blood Stem Cells (PBSCs) averaged 13.2%, while targeted integration in CRISPR/Cas9 treated PBSCs averaged 16.2% (gRNA, Cas9 mRNA) and 20.8% (RNP). Interestingly, Kuo and colleagues also showed that addition of adenoviral E4orf6/E1b55k H354 ''helper'' proteins mRNA, with the AAV CD40L cDNA donor consistently doubled cDNA donor integration rates in $CD34^+$ PBSCs assayed *in vitro* regardless of nuclease platform. However, despite significantly augmented rates of gene integration in HSCs *in vitro*, this benefit was not maintained long-term *in vivo* in murine studies. In particular, gene modification rates in samples of the input PBSCs cultured *in vitro* averaged 21%, 28%, 15%, and 28% in the TALEN/AAV donor, TALEN/helper/AAV donor, RNP/AAV donor, and RNP/helper/AAV donor samples, respectively, while gene integration rates detectable in the bone marrow 12–20 weeks after transplant ranged from 0.3% to 22% across all treatment groups.

# 2 SCOPE OF THE THESIS

Provide proof-of-efficacy of HIGM1 disease correction in the Cd40lg-/- mouse model: establishment of the therapeutic threshold levels of WT T cells and HSPC and of the transplant conditions required to achieve immune reconstitution and functional immunologic restoration with gene-corrected cells.

# 3 MATERIALS AND METHODS

## 3.1 Study design

The objectives of this study were to establish the conditions for safe and effective correction of HIGM in a mouse model of the disease. Kinetics and extent of reconstitution from limited input of WT HSPCs or T cells were analyzed in transplanted Cd40l-/- mice upon different types of conditioning. Mice were randomized to treatment groups, without blinding. Criteria applied for mouse termination before the established end point were in accordance with the Institutional Animal Care and Use Committee protocol of the San Raffaele Scientific Institute; no outliers were excluded.

## 3.2 Flow cytometry

All cytometric analyses were performed using the FACS Canto II (BD Pharmingen) equipped with DIVA Software and analyzed with the FSC express software (v. 6, De Novo Software). Peripheral Blood (PB): for immunostaining a known volume of whole blood (100 µl) was first incubated with anti-mouse FcγIII/II receptor (Cd16/Cd32) blocking antibodies for 10 min at room temperature and then incubated in the presence of monoclonal antibodies (for antibodies see table 3) for 20 min at 4°C in the dark. The LIVE/DEAD Fixable Dead Cell Staining (Thermo Fisher) was used to discriminate alive and dead cells according to manufacturer's instruction. Erythrocytes were removed by lyses with the TQ-Prep workstation (Beckman-Coulter) in the presence of an equal volume of FBS (100 µl) to protect white blood cells. Anti-murine immunoglobulin G (IgG) beads (BD Biosciences), single stained and Fluorescence Minus One (FMO) stained cells were used for single-staining controls. Bone marrow (BM), spleen, lymph nodes and thymus: BM cells were obtained by flushing the femurs in MACS buffer (PBS pH 7.2 0.5%

bovine serum albumin (BSA), 2Mm EDTA) and by passing cell suspension through a 40 μm nylon filter. Spleens were first smashed and lysed by Ammonium Chloride to remove erythrocytes. The obtained cells suspension was passed through 40 μm nylon filter and washed in cold PBS containing 2% FBS mM. Lymph nodes and thymus were smashed in MACS buffer and the obtained cell suspension was washed in PBS 2% FBS solution.

Table 3. List of antibodies for flow cytometry.

| **Antibody** | **Fluorochrome** | **Clone** | **Company** |
|---|---|---|---|
| CD16/32 | none | 2.4G2 | BD |
| CD11b | APC-Cy7 | M1/70 | Biolegend |
| CD150 | APC | TC15-12F12.2 | Biolegend |
| CD19 | PECy7 | 6D5 | Biolegend |
| CD19 | PB | 6D5 | Biolegend |
| CD25 | Percp5.5 | PC61 | BD |
| CD25 | APC | PC61 | BD |
| CD3 | PE | 145-2C11 | BD |
| CD8a | APC780 | 53-6.7 | eBioscience |
| CD4 | PB | RM4-5 | BD |
| CD44 | PECy7 | IM7 | BD |
| CD62L | APC | MEL-14 | BD |
| CD45RA/B220 | PE | RA3-6B2 | BD |
| CD45RA/B220 | PB | RA3-6B2 | BD |
| Lineage Cocktail | PE | | Biolegend |
| Sca1 | PECY7 | D7 | BD |

| | | | |
|---|---|---|---|
| GL7 | APC | 1D3 | eBioscience |
| PNA | FITC | | Vector Laboratories |
| CD45.1 | FITC | A20 | BD |
| CD45.2 | Percp5.5 | 104 | BD |

### 3.3 Mice experiments

C57Bl/6 Ly45.1 and Cd40lg-/- (B6.129S2-Cd40lgtm1Imx/J) were purchased respectively from Charles River Laboratory and Jackson Laboratory. C57Bl/6 Ly45.1 or C57Bl/6 Ly45.1/Ly45.2 obtained by crossing C57Bl/ 6 Ly45.2 and C57Bl/6 Ly45.1 mice at the San Raffaele Scientific Institute animal research facility, were used as donors for adoptive T cell transfer and HSPC transplant into Cd40lg-/- mice. All animal procedures were performed according to protocols approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute and communicated to the Ministry of Health and local authorities according to the Italian law.

### 3.4 Hematopoietic stem and progenitor cell (HSPC) transplantation studies

Donor mice between 6 and 10 weeks of age were euthanized by CO2, and BM cells were retrieved from femurs, tibias, and humeri. HSPCs were purified by Lin- selection using the mouse Lineage Cell Depletion Kit (Miltenyi Biotec) according to the manufacturer's instructions. Cells were then cultured in serum-free StemSpan medium (StemCell Technologies) containing penicillin, streptomycin, glutamine and a combination of mouse cytokines (20 ng/ml IL-3, 100 ng/ml SCF, 100 ng/ml Flt-3L, 50 ng/ml TPO all from Peprotech), at a concentration of $10^6$ cells/ml. For competitive transplants, C57BL/6-Ly5.1 and Cd40lg-/- (CD45.2) Lin- cells were

cultured for 16 hours in the medium described above, mixed at the indicated ratios, and transplanted at a total dose of $10^6$ cells/mouse into 8-week-old lethally irradiated Cd40lg-/- CD45.2) mice. Serial collections of blood from the mouse tail were performed to monitor the hematological parameters and donor cell engraftment. At the end of the experiment, BM, thymus and spleen and lymph nodes were harvested and analyzed.

### 3.5 Adoptive T cell transfer studies

For adoptive T cell transfer of naïve T cells, CD3+ lymphocytes were harvested from the spleen of 8 weeks-old male C57Bl/6 Ly45.1 mice by immune-magnetic separation (Pan T cell isolation kit, Miltenyi Biotec). Right after purification, $2x10^6$, $10^7$ or $2x10^7$ naive T cells were intravenously injected in Cd40lg-/- mice pre-conditioned or not with 300µg/g of cyclophosphamide (CPA, Baxter) 1 day before T cell infusion. For adoptive T cell transfer of activated T cells, CD4+ lymphocytes were harvested from the spleen of 8 weeks- old C57Bl/6 CD45.1+ mice by immune-magnetic separation (CD4+ T cell isolation kit, Miltenyi Biotec) and subsequently activated using magnetic beads (ratio cell:bead 1:1) conjugated with anti-CD3/anti-CD28 antibodies (Dynabeads mouse T-activator CD3/CD28; Thermo Fisher). T cells were cultured in RPMI supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml), 1% glutamine, IL2 (30 U/ml, Proleukin, Novartis Pharma), IL7 (5 ng/ml), IL15 (5 ng/ml), Sodium Pyruvate (1 mM Thermo Fisher Scientific), Hepes (20 mM Thermo Fisher Scientific), MEM Non-Essential Amino Acids (Thermo Fisher Scientific 1µM) and Beta-Mercaptoethanol (0.05 mM Thermo Fisher Scientific). Activated CD4+ T cells were expanded in culture for 7 days prior to infusion in mice at a dose of $10^7$ cells. Mice were pre-treated with intraperitoneal (i.p.) injection of 200 or 300µg/g of CPA. CPA was administered in one single large dose or a preliminary ("priming") dose of CPA at 50 µg/g was given 7 days before a single large dose of 150 µg/g or 250 µg/g to reduce lethality in mice (Collis, Wilson and Jones, 1980). The large dose was administered 1 day before T cell infusion. 50 µg anti-CD4 antibody (BioXcell) or 250 µg ALS

(Thymocyte Antibody, LSBio) were given i.p. 7 days before T cell infusion. Serial collections of blood from the mouse tail were performed to monitor the hematological parameters and donor T cell engraftment. At the end of the experiment spleen and lymph nodes were harvested and analyzed.

### 3.6 In vivo immunization and IgG quantification

Mice were immunized i.p. with 100 μg of TNP-KLH (Lgc Biosearch Technologies) in Imject Alum Adjuvant (1:2) (Thermo Fisher Scientific) or with 40 μg OVA (Sigma-Aldrich) in Freund's Adjuvant, Incomplete (Sigma-Aldrich). Serum was collected at day 0, 7, 14 and 21 after immunization. Mice were boosted as described above on day 27 or 28, and serum was collected on day 7 after re-challenge. For IgG quantification, the concentration of antigen-specific IgGs in mouse sera was determined by an enzyme- linked immunosorbent assay (ELISA). Plates were coated with 100 μL/well of either 5 μg/mL TNP-KLH or 2 μg/mL OVA in carbonate buffer. Following incubation, plates were washed 3 times in PBS containing 0.05% Tween20 (Sigma-Aldrich) (Wash Buffer). The plates were then blocked for 1 hour using 100 μL/well of PBS containing 1% Bovine Serum Albumine (BSA), followed by a washing step, as described above. Serum samples were serially diluted in wash buffer and 100 μL per well of each diluted sample was added into the plate and incubated for 2 hours at room temperature. For determination of the plate background optical density (OD) values, some wells were incubated with wash buffer alone. Following incubation, plates were washed and 100 μL/well of HRP-conjugated goat anti-mouse (Southern Biotech 1:10,000) was added and incubated for 1 hour at room temperature. After washing, the plates were incubated for 5' with 3,3',5,5'-tetra-methyl benzidine (TMB, Sigma-Aldrich) substrate at room temperature. The reaction was stopped by the addition of 50 μL of 1 M H2SO4. The OD values at 450 nm were determined for each well using a using a Multiskan GO microplate reader (Thermo Fisher Scientific) and normalized

to IgG1 standard curves. Results were expressed as mean of duplicate determinations.

### 3.7 ELISPOT assay

The ELISPOT was performed in nitrocellulose membrane 96-well flat-bottomed plate (Millipore) coated with 10 μg/ml of TNP-KLH. After blocking with PBS 1% BSA, serial dilutions of total splenocytes (from $2x10^5$ to $2.5 \times 10^3$ cells/well) were incubated overnight at 37 °C/5%$CO_2$. Plates were washed and antigen specific IgG–producing cells were detected by isotype specific secondary detection monoclonal antibody and streptavidin-HRP, and finally developed with 3-amino-9-ethylcarbazole (Sigma-Aldrich) as chromogenic substrate. Number of spots per well were counted by ImmunoSpot® S6 Fluorescent Analyzer (Cellular Technology Limited).

### 3.8 RNA extraction and droplet digital PCR

RNA extraction was performed using the RNeasy Plus mini Kit (Qiagen) according to manufacturer's instructions and retro-transcribed using the SuperScript Vilo kit (Invitrogen). Droplet digital PCR (ddPCR) against P. *murina* ribosomal RNA was used to assess levels of P. *murina* infection in the lung using PCR primers 5′-ATGAGGTGAAAAGTCGAAAGGG-3′ and 5′-TGAGGTCTCAGATGAAAAACCTCTT-3′. The probe labeled with 6FAM had the sequence 5′-6FAM-AACAGCCCAGAATAATGAATAAA-BHQ1-3′

### 3.9 Protection against P. murina

To assess the immune responses to a live pathogen relevant to HIGM1 disease, P. *murina* organisms were isolated from lungs of Cd40lg-/- mice previously

inoculated with P. *murina* or by cohousing them with a Cd40lg-/- seeder mouse infected with a large load of *P. murina*. P. *murina* organisms were purified by centrifugation. Briefly, lungs were homogenized in 10 ml homogenization buffer (58.5 mM disodium phosphate, 1.5 mM monopotassium phosphate, 43.5 mM sodium chloride, 10 mM trisodium citrate, 10 mM dithiothreitol and 2.7 mM potassium chloride, pH 7.4) or PBS, in Mylteni gentleMACs dissociator filtered through a 40 µm nylon filter and centrifuged at 3400 rpm for 10 min to remove large debris. Pellet was resuspended in 1 ml RPMI-1640 with 10% FBS and 7,5% DMSO for cryopreservation. Competitive transplanted CD40L-knockout mice were infected intranasally with P. murina organisms in 50 µl PBS. At 4,5 months after infection, mice were sacrificed and body weight and lung weight were measured. Pulmonary lesions suggesting P.*murina* pneumonia and P.*murina* cystis themselves were detected on formalin-fixed, paraffin-embedded left lung specimens by combining Hematoxylin and Eosin (H&E) staining with immunohistochemistry (IHC). 5- to 10-µm lung sections were labeled with rabbit anti-Msg hyperimmune serum (Bishop et al., 2012) and stained with DAPI (Life Technologies). Biotinylated goat anti-rabbit IgG (Thermo Fisher Scientific) was used as secondary antibody for the anti-Msg antibody. The remaining lung tissue was homogenized for the detection of P. *murina* ribosomal RNA by Droplet Digital PCR (ddPCR) and to identify P. *murina* cystis by indirect immunofluorescence staining (IF) using rabbit anti-Msg hyperimmune serum and Alexa Fluor 488–labeled goat anti-rabbit IgG (Thermo Fisher Scientific) as secondary antibody.

# 4 RESULTS

## 4.1 Feasibility of adoptive T cell transfer in the HIGM mouse model

### 4.1.1 The HIGM mouse model

To support the scientific rationale of CD40L gene correction, we performed preclinical studies on a suitable mouse model of the HIGM syndrome. In particular we exploited a Cd40lg-/- mouse model in which exons 3 and 4 of the Cd40 ligand gene were replaced with a neomycin cassette (Jax) (Renshaw BR et al, 1994) which abrogates Cd40l gene expression (Figure 4.1 A).

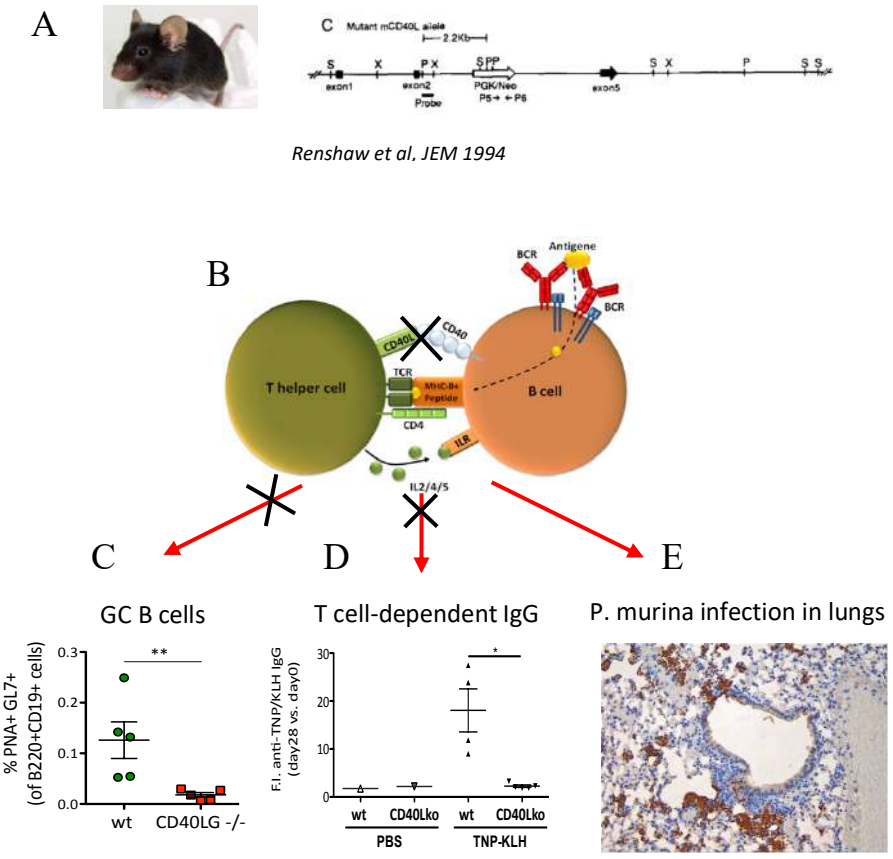



Fig. 4.1 (A) Schematic diagram of the mutant Cd40l allele in HIGM mouse model (Renshaw et al. 1994). (B) T cell-dependent B cell activation mediated by CD40L-CD40 interaction. (C) Detection of germinal centers B cells in the spleen. Splenocytes were harvested from WT and Cd40lg-/- mice, B cells were stained for the GC markers peanut agglutinin (PNA) and GL7 and analyzed by flow

cytometry (each symbol represents an individual subject). (D) Immunoglobulin subclass switching in response to a thymus-dependent antigen (TNP-KLH). Fold increase in TNP-KLH specific IgG levels 28 days after vaccination (compared with day 0) measured by ELISA (each symbol represents an individual subject). (E) P. *murina* infection in CD40LG-/- mouse. Lung histology 4 months after intratracheal challenge with P.*murina*. Brown areas represent P.*murina* organisms.

By immunophenotypical and histological characterization, we confirmed that the loss of the CD40L-CD40 interaction in Cd40lg-/- mice (Figure 4.1 B) leads to the inability to form germinal centers in the spleen and to mount secondary antigen-specific responses to primary and secondary immunization with a thymus-dependent antigen, trinitrophenol-conjugated keyhole limpet hemocyanin (TNP-KLH). Flow cytometric analysis of the spleen of WT and Cd40lg-/- mice in figure 4.1 C indeed, confirms the presence of PNA+GL7+ Germinal Center B cells in the spleen of WT mice suggesting their ability to form Germinal Centers in secondary lymphoid organs even in the absence of antigenic challenge. On the contrary, Germinal Center B cells are absent or almost absent in Cd40lg-/- mice. Figure 4.1 D instead, shows the antigen-specific antibody response of WT and Cd40lg-/- mice to vaccination with TNP-KLH measured by ELISA assay. 21 days after vaccination, experimental mice were boosted with a second dose of antigen and sera were collected before (day 0), 14 and 28 days after primary immunization. As expected, all WT mice show a fold increase in antibody levels 28 days after vaccination (compared with day 0) whereas Cd40lg-/- and PBS treated mice failed to produce antigen-specific antibodies in response to TNP-KLH mediated challenge. In addition, the mouse model of HIGM also showed high susceptibility to the opportunistic infection mediated by *Pneumocystis murina*, thus mimicking findings typical of the human phenotype. In figure 4.1 E a tissue section of the infected lung of a Cd40lg-/- mouse, obtained from the paraffin blocks, was stained with Hematoxylin and Eosin (H&E) and immunohistochemistry with a rabbit anti-Msg hyperimmune serum generated to the major surface glycoprotein (Msg) of *Pneumocystis* (Bishop et al., 2012).

### 4.1.2 WT donor CD3+ lymphocytes engraft at low levels and survive at long term in vivo

To study whether adoptive transfer of edited functional T cells could represent a potential therapeutic option for HIGM1 patients, we first assessed the feasibility of this approach in the Cd40l-/- mouse model, which faithfully recapitulates the human disease phenotype (Renshaw et al. 1994). Since corrected CD40 ligand-expressing T cells could lead to undesirable immunogenicity in patients who typically lack its expression, we tested persistence and possible graft rejection of functional T lymphocytes by transplanting two doses of spleen-derived WT CD3+ T cells into Cd40lg-/- or WT mice (Fig. 4.2 A).

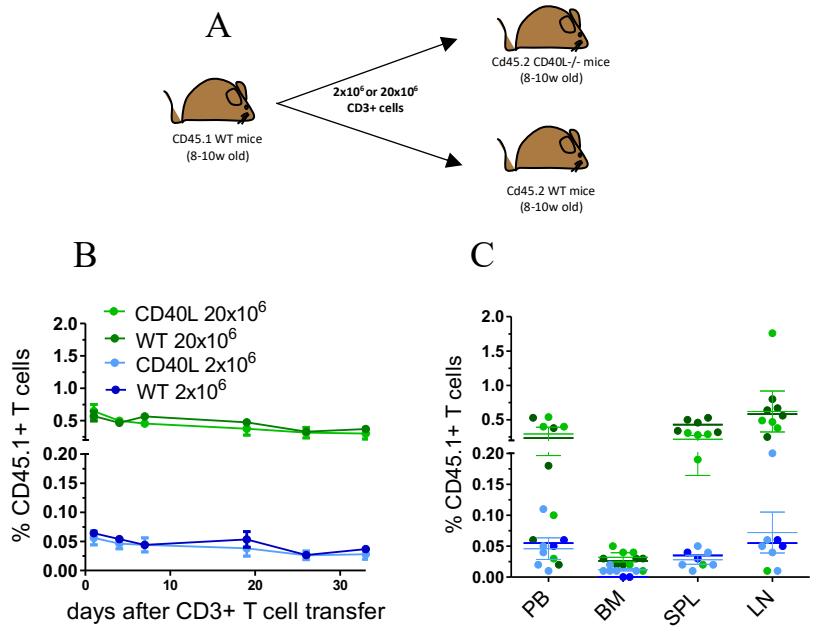


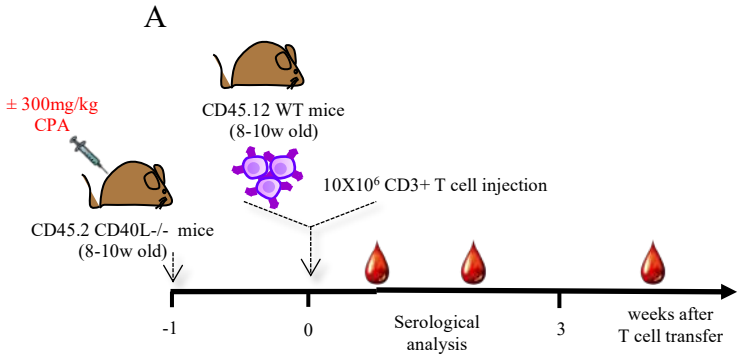

Fig. 4.2 (A) Schematic representation of adoptive T cell transfer. 8 weeks old CD45.2 Cd40lg -/- or WT mice received adoptive transfer of $2x10^6$ or $20x10^6$ spleen derived CD45.1 WT CD3+ T cells by lateral tail vein administration. (B) Recipient mice were followed overtime by weekly bleedings and euthanized at 5 weeks. Mean with SEM of circulating levels of engrafted donor CD3+ T cells at indicated times after T cell transfer. *(*C) Percentages of engrafted WT donor T cells in Peripheral Blood (PB), Bone Marrow (BM), Spleen (SPL) and Lymph Nodes (LN). Each data point represents the percentage of one mouse.

We then monitored circulating levels of donor T cells overtime and one month after adoptive T cell transfer, we evaluated percentages of engraftment in Peripheral

Blood (PB) and secondary lymphoid organs of recipient animals (Fig. 4.2 B,C). Given the same dose of input WT cells, we found proportional and comparable levels of engrafted donor lymphocytes in both groups of mice, thus confirming that WT T cells could survive at long term in Cd40lg-/- mice.

### 4.1.3 Pre-conditioning with CPA enhances engraftment of donor T cells in Peripheral Blood, Spleen and Lymph Nodes and leads to preferential expansion of CD8+ donor T cell population in vivo

Then, in order to assess the efficacy of adoptive T cell transfer in rescuing the disease phenotype, we evaluated the impact of a conditioning regimen on immune reconstitution capacity. By infusing $10x10^6$ spleen-derived WT CD3+ T cells into Cd40lg-/- mice pre-treated or not with a single dose of 300 mg/kg cyclophosphamide (CPA), intraperitoneally injected the day before T cell transfer. We followed experimental mice by weekly bleedings to assess CPA-mediated depletion in Cd40lg-/- recipients as well as circulating levels of injected donor T cells and in order to characterize the overtime phenotype of CD4+ and CD8+ T cells. Figure 4.3 presents a longitudinal analysis of the proportions and absolute values of recipient and donor CD3+ lymphocytes as well as of the proportions and absolute values of recipient and donor CD4+ and CD8+ cells in both groups of pre-conditioned and not pre-conditioned Cd40lg-/- mice.



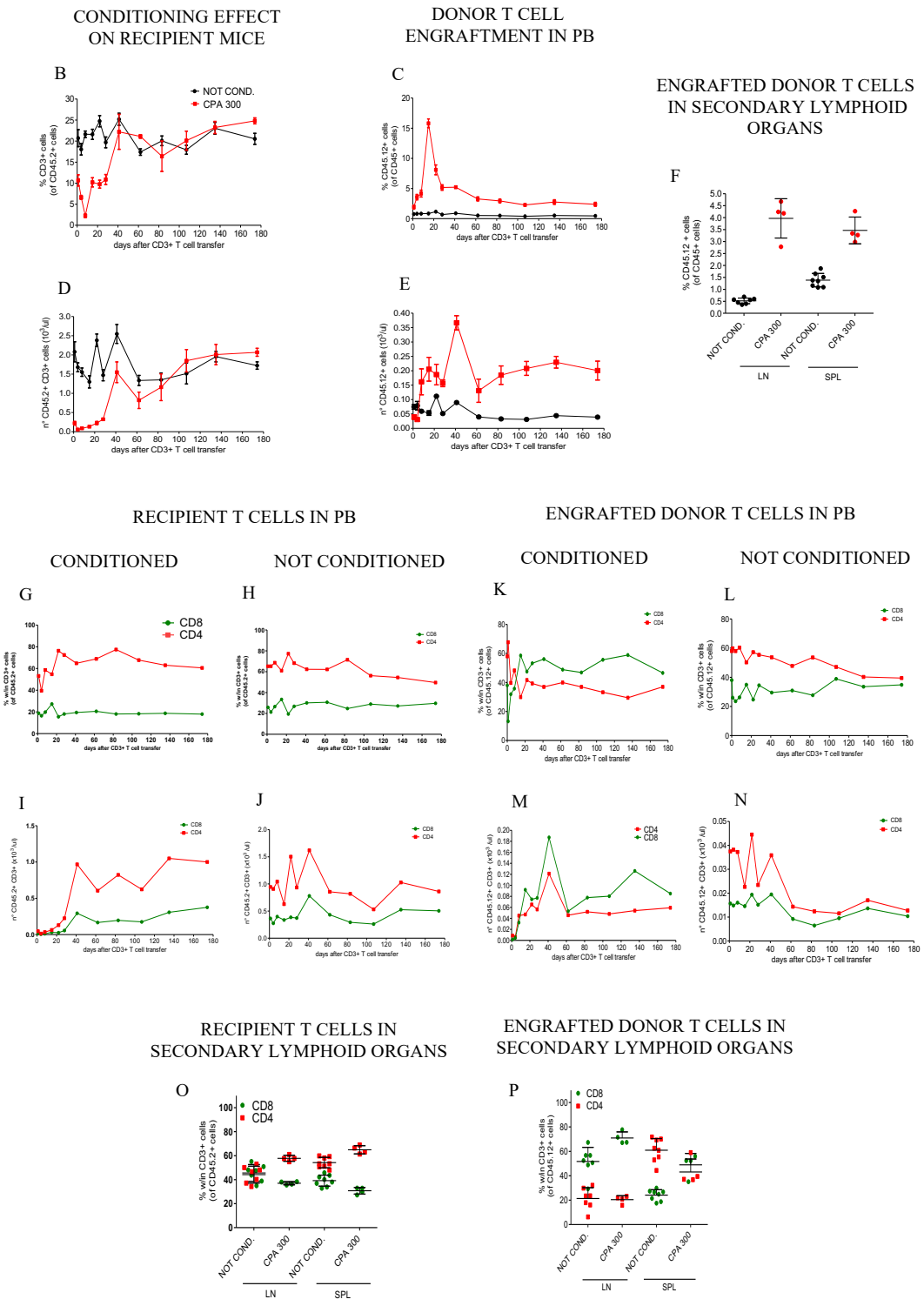



Fig 4.3 (A) Experimental design: 8 weeks old CD45.2 Cd40lg-/- mice pre-conditioned or not with 300mg/kg CPA received adoptive transfer of $10x10^6$ spleen derived CD45.12 WT CD3+ T cells by lateral tail vein administration. Recipient mice were followed overtime by weekly bleedings and euthanized at 26 weeks. Mean with SEM of circulating recipient CD45.2+ CD3+ T cell percentages (B) and absolute numbers (D) and of engrafted donor CD45.12+ CD3+ T cell percentages (C) and absolute numbers (E) at indicated times after T cell transfer in mice pre-conditioned (red curves) or

not (black curves) with the indicated dose of CPA. Donor T cell engraftment levels were also assessed in spleen and lymph nodes of both experimental groups (F). Longitudinal changes of recipient (G,H,I,J) and donor (K,L,M,N) $CD3^+CD4^+$ (red curves) and $CD3^+CD8^+$ (green curves) cell percentages (upper panels) and absolute numbers (lower panels) were assesses in peripheral blood of both, pre-conditioned (G,I, K,M ) and not pre-conditioned (H,J,L,N) Cd40lg-/- mice. Detection of CD4+ and CD8+ cell percentages within CD3+ recipient (O) and donor (P) T cell population of lymph nodes and spleen in both experimental groups.

Results show that the transient depletion of recipient T cells mediated by CPA treatment (Fig. 4.3 red curve on panels B,D), allows higher levels of donor T cell engraftment in PB, both in terms of percentage and absolute number of cells (Fig. 4.3 red curve on panels C,E), compared with the not conditioned group of mice (Fig. 4.3 black curve on panels C,E). Similar levels of engraftment were observed also in LN and SPL (Fig. 4.3 F). Taken together these data indicate that lymphodepletion effectively removes host inhibitory cells, thus opening up available space (the so-called Lebensraum effect) for adoptively transferred T cell expansion and function (Anasetti and Mulé, 2007). Interestingly, despite observed initial proportions of CD4+ versus CD8+ donor cells, a preferential expansion of the engrafted CD8+ cell population (Fig. 4.3 green curve on panels K,M) at the expense of the CD4+ cell population (Fig. 4.3 red curve on panels K,M) was induced in the pre-conditioned group of transplanted mice. The same CPA-mediated inversion of the CD4+ versus CD8+ donor T cell proportion was detected also in secondary lymphoid organs (P). On the contrary, in the absence of conditioning, frequencies and absolute values of engrafted CD4+ T cells remained stable at higher levels with respect to donor CD8+ T cells overtime. Looking at recipient T cell compartment in PB (G,H,I,J) LN and SPL (O), no significant differences were observed in the percentages (G,H) and absolute numbers (I,J) of CD4+ versus CD8+ lymphocytes between conditioned and not conditioned mice, indicating that the treatment with CPA did not lead to changes in the CD4:CD8 recipient T cell ratio.

### 4.1.4 Engrafted CD8+ T cells and CD4+ T cells progressively develop into memory and effector cells

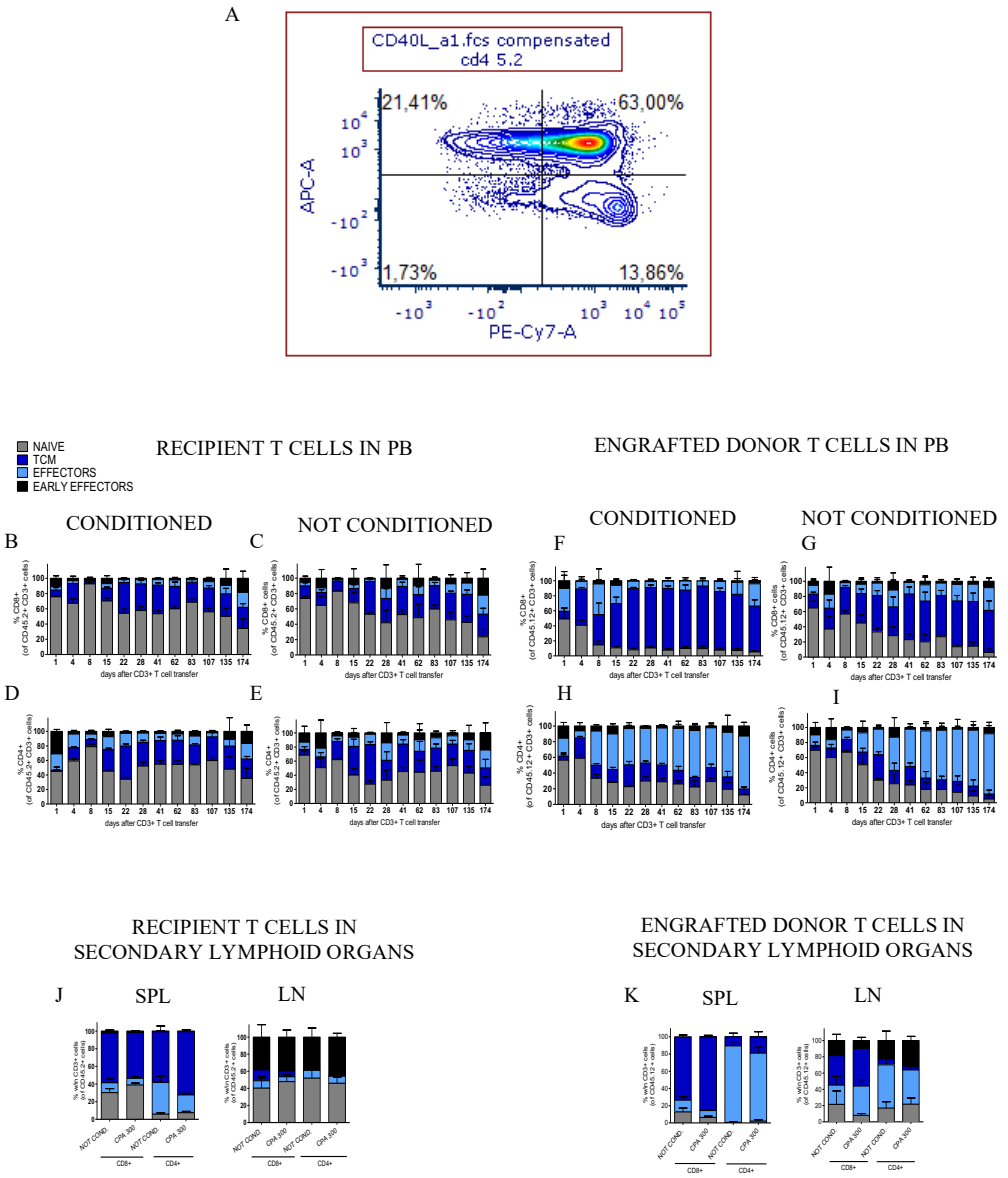



Fig. 4.4 (A) Example of a flow-cytometry analysis plot, showing the four main T cell subpopulations, marked for CD62L and CD44, our reference surface markers. Longitudinal changes of $CD44^{-}CD62L^{+}$ naive, $CD44^{+}CD62L^{+}$ central memory (Tcm), $CD44^{+}CD62L^{-}$ effector, and CD44 $CD62L^{-}$ early effector cell percentages within recipient (B,C,D,E) and donor (F,G,H,I) CD8+ (upper panels) and CD4+ (lowers panels) cell populations in both experimental groups. Same T cell subsets in secondary lymphoid organs (J,K). All values are given as percentage of $CD4^{+}$ or CD8+ cells within CD3+ cells.

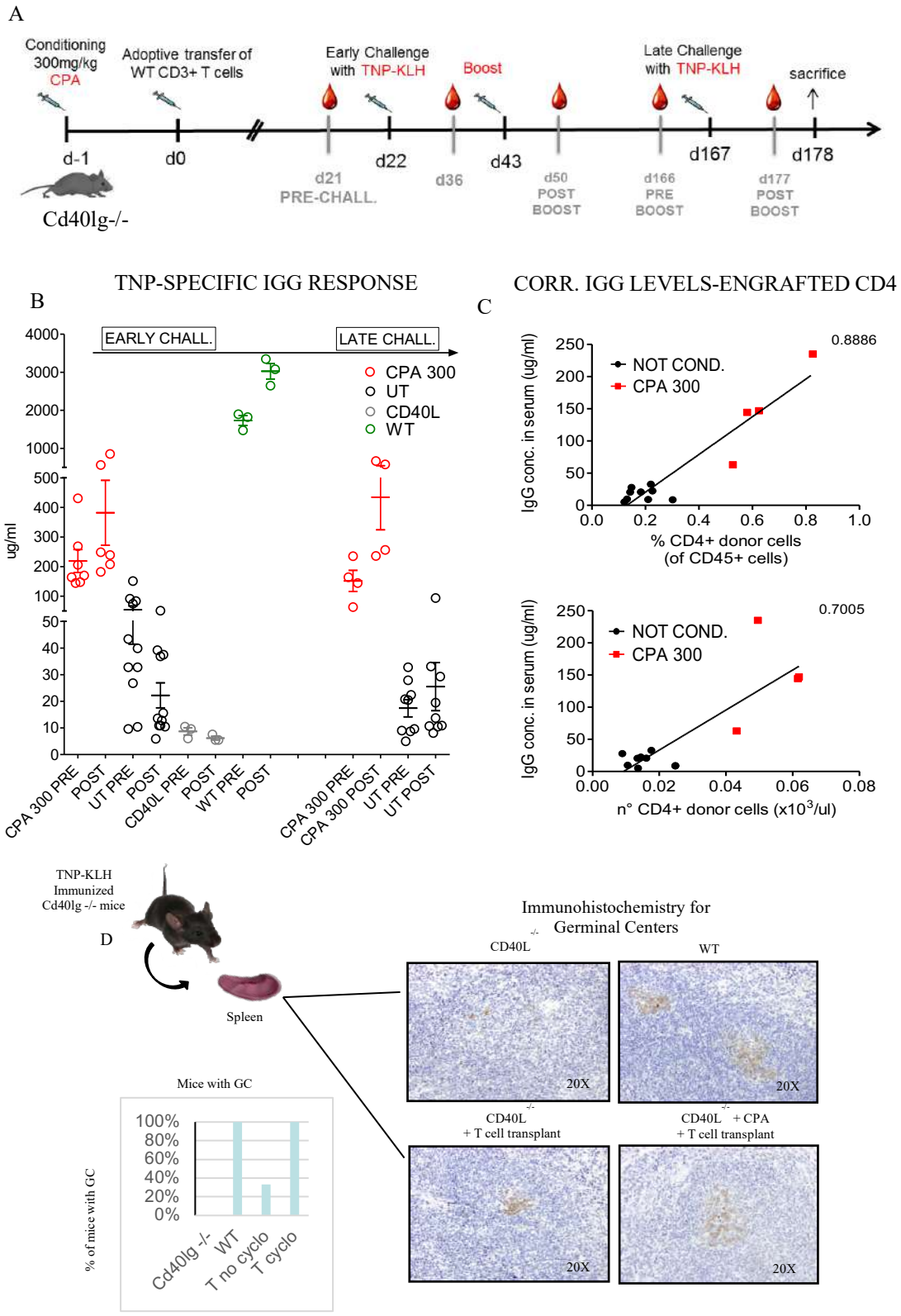
We then compared the differences in the overtime T-cell phenotype between mice pre-conditioned or not with CPA (Fig. 4.1.4). To characterize WT donor and Cd40lg -/- recipient $CD4^+$ T cells and CD8+ T cells, the distribution of naïve ($CD62L^+CD44^-$), TCM ($CD62L^+CD44^+$), Effector ($CD62L^-CD44^+$) and early Effector ($CD62L^-CD44^-$) cells were analyzed using flow cytometry (A). Recipient T cells (B,C,D,E) maintained an almost stable overtime distribution of the different subsets within CD4+ and CD8+ cell populations, with a slight progressive regression of the naïve T cell compartment. Recipient T cells in the spleen were predominantly central memory and naïve within the CD8+ T cell compartment, while an expansion of the effector T cell compartment at the expense of the naïve T cell compartment was observed within the CD4+ T cell population. A larger fraction of early effector T cells, at the expense of the central memory T cell fraction, was detected in the lymph nodes (J). No significant differences were observed in recipient T cell phenotype between conditioned and not conditioned mice, neither in peripheral blood overtime analysis nor in secondary lymphoid organs. On the contrary, looking at the engrafted donor T cell population, we could detect a progressive expansion of the central memory compartment at the expense of naïve and effector compartments, within the CD8+ cell population (F,G) while donor CD4+ T cells mainly develop into effector cells (H,I). Even in this case, the treatment with CPA did not appear to affect the distribution of the T cell subsets within both, CD8+ and CD4+ T cell populations, as compared to the not conditioned mice. As observed in peripheral blood, also in spleen and lymph nodes engrafted CD8+ donor T cells were predominantly central memory, while donor CD4+ T lymphocytes mainly effectors (K).

## 4.2 Efficacy of HIGM disease correction in the mouse model

As previously described, the Cd40lg-/- mouse model shows inability to form germinal centers in the spleen and to produce class-switched antibodies against TNP-KLH. Thus, to determine the ability of engrafted T cells to restore the defective immune function, we vaccinated transplanted mice with TNP-KLH and assayed for the presence of germinal centers B cells by immunohistochemistry and antigen-specific IgGs by ELISA (Fig. 4.2.1 A).

### 4.2.1 CPA-mediated higher engraftment results in increased partial rescue of TNP-specific IgG response and germinal center formation

In particular, 22 days after adoptive transfer of WT T cells we challenged the mice by intraperitoneal injection of TNP-KLH using alum adjuvant to enhance efficacy of the vaccine antigen. 3 weeks later, experimental mice were boosted with a second dose of antigen and sera were collected the day before primary immunization, 14 days after and 7 days after boost to measure the TNP-specific IgG response by ELISA assay. Results reveal that, compared to the not transplanted group of Cd40lg-/- mice, all the mice transplanted upon pre-conditioning and most of the mice transplanted without pre-conditioning, were able to partially restore the antigen specific IgG response upon immunization. Of note, this response was quantitatively greater in mice transplanted after pre-conditioning with CPA (Fig. 4.2.1 B). The positive correlation observed between percentages and absolute counts of engrafted CD4+ T cells and TNP-specific IgG concentrations in sera confirmed that the transient depletion effect mediated by the chemotherapeutic agent allowed higher engraftment levels of adoptively transferred functional T cells, thus leading to an increased IgG response (Fig. 4.2.1 C).


A
Conditioning
300mg/kg
CPA
Adoptive transfer of
WT CD3+ T cells
Early Challenge
with TNP-KLH
Boost
Late Challenge
with TNP-KLH
sacrifice
d-1
d0
d21
PRE-CHALL.
d22
d36
d43
d50
POST
BOOST
d166
PRE
BOOST
d167
d177
POST
BOOST
d178
Cd40lg-/-
TNP-SPECIFIC IGG RESPONSE
B
EARLY CHALL.
LATE CHALL.
CPA 300
UT
CD40L
WT
ug/ml
CPA 300 PRE
POST
UT PRE
POST
CD40L PRE
POST
WT PRE
POST
CPA 300 PRE
CPA 300 POST
UT PRE
UT POST
CORR. IGG LEVELS-ENGRAFTED CD4
C
NOT COND.
CPA 300
0.8886
IgG conc. in serum (ug/ml)
% CD4+ donor cells
(of CD45+ cells)
0.7005
n° CD4+ donor cells (x10$^3$/ul)
TNP-KLH
Immunized
Cd40lg -/- mice
D
Spleen
Immunohistochemistry for
Germinal Centers
CD40L
WT
20X
CD40L -/-
+ T cell transplant
CD40L -/- + CPA
+ T cell transplant
Mice with GC
% of mice with GC
100%
80%
60%
40%
20%
0%
Cd40lg -/-
WT
T no cyclo
T cyclo

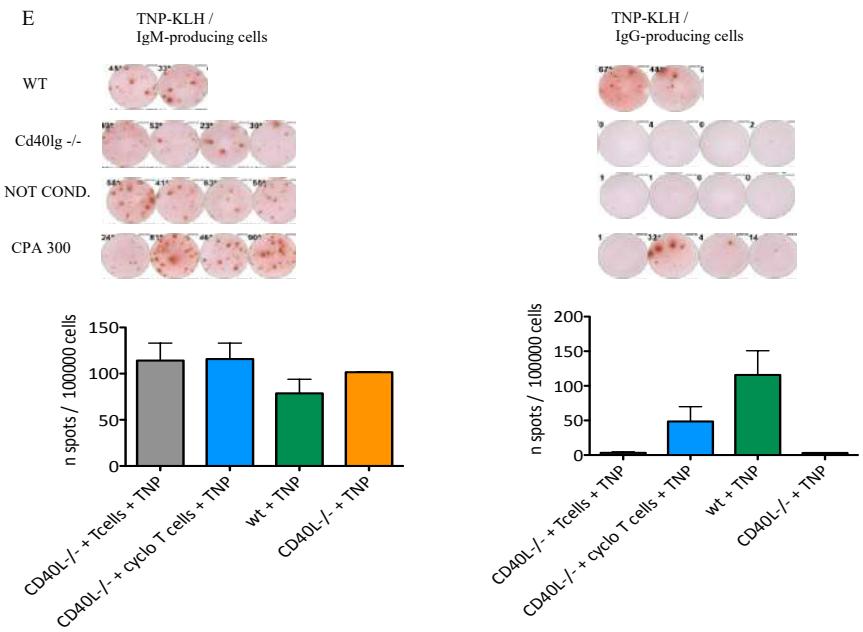



**Fig. 4.5 (**A) Cd40lg-/- mice pre-conditioned or not with CPA the day before T cell transfer were challenged by intraperitoneal immunization with TNP-KLH, using alum as adjuvant, at day 22 and 43 upon T cell infusion. 167 days after T cell transfer experimental mice were boosted with a third dose of TNP-KLH + Alum and euthanized 10 days later. (B) Sera from vaccinated Cd40lg-/- mice transplanted with or without pre-conditioning and not transplanted Cd40lg-/- mice were collected at indicated times and TNP-specific IgG concentrations were determined by ELISA in two independent experiments. Sera from vaccinated WT mice were used as positive controls. Each data point represents the titer of one mouse. Results are expressed as mean ± SEM. (C) Correlation between percentage and absolute number of engrafted CD4+ T cells and IgG concentration in sera. (D) Peanut agglutinin (PNA) immunohistochemical staining of spleen sections from mice immunized 10 days earlier with TNP-KLH. Brown (PNA+) areas represent germinal centers (GCs). (E) Detection of splenic IgG-secreting cells. The fractions of TNP-specific IgG and IgM secreting B cells among the splenocytes was analyzed by ELISPOT assay. Spots were counted by an ELISPOT Reader using a size range of 0.005–1 mm. Results are expressed as mean ± SD. Horizontal bars identify mean values.

Interestingly, TNP-specific antibody concentrations increased upon booster immunization only in the pre-conditioned group of mice. After boosting, a decline in antibody titres occurred in the not pre-conditioned mice, indicating that the higher concentrations in the mice treated with CPA translated into greater persistence of long-term protection. In fact, 144 days post primary immunization, TNP-specific IgGs in this latter group were still detectable at comparable levels. Some mice were capable of responding to a second booster immunization, which

was administered 167 days after T cell transfer and this effect was particularly evident in pre-conditioned mice.

In addition to sera collection, 10 days after last booster immunization, spleens of all experimental mice were collected and analysed to investigate the ability of the transplanted mice to form germinal centers (GCs) after vaccination. Immunostaining of splenic sections with Anti-Peanut Agglutinin (PNA), which binds to PNA-reactive glycans expressed by germinal center B cells, revealed that, while none of the not transplanted Cd40lg-/- mice was capable of forming GCs, 100% of WT mice as well as 100% of mice transplanted after pre-conditioning and 30% of the mice transplanted without conditioning formed PNA positive nodules within their spleens (Fig. 4.5 D). Furthermore, to identify the presence of TNP-specific IgG-secreting cells within the splenic B cell compartment of transplanted mice, we also performed and ELISPOT assay and found antigen-specific spot-forming cells in the spleen of 50% of pre-conditioned mice. No IgG secreting cells were detected in the spleen of not pre-conditioned mice (Fig. 4.5 E).

### 4.2.2 Lowering the dose of CPA or increasing the dose of input T cells in the absence of pre-conditioning results in decreased engraftment and IgG response

Next, we tested whether we could achieve same or higher levels of TNP-specific IgG response in adoptively transferred Cd40lg-/- mice by lowering the dose of administered CPA or by increasing the dose of input donor T cells given without pre-conditioning recipient mice (Fig.4.6). To this purpose, we performed an adoptive transfer of $10x10^6$ spleen derived WT CD3+ T cells into Cd40lg-/- mice pre-conditioned with 300mg/kg CPA or 100mg/kg CPA and in parallel we also infused $10x10^6$, $25x10^6$ or $50x10^6$ WT CD3+ T cells into not pre-conditioned Cd40lg-/- mice. As expected, the transient CD3+ T cell depletion induced by the treatment with the lower dose of CPA was milder as compared to the higher dose (Fig. 4.6 A), but unfortunately not sufficient to permit levels of engraftment (Fig.4.6

B) and TNP-specific IgG response (4.6 C) comparable to those observed in mice pre-conditioned with the high dose of CPA. Furthermore, transplanting increasing doses of input cells without pre-conditioning, did not lead to significant differences in the corresponding percentages (data not shown) and absolute numbers of engrafted T cells (Fig. 4.6 B), thus reflecting the achievement of similar levels of TNP-specific IgG response (Fig.4.6 C). Taken together these data suggest that even if pre-conditioning the mice with a mild dose of CPA permitted a slight increase in the antigen-specific antibody production as compared to the not conditioned groups of mice, highest amounts of antigen specific antibodies and ability to partially respond to a booster immunization were achieved only in the group of mice pre-conditioned with the higher dose of CPA (Fig. 4.6 C). Interestingly, we could confirm an inversion of the CD4:CD8 donor T cell ratio in mice pre-conditioned with the full dose of CPA (4.6 D) but not with the lower dose of chemotherapeutic agent (Fig. 4.6 E).

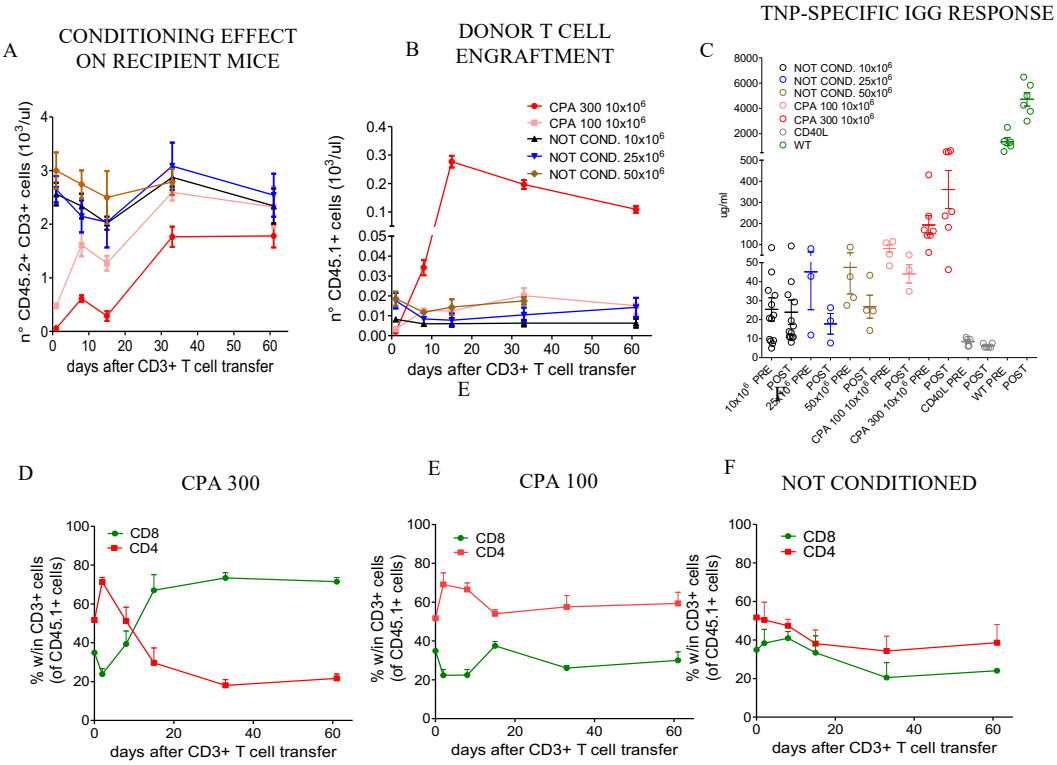



Fig. 4.6 8 weeks old CD45.2 Cd40lg-/- mice pre-conditioned or not with 300mg/kg CPA or 100mg/kg CPA received adoptive transfer of $10x10^6$ spleen derived CD45.1 WT CD3+ T cells by

lateral tail vein administration. Two other groups of not-preconditioned Cd40lg-/- mice were respectively infused with $25x10^6$ or $50x10^6$ WT CD3+ T cells. Recipient mice were followed overtime by weekly bleedings and sera were collected upon primary immunization and booster immunization with TNP-KLH + alum. Mean with SEM of circulating recipient CD3+ T cell absolute numbers (A) and of engrafted donor CD3+ T cell absolute numbers (B) at indicated times after T cell transfer. (C) TNP-specific IgG response post prime-boost vaccination. Longitudinal changes of percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ donor T cells in peripheral blood of mice pre-conditioned with a full dose of CPA (D), a low dose of CPA, or in the absence of chemotherapeutic treatment.

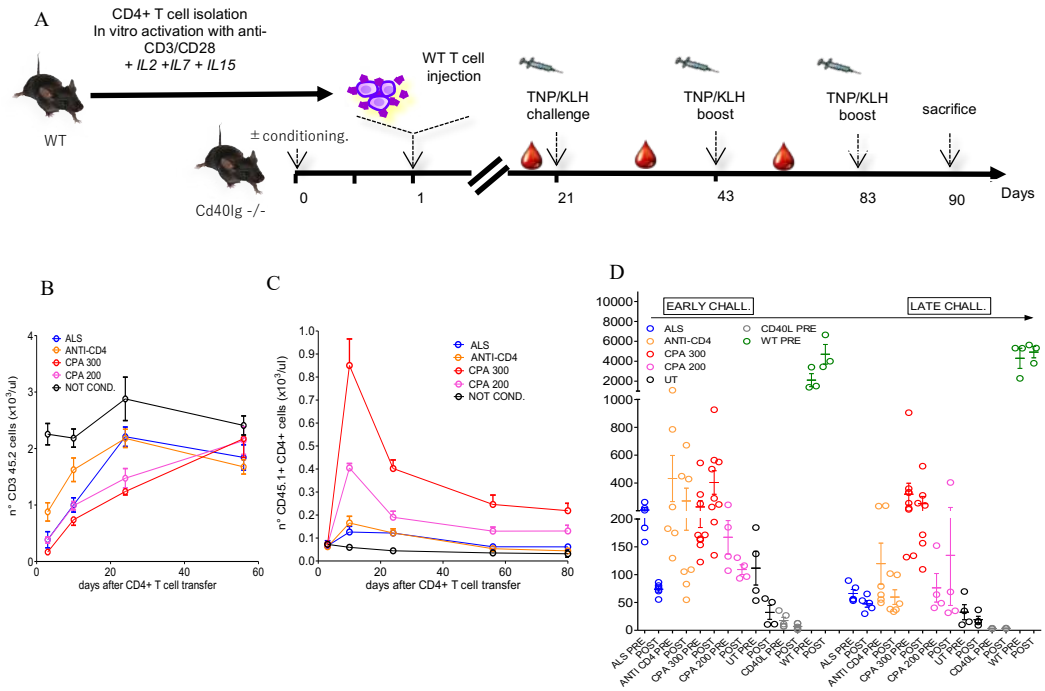
## 4.3 Adoptive transfer of activated CD4+ T cells

Since T cells require to be activated in vitro for efficient genetic engineering (Chen, 2015), we investigated the possible impact of culture manipulation on T cell fitness by stimulating and expanding spleen-derived donor T cells with anti-CD3/CD28 beads in the presence of interleukins 2,7 and 15 prior to infusion (Fig. 2A). Finally, having established the importance of conditioning in promoting the rescue of the immune function, we also evaluated the effect of several clinically relevant lymphodepleting regimens on T cell engraftment and immune reconstitution. However, because we previously observed a preferential expansion of engrafted CD8+ T cells after pre-conditioning with CPA, we adoptively transferred only purified CD4+ donor T cells in order to enhance engraftment of major mediators of CD40L function.

### 4.3.1 Impact of different lymphodepleting regimens and input doses of activated T cells on engraftment, IgG response and germinal center formation

In addition to the treatment with two different doses of CPA we also tested Anti-lymphocyte Serum (ALS) and anti-CD4 antibody. Longitudinal analysis of recipient CD3+ T cells showed that both doses of CPA led to a greater degree of depletion compared with ALS and anti-CD4 resulting in higher and depletion degree-dependent engraftment in peripheral blood, spleen and lymph nodes. Again, highest levels of depletion, engraftment and antigen-specific IgG response post

immunization with TNP-KLH were detected in mice pre-conditioned with the full dose of CPA (300mg/kg), while only a mild increase of response was observed in mice pre-conditioned with the lower dose of CPA (200mg/kg) or with ALS as compared with the not conditioned group of mice. Surprisingly, despite the low engraftment of donor CD4+ T cells observed in mice pre-conditioned with the anti-CD4 antibody, some mice in this group showed levels of response that were similar to those detected in mice pre-conditioned with the full dose of CPA. However, only the latter group revealed a mild boosting effect after immunization with a second dose of antigen and maintained same amounts of antigen-specific antibodies at long term follow-up after first immunization (Fig. 4.7 A,B,C,D). Moreover, immunohistochemistry of the spleen sections of transplanted mice (Fig. 4.7 E) showed a rescue of the ability to form germinal centers in all the conditioned groups, even if at different frequencies. None of the Cd40lg-/- mice transplanted without conditioning was able to generate PNA positive nodules within their spleen.



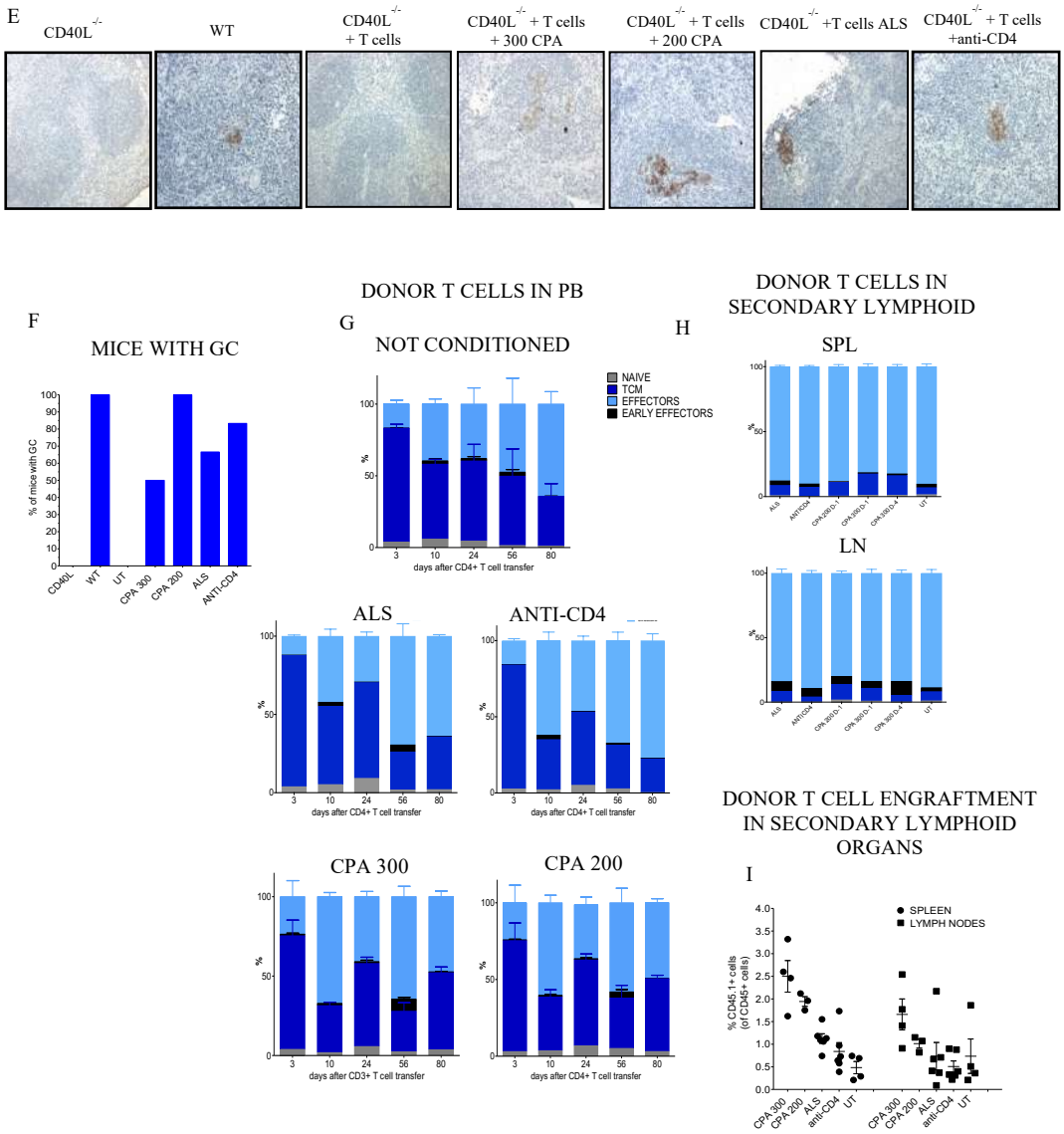



Fig. 4.7 (A) Schematic representation of adoptive transfer of $10x10^6$ activated WT CD4+ T cells into Cd40lg-/- mice pre-conditioned or not with CPA, ALS or anti-CD4 antibody. CD4+ T cells isolated from the spleen of WT donor mice were activated in vitro with anti-CD3/ anti CD28 antibody coated beads and maintained in culture for 7 days in presence of IL2, IL7 and IL15. Experimental mice were followed by weekly bleedings and vaccinated with TNP-KLH + alum at indicated times after T cell transfer. Sera were collected after primary, secondary and tertiary immunization with TNP-KLH. Assessment of recipient CD3+ T cell depletion (B) and engraftment of donor CD4+ T cells (C) at indicated times upon T cell transfer. (D) IgG-TNP-specific antibodies in serum measured by ELISA. (E) Spleens from mice immunized 7 days earlier with TNP-KLH were sectioned and stained with PNA. (F) Percentages of mice with GC within each experimental group. (G) Longitudinal flow cytometry analysis (histograms) of PB percentages of $CD44^-$ $CD62L^+$ naive, $CD44^+CD62L^+$ central memory (Tcm), $CD44^+CD62L^-$ effector, and CD44 $CD62L^-$ early effector cells within engrafted CD4+ T cell population of Cd40lg-/- mice pre-

conditioned or not with different lymphodepleting regimens. The same T cell subsets (H) as well as donor T cell engraftment (I) were also assessed in spleen and lymph nodes.

Longitudinal analysis of T cell subsets within the engrafted T cell population did not reveal significant differences between the different tested conditions. Activated CD4+ lymphocytes were mostly memory and effector cells and tended to develop an effector phenotype overtime, especially in the not conditioned group of mice and in mice pre-conditioned with ALS and anti-CD4 (Fig.4.7 G). The predominant type of subpopulation detected within spleen and lymph nodes was the effector T cell subpopulation (Fig. 4.7 H) and engraftment levels in these organs were comparable to those observed in periphery (Fig. 4.7 I).

Finally, to test if increasing the dose of adoptively transferred activated CD4+ T cells could further improve the ability to produce TNP-specific IgGs, in parallel to $10x10^6$ wild type CD4+ cells we adoptively transferred also $50x10^6$ CD4+ lymphocytes into mice pre-conditioned or not with ALS or a full dose of CPA (Figure 4.8).

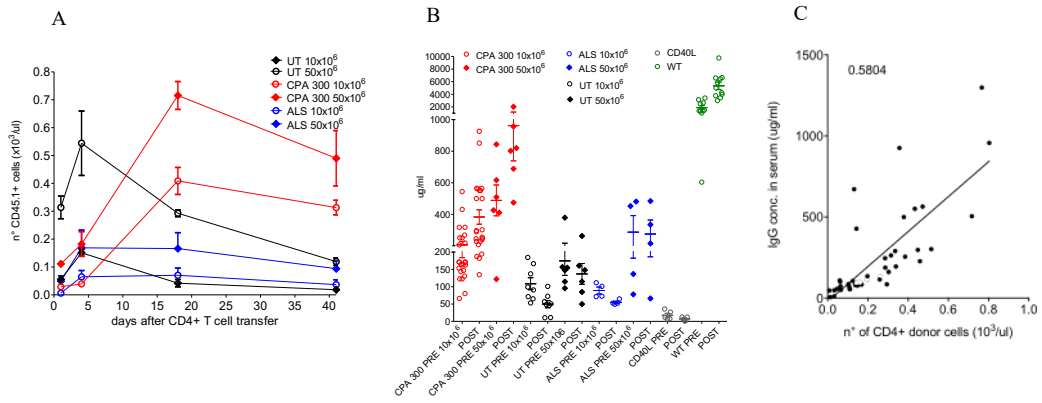
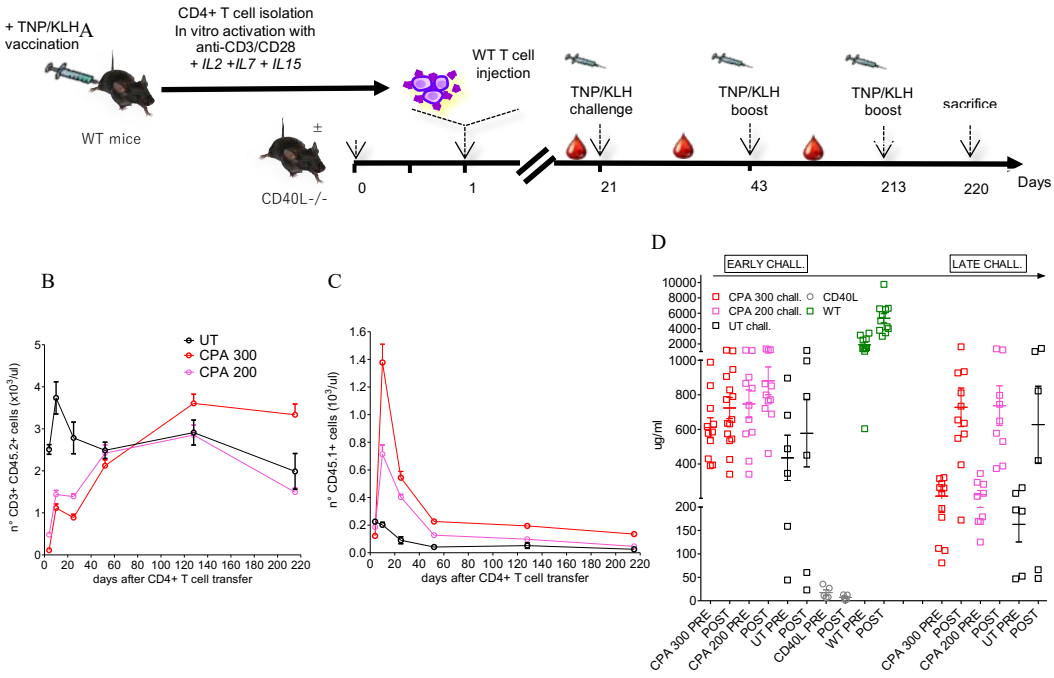



Fig. 4.8 8 weeks old CD40L-/- mice pre-conditioned or not with full dose of CPA or ALS received adoptive transfer of $10x10^6$ or $50x10^6$ activated WT CD4+ T cells by lateral tail vein administration. Recipient mice were followed by weekly bleedings and sera were collected upon primary immunization and booster immunization with TNP-KLH + alum. Mean with SEM of circulating CD3+ recipient (A) and CD4+ donor T cell numbers (B) at indicated times after T cell transfer. B) TNP-specific IgG response post prime-boost vaccination (C) correlation between absolute number of engrafted CD4+ donor T cells and IgG concentration in serum.

Results show a dose dependent engraftment (figure 4.8 A) and corresponding IgG response (Figure 4.8 B) within each experimental group of transplanted mice. We could also confirm that, comparing experimental groups transplanted with the same input dose of cells, highest levels of engraftment and rescue of the immune response were obtained upon treatment with CPA. Again, a boosting effect was detected only this latter group, which was more evident in mice infused with the highest dose of WT cells.

### 4.3.2 In vivo priming of donor CD4+ T cells results in increased partial rescue of the antigen specific IgG response even in the absence of conditioning

Finally, since T cells from patients with pre-existing infections are already primed for antigens, we aimed to model the immune response in this context by infusing into mice pre-conditioned or not with 300 mg/kg or 200mg/kg CPA, $10x10^6$ CD4+ T cells derived from previously TNP-KLH-immunized WT donor mice (Fig 4.9 A).



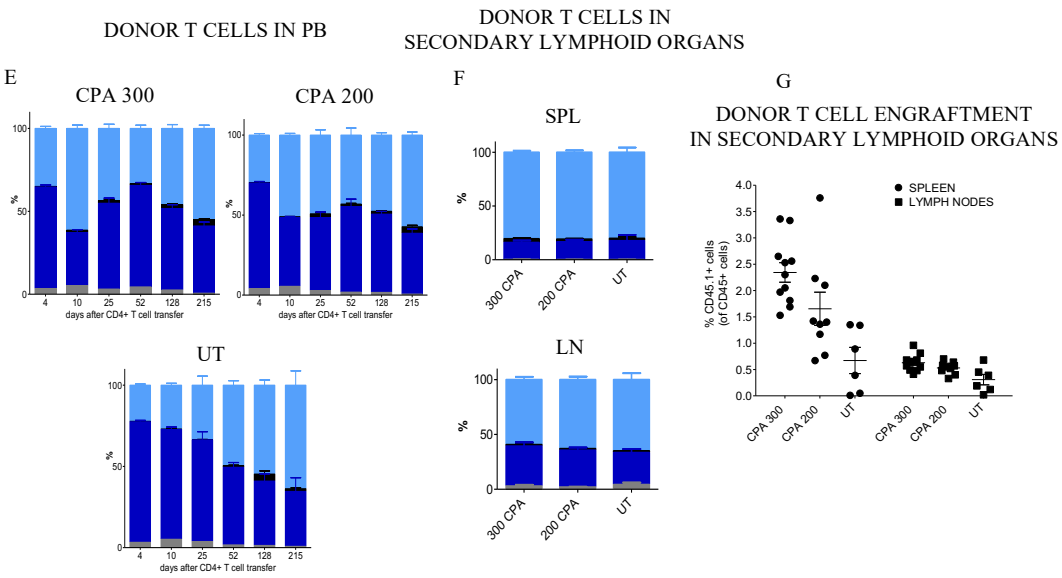



Fig. 4.9 (A) Schematic representation of adoptive transfer of $10x10^6$ activated WT CD4+ T cells into Cd40lg-/- mice pre-conditioned or not with CPA. WT donor mice were challenged by intraperitoneal injection of TNP-KLH + alum 14 days before purification of donor CD4+ T cells from spleens. Isolated CD4+ lymphocytes were activated in vitro with anti-CD3/ anti CD28 antibody coated beads and maintained in culture for 7 days in presence of IL2, IL7 and IL15. Experimental mice were followed by weekly bleedings and vaccinated with TNP-KLH + alum at indicated times after T cell transfer. Assessment of recipient CD3+ T cell depletion (B) engraftment of donor CD4+ T cells (C) and TNP-specific IgG response (D) at indicated times upon T cell transfer. E) Longitudinal changes of circulating levels of different T cell subsets within engrafted CD4+ donor T cell population isolated from TNP-KLH primed WT mice. T cell phenotype F) and G) engraftment in spleen and lymph nodes

Figure 4.9 shows that, although doses of CPA treatment, depletion degree (Fig. 4.9 B) and engraftment levels (Fig. 4.9 C) of activated CD4+ T cells were comparable to those observed in our previous experiments in which, the same input dose of activated donor T cells was infused in the same experimental groups, the amounts of TNP-specific antibodies detected in sera were overall higher and, unlike our previous results, comparable levels of IgG response were detected in all the tested conditions, notably, also in mice adoptively transferred without conditioning (Fig.4.9 D). As observed in our previous experiment, longitudinal analysis of T cell subsets within the engrafted T cell population did not reveal significant differences between the different tested conditions. Activated CD4+ lymphocytes were mostly

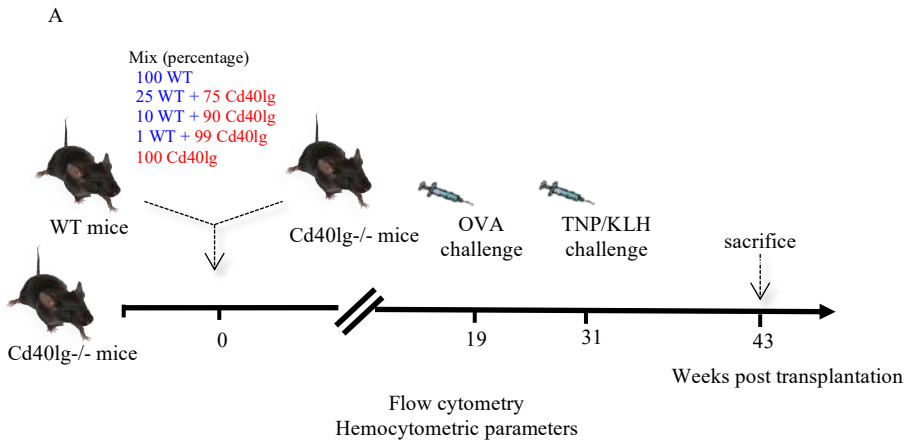
memory and effector cells and tended to develop an effector phenotype overtime, especially in the not conditioned group of mice (Fig.4.9 E). Again, the predominant phenotype of engrafted CD4+ T cells in spleen and lymph nodes was the effector T cell phenotype (Fig. 4.9 F), particularly in the spleen. Engraftment levels in these organs were comparable to those observed in peripheral blood (Fig. 4.9 G)

## 4.4 Establishing the threshold proportion of functional HSPC to ensure reconstitution of the immune function in Cd40lg-/- mice

To assess the dose of cells required to ensure rescue of the immune function with corrected HSPCs we performed co-infusions of WT HSPC mixed with *Cd40lg-/-* HSPC into HIGM recipients conditioned by total body irradiation (TBI) and monitored lymphohematopoietic reconstitution over time. In particular, these competitive transplantations were designed to compare doses of 0 (0%) versus 10,000 (1%) versus 100,000 (10%) versus 250,000 (25%) versus 500,000 (50%) versus 1,000,000 (100%) of WT HSPCS (Fig. 4.10 A). By observing the hematopoietic chimerism of WT and Cd40lg-/- cells within CD19+ B cells, CD4+ T cells, CD8+ T cells and CD11b myeloid cells, we found a mild selective advantage of WT T lymphocytes over defective T cells in PB and SPL (fig. 4.10 B,C). This advantage was more evident within the CD4+ T cell compartment compared with the CD8+ T cell compartment, as expected for a cell type that is dependent on Cd40lg signaling for its function. Conversely, the WT chimerism observed within mature B cells and myeloid cells closely mirrored that of the HSPC. Overall, B, T and myeloid cell reconstitution was comparable among different experimental groups, measured both as percentage and absolute counts of engrafted cells (fig. 4.10 D,E).

To determine the proportion of functional cells required to restore the antigen-specific IgG response, we vaccinated transplanted mice with Ovalbumine (OVA) and TNP-KLH and evaluated their ability to produce switched antibodies against

these specific antigens after primary challenge and a recall immunization. Mice injected with increasing proportions of WT HSPCs displayed a dose-dependent CD4+ T cell engraftment (Fig. 4.10 F) and rescue of the immune function, measured both as serum levels of antigen-specific IgGs and percentages of splenic GC B cells. Whereas mice transplanted with 1% functional cells nearly failed to produce OVA or TNP-KLH specific IgGs and to engage B cells for germinal center formation (Fig. 4.10 G,H,I,J), mice transplanted with 10% or 25% of WT HSPCs partially rescued the switched-antibody response to primary or recall vaccinations and GC formation in the spleen. Importantly, the administration of 10% or 25% of WT HSPCs led to levels of TNP-specific IgG response comparable to those obtained in Cd40lg-/- mice transplanted either with WT T cells after CPA treatment or with primed WT lymphocytes with or without pre-conditioning (Fig. 4.10 K) Overall, similar levels of disease correction were achieved with both approaches.



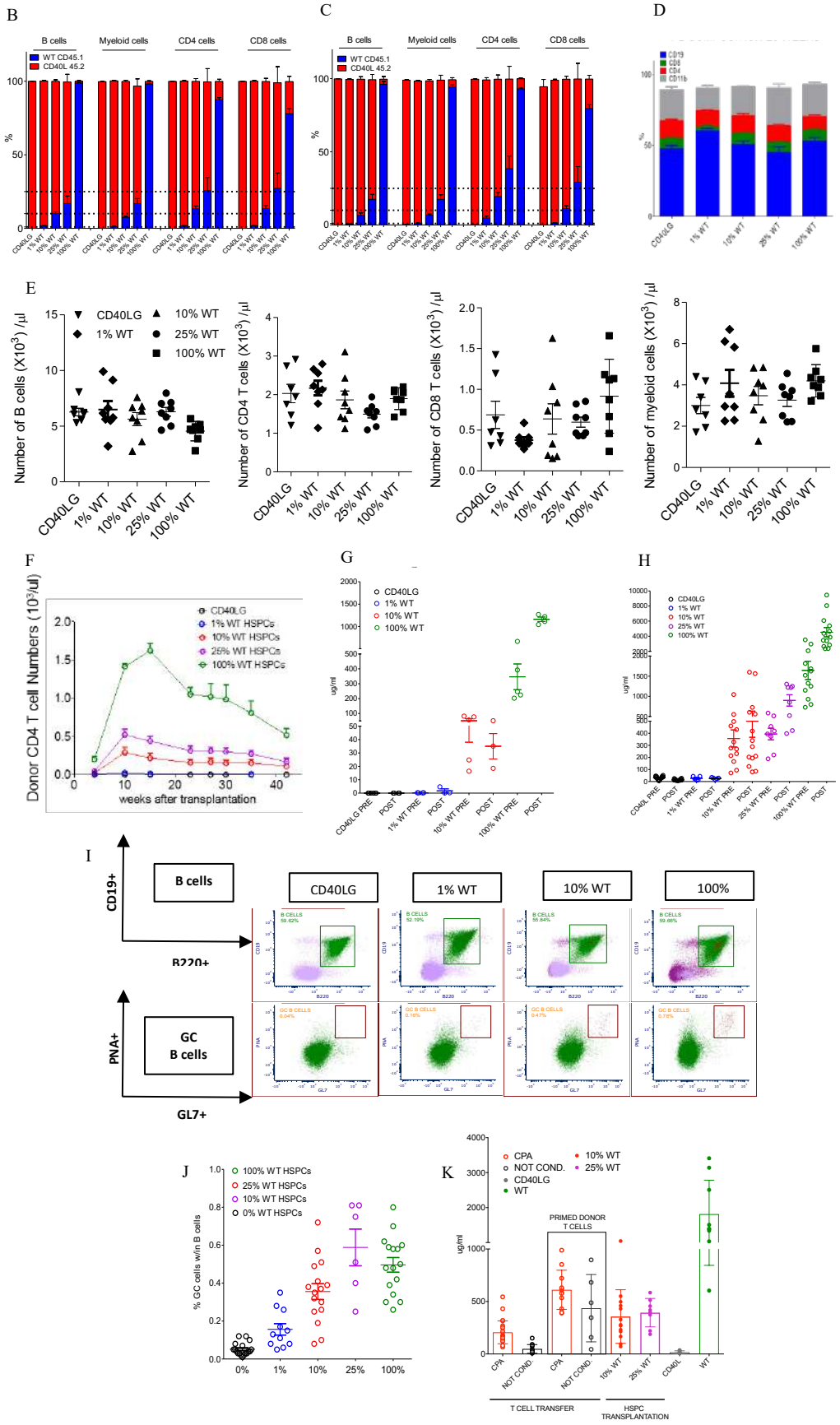

B
B cells
Myeloid cells
CD4 cells
CD8 cells
WT CD45.1
CD40L 45.2
%
C
D
CD19
CD8
CD4
CD11b
E
CD40LG
1% WT
10% WT
25% WT
100% WT
Number of B cells (X10³) /µl
Number of CD4 T cells (X10³) /µl
Number of CD8 T cells (X10³) /µl
Number of myeloid cells (X10³) /µl
F
Donor CD4 T cell Numbers (10³/ul)
CD40LG
1% WT HSPCs
10% WT HSPCs
25% WT HSPCs
100% WT HSPCs
weeks after transplantation
G
ug/ml
CD40LG PRE
POST
1% WT PRE
10% WT PRE
100% WT PRE
H
I
CD19+
B220+
B cells
PNA+
GL7+
GC B cells
CD40LG
1% WT
10% WT
100%
J
100% WT HSPCs
25% WT HSPCs
10% WT HSPCs
0% WT HSPCs
% GC cells w/in B cells
K
CPA
NOT COND.
CD40LG
WT
10% WT
25% WT
PRIMED DONOR T CELLS
T CELL TRANSFER
HSPC TRANSPLANTATION

**Fig. 4.10** (A) Schematic representation of competitive transplant at different ratios of WT (blue) and Cd40lg -/- HSPC (red) into lethally irradiated HIGM recipients. (B,C) Chimerism of WT and Cd40lg-/- cells observed within $CD19^+$ B cells, $CD4^+$ T cells, CD8+ T cells and $CD11b^+$ myeloid cells 43 weeks after transplant in peripheral blood and spleen (n = 6 or 8 per group, pooled from three independent experiments). (D) Percent composition of myeloid and lymphoid lineages in PB 25 weeks after transplant. (E) Total counts of $CD19^+$ B cells, $CD4^+$ T cells, CD8+ T cells and $CD11^+$ myeloid cells in PB 25 weeks after transplant. (F) CD4+ donor T cell engraftment within the different experimental groups. (G, H) Sera from competitive transplanted Cd40lg-/- mice, vaccinated with TNP-KLH and OVA were collected at indicated times and antigen-specific IgG concentrations were determined by ELISA. Each data point represents the titer of one mouse. (I) Example of a flow-cytometry analysis plot, showing B220+PNAhigh GC B cell percentages within the different experimental groups. At day 7 post tertiary immunization, splenic mononuclear cells were stained with B220, CD19, PNA, and GL7. PNA+GL7+ GC B cells are derived from B220+CD19+ gated splenocytes. (J) Percentages of PNA+GL7+ GC B cells within different experimental groups. Each data point represents one mouse (n = 6 or 8 per group, pooled from three independent experiments. (K) Comparison between the TNP-specific IgG production after T cell transfer of activated WT CD4+ T cells (in vivo primed or not) and after transplantation of 10% or 25% of WT HSPC.

## 4.5 Establishing the rationale for the enrichment of corrected HSPC in HIGM gene editing

Finally, we performed another experiment in which we infused a higher dose of competitive transplanted WT HSPC and found that 50% of functional HSPC was sufficient to completely restore the TNP-specific IgG response. Since 50% of gene editing efficiency is difficult to achieve with current HSPC gene editing protocols, we wondered whether we could enhance the therapeutic potential of our gene editing approach by using a selection strategy to enrich for corrected HSPC prior to transplantation. To assess this, we also modeled a selection-like procedure in which, as in our previous experiments, WT HSPC were co-transplanted at different ratio with Cd40lg-/- HSPC but in addition, the same numbers of functional cells were also infused without the respective fraction of defective cells. Figures 4.11 A

and C show that the WT chimerism observed within each group closely mirrored that of the input dose of cells and that B, T and myeloid cell reconstitution was comparable among different experimental groups, respectively. Taken together our results reveal that Cd40lg-/- mice reconstituted with increasing proportions of WT HSPC display a dose-dependent rescue of the T cell mediated immune response to TNP-KLH immunization, that culminates in a complete restoration of the IgG response upon infusion of 50% of functional HSPC. In addition, if as expected, co-infusing 25% of functional cells along with 75% of defective cells was sufficient to ensure only a partial rescue of the antigen-specific immune response, the transplantation of the same dose of WT HSPC alone completely restored this response (Fig. 4.11 B).

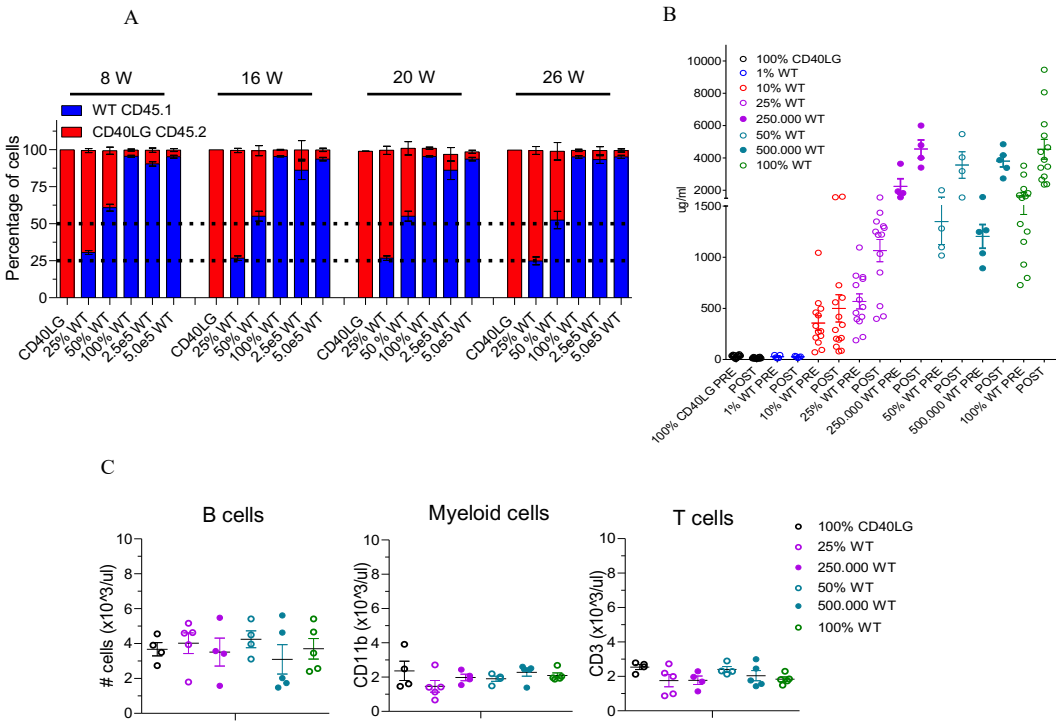



Figure 4.11 (A) Chimerism of WT and Cd40lg -/- cells observed within CD11b+ myeloid cells at long term follow up after transplant of WT Lin− cells (n = 6 or 8 per group). B) TNP-specific IgG response within different experimental groups. (C) Total counts of $CD19^{+}$ B cells, $CD11^{+}$ myeloid cells and T cells (lower in PB 26 weeks after transplant.

## 4.6 Generating a model of *Pneumocystis murina* acute airways infection in Cd40lg-/- mice

To assess whether the partial rescue of the immune function that we observed upon transplantation could be sufficient to clear a disease relevant pathogen, we developed a model of P. *murina* airways infection in Cd40lg-/- mice. To determine suitable conditions for inducing appropriate acute airway infection, we challenged Cd40lg -/- and WT mice with P. *murina* via intratracheal or intranasal injection or by placing the animals in co-housing with infected Cd40lg-/- mice. Starting from 3 weeks after infection, change in body weights were monitored weekly. In Cd40lg-/- mice, infection led to 50% mortality within 4-6 months. The week before animals' death, typical signs of the pathology started to appear, including weight loss of approximately 30%, hunched-back posture, listlessness and rapid shallow breathing. By contrast, all WT mice survived the infection. Cd40lg-/- mice showing symptoms of P. *murina* induced pneumonia were sacrificed and murine lungs were collected to assess infection. We observed a massive increase in fungal load in the Cd40lg-/- mice infected with P.*murina* compared to WT mice, confirming a severe defect in P. *murina* clearance in Cd40lg-/- mice and thus mimicking hallmarks of the human disease.

### 4.6.1 Analysis of lungs after P. murina infection

To better characterize the molecular and histopathological features of P.*murina* infection, lungs of infected mice displaying first symptoms of the disease were homogenized for the detection of P. *murina* ribosomal RNA by Droplet Digital PCR (ddPCR) and to identify P. *murina* cystis by indirect immunofluorescence staining (IF). Furthermore, pulmonary lesions suggesting P.*murina* pneumonia and P.*murina* cystis themselves were detected on formalin-fixed, paraffin-embedded

lung specimens by combining Hematoxylin and Eosin (H&E) staining with immunohistochemistry (IHC). Under high magnification, stained organisms had a cystic structure and nucleus, indicative of P. *murina* (Fig. 4.12 E). Lungs of infected mice typically present acidophilic macrophagic pneumonia (AMP), peri-bronchial and peri-vasal lymphocytic infiltrates. Nonetheless, only heavily infected mice showed P. murina-associated interstitial pneumonia, characterized by the accumulation of macrophages in alveolar spaces and interstitial inflammation.

### 4.6.2 Attenuation of P. murina infection in competitive transplanted Cd40lg-/- mice

To determine whether the transplantation of low doses of functional HSPCs is sufficient to confer protection against P.murina pneumonia in experimental mice, Cd40lg-/- mice competitive transplanted with 0%, 10%, 25% and 100% WT HSPCs were challenged by intranasal injection of P. murina organisms. 4,5 months after inoculation, signs of the pathology started to appear in the group of Cd40lg-/- mice transplanted with 0% functional HSPCs, all experimental mice were euthanized, and lungs collected to evaluate the establishment of infection.

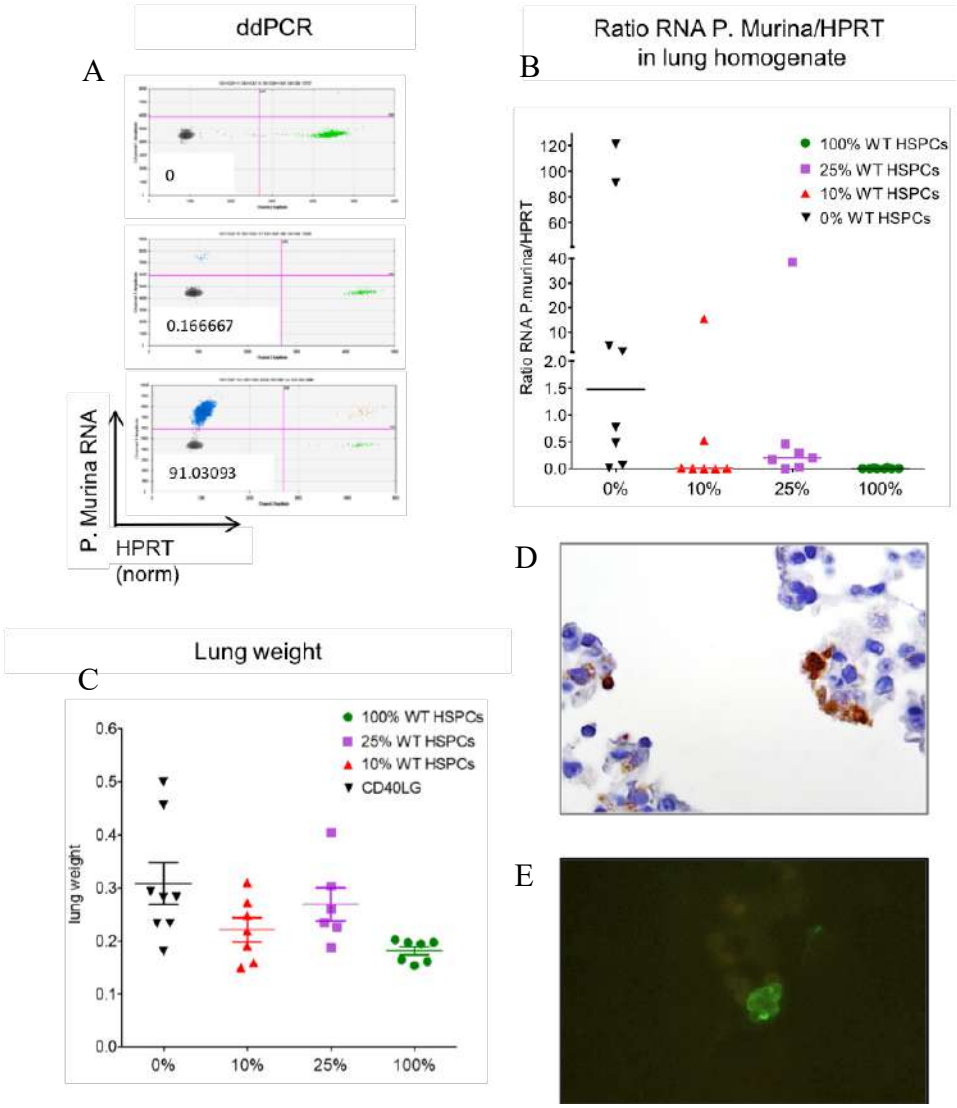



Fig. 4.12 (A) Representative 2D scatter plot of ddPCR results, corresponding to lung homogenates from cases 1A2, 5C4 and 7D2 respectively. The *y-axis* shows the fluorescence amplitude of the FAM probe, designed to hybridize only to P.murina cDNA (*blue*). The HEX probe, which hybridizes only to reference gene HPRT (*green*), is plotted on the *x-axis*. Double-positive droplets carrying both types of molecules are represented in *orange*, while double-negative droplets (no amplification) are shown in *grey*. HPRT Hypoxanthine Phosphoribosyltransferase, *ddPCR* droplet digital PCR (B) Quantitation of P. murina ribosomal RNA of infected lung homogenate using ddPCR. Results are expressed in P.murina/HPRT RNA copies per 20 ul well. Each circle represents one mouse (C) Lung weight from mice infected with P. murina (D) Representative picture of IHC finding from case 7D1. Five µm-thick tissue sections obtained from the paraffin blocks were stained with Hematoxylin and Eosin (H&E) and immunohistochemistry with a rabbit polyclonal antibody. Brown areas represent P.murina organisms. (E) Representative picture at high magnification of P.murina cystis from case 3B2 detected by immunofluorescence.

Different dilutions of lungs homogenates were stained with an indirect immunofluorescence technique.

As reported in figure 4.12 and in table 4, lung sections of all infected mice showed accumulation of lymphocytes around blood vessels and bronchi. 4 out of 8 Cd40lg-/- mice transplanted with 0% functional HSPCs showed substantial accumulation of inflammatory lymphocytes and macrophages, intrabronchial crystals and, in one case, also exudation of amorphous foamy material into many of the alveoli, suggesting severe pneumonia. Typical features of interstitial pneumonia were also detected in 1 out of 8 mice transplanted with 10% WT HSPCs and 1 out of 8 mice transplanted with 25% functional cells. In contrast, lung sections from Cd40lg-/- mice transplanted with 100% WT HSPCs showed very limited evidence of inflammation. Consistent with these data, heavily infected mice described in table 4, namely cases 3B1 (10%) , 5C3 (25%) , 7D2 and 7D5 (0%) showed high levels of P. *murina* ribosomal RNA in lung homogenate (Fig. 4.12 A,B) substantial accumulation of immuno-stained organisms in alveoli and increased lung weight (Fig. 4.12 C). 2 out of 8 mice of the group transplanted with 10% functional HSPCs, 3 out of 8 mice of the 25% transplanted group and 5 out of 8 mice transplanted with 100% defective cells showed a decreased area of organisms in alveoli (Fig. 4.12 D, E) and minimal to moderate levels of ribosomal RNA, while no P. murina cystis and almost undetectable levels of ribosomal RNA were observed in lungs of the remaining mice belonging to the above mentioned groups. As expected, cystis were absent in mice transplanted with 100% WT HSPCs, except for one mouse, which presented very few immunofluorescent organisms in the lungs. Mice of this latter group showed absent to almost undetectable levels of P.*murina* RNA. Taken together these results indicate that, infusion of 10% and 25% of WT HSPCs is sufficient to prevent or at least attenuate infection in lungs of Cd40lg-/- mice challenged with P.*murina*.

| # | IF* | IHC** | ddPCR | Other lesions (severity) |
|---|---|---|---|---|
| 1A0 | - | - | 0,046 | Peribronchial/perivasal lymphocytic infiltrates, +++ |
| 1A1 | - | - | 0 | Perivasal lymphocytic infiltrates, ++ |
| 1A2 | - | - | 0 | Peribronchial/perivasal lymphocytic infiltrates, ++ |
| 1A3 | - | - | 0,009 | Peribronchial/perivasal lymphocytic infiltrates, +++ |
| 1A4 | - | - | 0 | Peribronchial/perivasal lymphocytic infiltrates, +++ |
| 1A5 | - | - | 0,039 | - |
| 2A0 | + | - | 0,029 | Peribronchial/perivasal lymphocytic infiltrates, +++ |
| 2A1 | - | - | 0 | • Lymphoma (peribronchial)<br>• AMP (+). |
| 3B0 | - | - | 0,03 | • Peribronchial/perivasal lymphocytic infiltrates, ++<br>• AMP, ++ |
| 3B1 | +++ | +++ | 14,987 | • Peribronchial/perivasal lymphocytic infiltrates, ++<br>• AMP, +++<br>• Interstitial pneumonia (lymphocytes, macrophages), + |
| 3B2 | + | + | 0,567 | • Peribronchial/perivasal lymphocytic infiltrates, +<br>• AMP, + |
| 3B3 | + | - | 0,013 | • Peribronchial/perivasal lymphocytic infiltrates, +<br>• AMP, + |
| 4B0 | - | - | 0,01 | - |
| 4B1 | - | - | 0,005 | Peribronchial/perivasal lymphocytic infiltrates, ++ |
| 4B3 | - | - | 0 | Peribronchial/perivasal lymphocytic infiltrates, ++ |
| 5C0 | - | - | 0,089 | • Peribronchial/perivasal lymphocytic infiltrates, ++<br>• AMP, + |
| 5C3 | +++ | +++ | 38,564 | • Peribronchial/perivasal lymphocytic infiltrates, ++<br>• AMP, ++<br>• Interstitial pneumonia (lymphocytes, macrophages), +++ |
| 5C4 | - | - | 0,167 | - |
| 6C0 | + | + | 0,238 | • Peribronchial/perivasal lymphocytic infiltrates, +<br>• AMP, ++ |
| 6C1 | + | - | 0,272 | • Peribronchial/perivasal lymphocytic infiltrates, +++<br>• AMP, ++ |
| 6C2 | - | - | 0,007 | Peribronchial/perivasal lymphocytic infiltrates, +++ |
| 6C3 | + | + | 0,419 | • Peribronchial/perivasal lymphocytic infiltrates, ++<br>• AMP, ++<br>• Interstitial pneumonia (lymphocytes, macrophages), + |
| 7D0 | + | - | 1,943 | - |
| 7D1 | + | + | 4,379 | • Peribronchial/perivasal lymphocytic infiltrates, ++<br>• AMP, ++<br>• Interstitial pneumonia (lymphocytes, macrophages), + |
| 7D2 | +++ | +++ | 91,03 | • Interstitial pneumonia with intra alveolar foamy/granular material |
| 7D3 | + | + | 0,064 | • Peribronchial lymphocytic infiltrates, + |
| 7D4 | - | - | 0,01 | • Peribronchial/perivasal lymphocytic infiltrates, ++<br>• AMP, ++, with intrabronchial crystals |
| 7D5 | +++ | +++ | 121 | • Peribronchial/perivasal lymphocytic infiltrates, +<br>• AMP, +<br>• Interstitial pneumonia (lymphocytes, macrophages), +++ |

| | | | | |
|---|---|---|---|---|
| 8D0 | + | - | **0,512** | • Peribronchial/perivasal lymphocytic infiltrates, ++<br>• AMP, ++, with intrabronchial crystals |
| 8D1 | + | + | **0,856** | • Peribronchial/perivasal lymphocytic infiltrates, ++<br>• AMP, +++<br>• Interstitial pneumonia (lymphocytes, macrophages), + |

**Table 4 Summary of histological and molecular evaluation of lungs from competitive transplanted mice infected with Pneumocystis murina.** Interstitial pneumonia in the presence of finely granular intra-alveolar material can be considered a typical P. murina associated lesion.

* Immunofluorescence on homogenate from lung tissue: higher score obtained in spot 1 and 5

**amount of positive material with morphological pattern compatible with P. murina

Scoring system: - = absent; + = minimal; ++ =moderate; +++ severe (abundant)

*** in the presence of finely granular intra-alveolar material

AMP = Acidophilic macrophage pneumonia

100% WT HSPCs

10% WT HSPCs

100% CD40LG

25% CD40LG

# 5 DISCUSSION

## 5.1 *CD40LG* gene editing strategy for the treatment of HIGM

Current gene therapy strategies rely on the use of new generation vectors, with an improved design, that ensure safer integration profiles, thus substantially alleviating the risk of genotoxicity arising from semi-random integration of the vector into the genome. However, the use of reconstituted artificial promoters cannot guarantee the restoration of a physiological expression pattern of the therapeutic transgene. This issue is particularly relevant when the target gene is involved in cell differentiation and proliferation because its unregulated expression may trigger uncontrolled cell expansion with a possible oncogenic potential. A relevant example is *CD40LG* gene, which is expressed on activated T cells in a tightly controlled manner. In fact, HSC gene therapy was proposed as a potential treatment for HIGM1 syndrome, but even if preclinical studies showed results demonstrating efficacy, ectopic and unregulated expression of the gene in thymocytes or peripheral T cells caused T and B lymphoproliferations, most of which progressed to frank lymphomas (Brown et al., 1998; Sacco et al., 2000). A gene editing approach for the treatment of HIGM1 syndrome could overcome this hurdle by ensuring a physiological expression of the gene. Because the genetic defect of the CD40L gene is not lethal to developing T cells, we aim to apply our gene editing strategy to autologous T cells, thus providing immediate therapeutic benefit to patients by resolving pre-existing infections and allowing a definitive cure by hematopoietic stem/progenitor cell (HSPC) transplant. However, preclinical evidence of the feasibility and therapeutic efficacy of a T cell-based gene editing approach to treat HIGM1 syndrome is lacking. We therefore used a Cd40lg-/- mouse model of HIGM1 to assess i) the minimal dose of edited cells required to relieve the disease phenotype, ii) the need for a conditioning regimen, iii) the persistence

of the adoptively transferred cells over time and iv) the possibility of rejection of the corrected cells.

### 5.1.1 Adoptive transplant of gene corrected autologous T cells

Preclinical studies in the mouse model of the disease revealed that Cd40l-/- mice, either preconditioned or not with different lymphodepleting regimens prior to infusion of different doses of WT T cells, show long-term and stable T cell engraftment and partially rescue antigen-specific IgG response and germinal center formation in splenic follicles after vaccination with a thymus-dependent antigen (TNP-KLH), with the highest rate of response obtained in mice pretreated with a chemotherapy regimen (cyclophosphamide). Our study supports a rationale for moving toward clinical testing of T cell gene correction in HIGM1 patients. Administration of the autologous edited T cells could be performed upon conditioning, aiming to establish sufficient engraftment and persistence of corrected cells, thus improving the therapeutic efficacy of our approach. However, extrapolation of the mouse data to the human setting should also warrant the following considerations. Animals (including humans) are exposed to commensal and pathogenic microbes throughout their lives, and this physiological microbial exposure has a profound impact on immune competency and overall health. The use of laboratory mice housed under specific pathogen-free (SPF) conditions in the vast majority of immunology research has been an important step to improve experimental consistency, but these unusually clean living conditions have inadvertently left the laboratory mice with an underdeveloped immune system, far different from the immune system found in humans (Huggins et al., 2019), thus resulting in a possible underestimation of the real level of immune response to an antigenic challenge that could possibly be achieved in the human setting. In fact, since T cells from patients with pre-existing infections are already primed for antigens, to better recapitulate the clinical scenario, we also infused T cells derived from previously TNP-KLH-immunized

WT donor mice. Compared to recipients receiving naïve T cells, these adoptively transferred CD40L-/- mice produced increased amounts of circulating antigen-specific IgGs in response to vaccination and importantly, similar levels of response were observed also in the absence of conditioning. Another aspect that may be particularly noteworthy in this regard is the possibility to elicit in patients with pre-existing infections a fatal inflammatory disease after reconstitution with CD4+ T cells. This inflammatory reaction is referred to as Immune Reconstitution Inflammatory Syndrome (IRIS) and is a major adverse event of antiretroviral therapy in HIV infection that paradoxically occurs as HIV viremia is suppressed and CD4+ T cell numbers recover. In individuals with IRIS, opportunistic infections acquired during the period of immunodeficiency may have been previously diagnosed and treated, or they may be subclinical and revealed by the host's regained capacity to mount an inflammatory response (French, 2009) [7]. If immune function improves rapidly following the commencement of ART, systemic or local inflammatory reactions may occur at the site or sites of the preexisting infection. This inflammatory reaction is usually self-limited, especially if the preexisting infection is effectively treated. However, long-term sequelae and fatal outcomes may rarely occur, particularly when neurologic structures are involved (Berkeley, Nath and Pardo, 2008). The occurrence of a fatal hyperinflammatory reaction associated with T-cell transfer has also been reported in *scid* recipient mice with well-established *Pneumocystis murina* induced lung infection (Roths and Siedman, 1992). Thus, a future clinical application should take in consideration of this theoretic risk and be designed in order to minimize the occurrence of adverse events by performing dose escalation transplants in patients with acute infections or by introducing safety switching into the ex vivo engineered T cells.

### 5.1.2 Autologous transplant of gene corrected HSPC

To possibly provide an even broader and prolonged therapeutic benefit to the patients by correcting all the affected hematopoietic lineages and by providing a

life-long supply of edited cells, we then adapted our gene correction strategy to target autologous HSPC. The proportion of functional HSPCs required to correct the disease in our mouse model was determined by performing competitive transplants of different doses of cells expressing the WT murine Cd40lg and cells expressing a murine *Cd40lg-/-*. We found that as low as 10% of functional HSPC in the cell product is sufficient to partially restore serologic immunity against different antigens (TNP-KLH, OVA) and germinal center formation. This finding is encouraging from the perspective of a clinical translation because we found that by exploiting our recently optimized gene editing protocol it is possible to attain this threshold on human HSPC reaching up to 50% of *CD40LG* editing in the bulk treated CD34+ cells and 30% after xenotransplantation in NSG mice (Fig. 5.1).

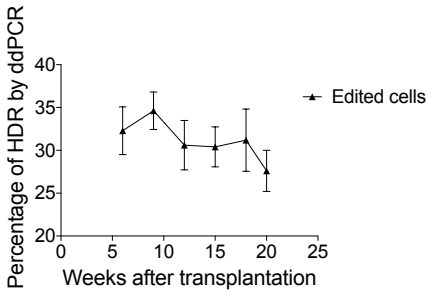



Fig. 5.1 Percentage of HDR within human cells measured by ddPCR.

In addition, our data show that similar levels of disease correction are achieved by transplanting T cells, thus further supporting a T-cell based gene editing approach for the treatment of HIGM syndrome. However, our studies in the mouse model showed that a complete rescue of the antigen-specific IgG response can be observed only after infusion of ~50% of functional HSPC. Thus, even if the engraftment of ≥10% corrected HSPC could be sufficient to provide therapeutic benefits in human patients, improving gene editing efficiency will be necessary to achieve full reconstitution of the immune function. Here we show that we could possibly overcome the challenge of achieving higher editing efficiency by adopting a selection strategy to enrich for corrected HSPC prior to transplantation. In fact, our

data show that disease correction can be achieved in the mouse model by performing a transplantation of a lower input dose of functional HSPC (that is the number of cells equivalent to 25% of competitively transplanted WT HSPC), but in the absence of competition with the defective fraction of cells. These data might suggest that in a competitive repopulation model, WT and Cd40lg-/- T cells compete for B cell activation within the same functional niche, thus indicating the strong therapeutic benefit that could be achieved in HIGM patients by selecting only genetically modified autologous HSPC prior to their transplantation.

### 5.1.3 Gene editing of human T cell and HSPC

In parallel to the studies on the murine model, I contributed to ongoing studies in our lab aimed to develop a gene correction approach on human T cells and HSPC. In particular this gene editing strategy, based on ribonucleoprotein electroporation and AAV6 transduction, targets the CD40L gene within its first intron and replaces the endogenous gene starting from exon 2 to exon 5 (Fig. 5.2).

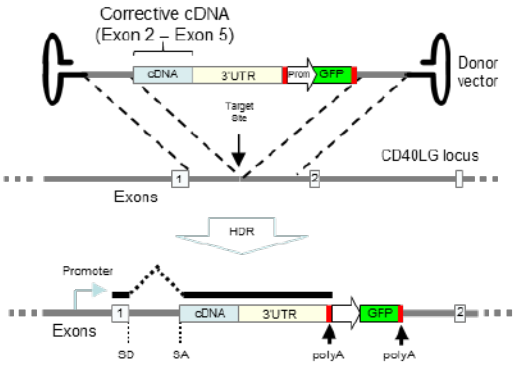



Fig. 5.2 Schematics of gene editing strategy and donor DNA template for intron 1 CD40LG target locus.

We selected the first intron as the target locus mainly for three reasons. First of all, this approach can lead to the correction of at least 95% of *CD40LG* mutations, thus

being an available option to treat the majority of affected patients. Then, not only HDR but also in sense NHEJ- mediated integration of the donor template can be corrective, because the targeting within an intron can be less precise than within an exon, where the ORFs must not be disrupted. Finally, for safety reasons, we didn't include into the donor construct neither any promoter sequence nor the full-length *CD40LG* cDNA. This choice is in contrast with the strategy published by Hubbard and colleagues in 2016. They targeted with a TALEN pair the 5'UTR of the gene and they delivered as AAV the donor template containing the full length *CD40LG* sequence diverged cDNA, followed by the 3' UTR and the putative polyA sequence. Notably, a part of the promoter sequences was included into the corrective construct (see chapter 1.4.8). Our approach should prevent the risk of genotoxicity and of adverse events due to an off-target integration of donor vector in the genome, especially when targeting HSPCs, thus causing unregulated expression of the entire gene maybe enhanced by its promoter sequences. Moreover, we maintained downstream to *CD40LG* cDNA its endogenous 3'UTR. Its inclusion could be relevant because it could be important for mRNA polyadenylation, translation, stability, localization and, remarkably, for the regulation of the gene expression due to the presence of miRNA binding sites. In addition, since as previously described, adverse events could occur into patients after transplantation of activated CD4+ T cells, we also developed a strategy based on cell surface expression of a modified version of the truncated form of human epidermal growth factor receptor (EGFR) that enables the elimination of infused cells by the clinical monoclonal antibody cetuximab, a chimeric antibody used to treat EGFR-expressing colorectal and head and neck cancer (Wang et al., 2011). By exploiting a stimulation protocol that preserves the long-term surviving T stem memory cells, we obtained ~35% of editing efficiency in both healthy donor and patients derived T cells reaching ~60% of the physiologic expression level of CD40L gene. T-B cells co-culture assays confirmed that this expression level is sufficient to restore CD4 T cells ability to provide normal contact dependent helper

function to B cells, as assessed by measuring *in vitro* proliferation, class switching, and IgG secretion.

## 5.2 Future directions

### 5.2.1 The clinical perspective of adoptive T cell therapy for HIGM

Up to date, immunoglobulin replacement therapy and bone marrow transplantation are available and established therapeutic options for the supportive treatment of HIGM syndrome. Immunoglobulin replacement permits to largely control the clinical consequences of the disease. However, the susceptibility to opportunistic infections remains problematic to manage (Davies et al., 2010); therefore, bone marrow transplantation (BMT) remains the only curative treatment for this disease. However, even if identical BMT results in good outcome, for most of the patients this option is not available. Thus, haploidentical or matched unrelated donor BMT remains the only available possibilities. However, non-identical BMT results in suboptimal prognosis, morbidity and mortality and in a higher risk of graft-versus-host disease development when compared to identical BMT. Infections revealed to be a major factor of mortality (Gennery et al., 2004), and some reports suggest that latent infections could reactivate after BMT even when they are apparently subclinical and negative at conventional testing (McLauchlin et al., 2003). In this context, autologous transplantation of corrected T cells may be a suitable "bridge therapy" for patients who have ongoing hardly manageable infections, prior to perform a BMT for the definitive cure. Our results pave the way toward clinical translation of targeted genome editing of T cells for the treatment HIGM, but still, some open questions need to be addressed. A first efficient scale up of the genome-editing process to clinically relevant numbers of cells will be necessary to make feasible the clinical translation. Then, it will be of primary importance to properly define the inclusion and exclusion criteria of the clinical trial. Indeed, our strategy of Intron 1 *CD40LG* gene correction would represent a treatment option for patients

who carry a mutation downstream to exon 1. However, not all the patients with this kind of mutations should be included in a gene editing clinical trial. Patients in critical conditions or that failed a first BMT and are waiting for a compatible matched donor urgently need a treatment. However, Conditioning regimens may be necessary to reduce the number of circulating T cells and to promote *in vivo* expansion of transferred cells by limiting the competition for cytokines (Gattinoni et al., 2005; Paulos et al., 2007), thus increasing engraftment and persistence (Klebanoff et al., 2005; Dudley et al., 2008; Uttenthal et al., 2012). Thus, the risk/benefit ratio of performing a pre-transplant conditioning to a patient affected by immunodeficiency, and possibly also ongoing infections, have to be properly assessed and will probably depend on the age, the clinical status and the co-morbidity of each patient. The administered cell dose is also difficult to be controlled. Typically, T cells are isolated from peripheral blood apheresis, cultured *in vitro* and re-infused as a defined number of cells per kilogram of body weight. However, the final number of cells and their phenotype and composition vary because of different patient-specific T cells replication and expansion rates both *in vitro* and after transplantation.

Currently, our efforts are aimed to demonstrate the ability of adoptively transferred Cd40lg-/- mice to clear or at least to attenuate a P. *murina* induced lung infection. Like individuals with HIGM1 syndrome, CD40L-/- mice are highly susceptible to the opportunistic infection mediated by *Pneumocystis murina* and several lines of evidence demonstrated that CD4+ T cells are required for resistance to Pneumocystis induced Pneumonia (PCP) and in particular, even if further characterization of the involved mechanisms needs to be conducted, the CD40-CD40L interaction seems to play a necessary role in PCP resolution (Wiley and Harmsen, 1995, Bishop et al., 2005). In our current study, we showed that the infusion of a low dose of WT HSPC into CD40L-/- recipient mice could be sufficient to enhance the ability of these immunodeficient mice to control an intratracheal challenge with P. *murina*. Previous studies have shown that PCP results in a decrease of body weight in CD40L-/- mice, an increase in lung weight

as well as in the accumulation of P. *murina* organisms and ribosomal RNA in the lungs of infected mice (Tahara et al., 2004). Attenuation of these parameters was observed in lungs of mice competitively transplanted with 10% of functional HSPC.

## 5.3 Conclusions

Taken together these results will hopefully help us to establish the key parameters for the design of a protocol for the clinical testing of an adoptive therapy based on the use of autologous gene corrected T cells to provide effective therapeutic benefit to HIGM1 patients. Indeed, not only long-term follow-up studies have demonstrated the persistence of gene-modified T cells in all memory and effector T cell compartments for up to 14 years after infusion (Oliveira et al., 2015), but the capacity of T-cells to expand in large number with well-established clinical grade procedures and their resistance to oncogenic transformation after genetic manipulation, make these cells suitable for a first-in-human testing and validation of a gene correction strategy based on engineered endonucleases. However, a major issue with clinical adoptive cell transfer therapy is the avoidance of senescent and exhausted states in the infused cells, the differentiation state of transferred T cells is indeed a critical parameter affecting their functionality. In addition, multiple administrations of edited T-cells may be required to reconstitute a long-lasting and broad T cell repertoire that can mediate efficient T cell help in response to a wide range of pathogens. To conclude, although *CD40LG* expression is mainly associated with CD4+ T cells, other cells such as activated B cells, platelets, NK cells, monocytes, basophils and eosinophils express it (Schonbeck and Libby, 2001). Therefore, a T cell-based gene editing strategy could also be adopted as an effective “bridge therapy” to the definitive cure that can likely be obtained only by administering corrected or WT HSCs, which can provide a much broader and prolonged therapeutic benefit to the patients. Overall our findings might open a path to a clinical translation of our *CD40LG* gene editing strategy, which may benefit HIGM1 patients while avoiding them the risks of allogeneic transplantation.

# 6 REFERENCES

Ahonen, C. L., A. Wasiuk, et al., (2008). "Enhanced efficacy and reduced toxicity of multifactorial adjuvants compared with unitary adjuvants as cancer vaccines." Blood 111(6): 3116-3125.

Aiuti, A., L. Biasco, et al., (2013). "Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome." Science 341(6148): 1233151.

Alberts B, Johnson A, Lewis J, et al., Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Renewal by Multipotent Stem Cells: Blood Cell Formation.

Alkhatib, G., C. Combadiere, et al., (1996). "CC CKR5: a RANTES, MIP-1alpha, MIP- 1beta receptor as a fusion cofactor for macrophage-tropic HIV-1." Science 272(5270): 1955-1958.

Allen, C. D., K. M. Ansel, et al., (2004). "Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5." Nat Immunol 5(9): 943-952.

Allen, C. D., T. Okada, et al., (2007). "Imaging of germinal center selection events during affinity maturation." Science 315(5811): 528-531.

Almarza, D., G. Bussadori, et al., (2011). "Risk assessment in skin gene therapy: viral- cellular fusion transcripts generated by proviral transcriptional read-through in keratinocytes transduced with self-inactivating lentiviral vectors." Gene Ther 18(7): 674- 681.

Ansel, K. M., L. J. McHeyzer-Williams, et al., (1999). "In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines." J Exp Med 190(8): 1123-1134.

Arumugam, P. I., T. Higashimoto, et al., (2009). "Genotoxic potential of lineage-specific lentivirus vectors carrying the beta-globin locus control region." Mol Ther 17(11): 1929- 1937.

Bainbridge, J. W., A. J. Smith, et al., (2008). "Effect of gene therapy on visual function in Leber's congenital amaurosis." N Engl J Med 358(21): 2231-2239.

Banatvala, N., J. Davies, et al., (1994). "Hypogammaglobulinaemia associated with normal or increased IgM (the hyper IgM syndrome): a case series review." Arch Dis Child 71(2): 150-152.

Banchereau, J., B. Dubois, et al., (1995). "Functional CD40 antigen on B cells, dendritic cells and fibroblasts." Adv Exp Med Biol 378: 79-83.

Banchereau, J., F. Bazan, et al., (1994). "The CD40 antigen and its ligand." Annu Rev Immunol 12: 881-922.

Bank, A., R. Dorazio, et al., (2005). "A phase I/II clinical trial of beta-globin gene therapy for beta-thalassemia." Ann N Y Acad Sci 1054: 308-316.

Barrangou, R., C. Fremaux, et al., (2007). "CRISPR provides acquired resistance against viruses in prokaryotes." Science 315(5819): 1709-1712.

Barrett, T. B., G. Shu, et al., (1991). "CD40 signaling activates CD11a/CD18 (LFA-1)- mediated adhesion in B cells." J Immunol 146(6): 1722-1729.

Bennett, J., M. Ashtari, et al., (2012). "AAV2 gene therapy readministration in three adults with congenital blindness." Sci Transl Med 4(120): 120ra115.

Benson, M. J., L. D. Erickson, et al., (2007). "Affinity of antigen encounter and other early B-cell signals determine B-cell fate." Curr Opin Immunol 19(3): 275-280.

Berger, C., C. A. Blau, et al., (2003). "CD28 costimulation and immunoaffinity-based selection efficiently generate primary gene-modified T cells for adoptive immunotherapy." Blood 101(2): 476-484.

Bergtold, A., D. D. Desai, et al., (2005). "Cell surface recycling of internalized antigen permits dendritic cell priming of B cells." Immunity 23(5): 503-514.

Berk AJ. 2007. Adenoviridae: the viruses and their replication. In *Fields Virology*, ed. DM Knipe, PM Howley, pp. 2355–94. New York: Lippincott Williams & Wilkins.

Berns KI, Parrish CR. 2007. Parvoviridae. In *Fields Virology*, ed. DM Knipe, PM Howley, pp. 2437–77. New York: Lippincott Williams & Wilkins.

Besser, M. J., R. Shapira-Frommer, et al., (2013). "Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies." Clin Cancer Res 19(17): 4792-4800.

Beumer, K. J., J. K. Trautman, et al., (2013). "Donor DNA Utilization During Gene Targeting with Zinc-Finger Nucleases." G3 (Bethesda) 3(4): 657-664.

Biasco, L. et al., (2011). "Integration profile of retroviral vector in gene therapy treated patients is cell-specific according to gene expression and chromatin conformation of target cell." EMBO Molecular Medicine. doi: 10.1002/emmm.201000108.

Biasco, L., A. Ambrosi, et al., (2011). "Integration profile of retroviral vector in gene therapy treated patients is cell-specific according to gene expression and chromatin conformation of target cell." EMBO Mol Med 3(2): 89-101.

Biffi, A. et al., (2013). "Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy." Science. doi: 10.1126/science.1233158.

Biffi, A., E. Montini, et al., (2013). "Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy." Science 341(6148): 1233158.

Blömer, U. et al., (1997). "Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector." Journal of virology.

Boitano, A. E., J. Wang, et al., (2010). "Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells." Science 329(5997): 1345-1348.

Bondanza, A., V. Valtolina, et al., (2006). "Suicide gene therapy of graft-versus-host disease induced by central memory human T lymphocytes." Blood 107(5): 1828-1836.

Bordignon, C. et al., (1995). "Transfer of the HSV-tk Gene into Donor Peripheral Blood Lymphocytes for In Vivo Modulation of Donor Anti Tumor

Immunity after Allogeneic Bone Marrow Transplantation. The San Raffaele Hospital, Milan, Italy." Human Gene Therapy. doi: 10.1089/hum.1995.6.6-813.

Boulais, P. E. and Frenette, P. S. (2015). "Making sense of hematopoietic stem cell niches." Blood. doi: 10.1182/blood-2014-09-570192.

Boulais, P. E. and P. S. Frenette (2015). "becker." Blood 125(17): 2621-2629.

Bourgeois, C., B. Rocha, et al., (2002). "A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory." Science 297(5589): 2060-2063.

Boztug, K. et al., (2010). "Stem-cell gene therapy for the Wiskott-Aldrich syndrome." New England Journal of Medicine. doi: 10.1056/NEJMoa1003548.

Boztug, K., M. Schmidt, et al., (2010). "Stem-cell gene therapy for the Wiskott-Aldrich syndrome." N Engl J Med 363(20): 1918-1927.

Bradford, G. B. et al., (1997). "Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment." Experimental Hematology.

Bradford, G. B., B. Williams, et al., (1997). "Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment." Exp Hematol 25(5): 445-453.

Braun, C. J. et al., (2014). "Gene therapy for Wiskott-Aldrich syndrome-long-term efficacy and genotoxicity." Science Translational Medicine. doi: 10.1126/scitranslmed.3007280.

Braun, C. J., K. Boztug, et al., (2014). "Gene therapy for Wiskott-Aldrich syndrome--long- term efficacy and genotoxicity." Sci Transl Med 6(227): 227ra233.

Breitfeld, D., L. Ohl, et al., (2000). "Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production." J Exp Med 192(11): 1545-1552.

Brentjens, R. J., J. B. Latouche, et al., (2003). "Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15." Nat Med 9(3): 279-286.

Broere, F, Apasov, S.G., Sitkovsky M.V. and Willem van Eden, Principle of Immunopharmacology, 3rd revised and extended edition, 2011.T cell subsets and T cell- mediated immunity.

Brouns, S. J., M. M. Jore, et al., (2008). "Small CRISPR RNAs guide antiviral defense in prokaryotes." Science 321(5891): 960-964.

Brown, B. D. and L. Naldini (2009). "Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications." Nat Rev Genet 10(8): 578-585.

Brown, B. D., M. A. Venneri, et al., (2006). "Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer." Nat Med 12(5): 585-591.

Bushman, F. et al., (2005). "Genome-wide analysis of retroviral DNA integration." Nature Reviews Microbiology. doi: 10.1038/nrmicro1263.

Bushman, F., M. Lewinski, et al., (2005). "Genome-wide analysis of retroviral DNA integration." Nat Rev Microbiol 3(11): 848-858.

Cao, O. et al., (2007). "Induction and role of regulatory CD4+CD25+ T cells in tolerance to the transgene product following hepatic in vivo gene transfer." Blood. doi: 10.1182/blood-2007-02-073304.

Carmona, E. M. et al., (2012). "Glycosphingolipids mediate Pneumocystis cell wall β-glucan activation of the IL-23/IL-17 axis in human dendritic cells." American Journal of Respiratory Cell and Molecular Biology. doi: 10.1165/rcmb.2011-0159OC.

Cathomen, T. and J. K. Joung (2008). "Zinc-finger nucleases: the next generation emerges." Mol Ther 16(7): 1200-1207.

Cattoglio, C. et al., (2010). "High-definition mapping of retroviral integration sites defines the fate of allogeneic t cells after donor lymphocyte infusion." PLoS ONE. doi: 10.1371/journal.pone.0015688.

Cattoglio, C., D. Pellin, et al., (2010). "High-definition mapping of retroviral integration sites identifies active regulatory elements in human multipotent hematopoietic progenitors." Blood 116(25): 5507-5517.

Cattoglio, C., G. Maruggi, et al., (2010). "High-definition mapping of retroviral integration sites defines the fate of allogeneic T cells after donor lymphocyte infusion." PLoS One 5(12): e15688.

Cavazza, A., A. Moiani, et al., (2013). "Mechanisms of retroviral integration and mutagenesis." Hum Gene Ther 24(2): 119-131.

Cavazza, A., Moiani, A. and Mavilio, F. (2013). "Mechanisms of retroviral integration and mutagenesis." Human Gene Therapy. doi: 10.1089/hum.2012.203.

Cavazzana-Calvo, M., E. Payen, et al., (2010). "Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia." Nature 467(7313): 318-322.

Cesana, D., J. Sgualdino, et al., (2012). "Whole transcriptome characterization of aberrant splicing events induced by lentiviral vector integrations." J Clin Invest 122(5): 1667-1676.

Cesana, D., M. Ranzani, et al., (2014). "Uncovering and dissecting the genotoxicity of self- inactivating lentiviral vectors in vivo." Mol Ther 22(4): 774-785.

Challita, P. M. and D. B. Kohn (1994). "Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo." Proc Natl Acad Sci U S A 91(7): 2567-2571.

Challita, P. M. and Kohn, D. B. (1994). "Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo." Proceedings of the National Academy of Sciences of the United States of America. doi: 10.1073/pnas.91.7.2567.

Chang, L. S. and T. Shenk (1990). "The adenovirus DNA-binding protein stimulates the rate of transcription directed by adenovirus and adeno-associated virus promoters." J Virol 64(5): 2103-2109.

Chang, L. S., Y. Shi, et al., (1989). "Adeno-associated virus P5 promoter contains an adenovirus E1A-inducible element and a binding site for the major late transcription factor." J Virol 63(8): 3479-3488.

Cho, S. W., S. Kim, et al., (2014). "Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases." Genome Res

24(1): 132-141.

Chylinski, K., A. Le Rhun, et al., (2013). "The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems." RNA Biol 10(5): 726-737.

Cieri, N., B. Camisa, et al., (2013). "IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors." Blood 121(4): 573-584.

Civin, C. I. et al., (1984). “Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells..” Journal of immunology (Baltimore, Md. : 1950)..

Civin, C. I., L. C. Strauss, et al., (1984). "Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells." J Immunol 133(1): 157-165.

Clark, E. A. and J. A. Ledbetter (1986). "Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50." Proc Natl Acad Sci U S A 83(12): 4494-4498.

Coito, S., D. Sauce, et al., (2004). "Retrovirus-mediated gene transfer in human primary T lymphocytes induces an activation- and transduction/selection-dependent TCR-B variable chain repertoire skewing of gene-modified cells." Stem Cells Dev 13(1): 71-81.

Collis, C. H., Wilson, C. M. and Jones, J. M. (1980). “Cyclophosphamide-induced lung damage in mice: Protection by a small preliminary dose.” British Journal of Cancer. doi: 10.1038/bjc.1980.167.

Cong, L., F. A. Ran, et al., (2013). "Multiplex genome engineering using CRISPR/Cas systems." Science 339(6121): 819-823.

Copelan, E. A. (2006). "Hematopoietic stem-cell transplantation." N Engl J Med 354(17): 1813-1826.

Copelan, E. A. (2006). “Hematopoietic stem-cell transplantation.” New England Journal of Medicine. doi: 10.1056/NEJMra052638.

Copley, M. R., P. A. Beer, et al., (2012). "Hematopoietic stem cell heterogeneity takes center stage." Cell Stem Cell 10(6): 690-697.

Cox, D. B., R. J. Platt, et al., (2015). "Therapeutic genome editing: prospects and challenges." Nat Med 21(2): 121-131.

Cushion, M. T., Ruffolo, J. J. and Walzer, P. D. (1988). “Analysis of the developmental stages of Pneumocystis carinii, in vitro.” Laboratory Investigation.

Davis, A. J. and D. J. Chen (2013). "DNA double strand break repair via non-homologous end-joining." Transl Cancer Res 2(3): 130-143.

Davis, K. M., V. Pattanayak, et al., (2015). "Small molecule-triggered Cas9 protein with improved genome-editing specificity." Nat Chem Biol 11(5): 316-318.

De la Rua, N. M. et al., (2016). “CD4+ T-cell-independent secondary immune responses to Pneumocystis pneumonia.” Frontiers in Immunology. doi: 10.3389/fimmu.2016.00178.

De Ravin, S. S., A. Reik, et al., (2016). "Targeted gene addition in human CD34(+) hematopoietic cells for correction of X-linked chronic granulomatous disease." Nat Biotechnol 34(4): 424-429.
de Witte, M. A., M. Coccoris, et al., (2006). "Targeting self-antigens through allogeneic TCR gene transfer." Blood 108(3): 870-877.

Deltcheva, E., K. Chylinski, et al., (2011). "CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III." Nature 471(7340): 602-607.

Deveau, H., R. Barrangou, et al., (2008). "Phage response to CRISPR-encoded resistance in Streptococcus thermophilus." J Bacteriol 190(4): 1390-1400.

Dever, D. P., R. O. Bak, et al., (2016). "CRISPR/Cas9 beta-globin gene targeting in human haematopoietic stem cells." Nature 539(7629): 384-389.

Didigu, C. A., C. B. Wilen, et al., (2014). "Simultaneous zinc-finger nuclease editing of the HIV coreceptors ccr5 and cxcr4 protects CD4+ T cells from HIV-1 infection." Blood 123(1): 61-69.

Diehl, L., A. T. den Boer, et al., (1999). "CD40 activation in vivo overcomes peptide- induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy." Nat Med 5(7): 774-779.

Dobrzynski, E. et al., (2004). “Induction of antigen-specific CD4+ T-cell anergy and deletion by in vivo viral gene transfer.” Blood. doi: 10.1182/blood-2004-03-0847.

Dooner, G. J. et al. (2008). “Gene expression fluctuations in murine hematopoietic stem cells with cell cycle progression.” Journal of Cellular Physiology. doi: 10.1002/jcp.21273.

Dooner, G. J. et al., (2008). “Gene expression fluctuations in murine hematopoietic stem cells with cell cycle progression.” Journal of Cellular Physiology. doi: 10.1002/jcp.21273.

Dudley, M. E., J. C. Yang, et al., (2008). "Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens." J Clin Oncol 26(32): 5233-5239.

Dutton, R. W., Bradley, L. M. and Swain, S. L. (1998). “T CELL MEMORY.” Annual Review of Immunology. Annual Reviews, 16(1)., pp. 201–223. doi: 10.1146/annurev.immunol.16.1.201.

Elgueta, R., M. J. Benson, et al., (2009). "Molecular mechanism and function of CD40/CD40L engagement in the immune system." Immunol Rev 229(1): 152-172.

Emiloju, O. E. et al., (2019). “Hemophagocytic Lymphohistiocytosis in an AIDS Patient with Kaposi Sarcoma: A Treatment Dilemma.” Case Reports in Hematology. doi: 10.1155/2019/7634760.

Erickson, L. D., B. G. Durell, et al., (2002). "Short-circuiting long-lived humoral immunity by the heightened engagement of CD40." J Clin Invest 109(5): 613-620.

Essers, M. A., S. Offner, et al., (2009). "IFNalpha activates dormant haematopoietic stem cells in vivo." Nature 458(7240): 904-908.

Evans, H. M. et al., (2017). “The trophic life cycle stage of the opportunistic fungal pathogen Pneumocystis murina hinders the ability of dendritic cells to stimulate CD4+ T cell responses.” Infection and Immunity. doi: 10.1128/IAI.00396-17.

Evans, H. M., Bryant, G. L. and Garvy, B. A. (2016). “The life cycle stages of Pneumocystis murina have opposing effects on the immune response to this opportunistic fungal pathogen.” Infection and Immunity. doi: 10.1128/IAI.00519-16.

Fares, I. et al., (2017). “EPCR expression marks UM171-expanded CD34+ cord blood stem cells.” Blood. doi: 10.1182/blood-2016-11-750729.

Fares, I., J. Chagraoui, et al., (2017). "EPCR expression marks UM171-expanded CD34+ cord blood stem cells." Blood 129(25): 3344-3351.

Felice, B. et al., (2009). “Transcription factor binding sites are genetic determinants of retroviral integration in the human genome.” PLoS ONE. doi: 10.1371/journal.pone.0004571.

Felice, B., C. Cattoglio, et al., (2009). "Transcription factor binding sites are genetic determinants of retroviral integration in the human genome." PLoS One 4(2): e4571.

Ferrua, F. et al., (2019). “Hematopoietic stem cell transplantation for CD40 ligand deficiency: Results from an EBMT/ESID-IEWP-SCETIDE-PIDTC study.” Journal of Allergy and Clinical Immunology. doi: 10.1016/j.jaci.2018.12.1010.

Fonfara, I., A. Le Rhun, et al., (2014). "Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems." Nucleic Acids Res 42(4): 2577-2590.

Foy, T. M., D. M. Shepherd, et al., (1993). "In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39." J Exp Med 178(5): 1567- 1575.

Foy, T. M., F. H. Durie, et al., (1994). "The expansive role of CD40 and its ligand, gp39, in immunity." Semin Immunol 6(5): 259-266.

Foy, T. M., J. D. Laman, et al., (1994). "gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory." J Exp Med 180(1): 157-163.

Frentsch, M., R. Stark, et al., (2013). "CD40L expression permits CD8+ T cells to execute immunologic helper functions." Blood 122(3): 405-412. Friedmann, T. and R. Roblin (1972). "Gene therapy for human genetic disease?" Science 175(4025): 949-955.

Fu, Y., J. A. Foden, et al., (2013). "High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells." Nat Biotechnol 31(9): 822-826.

Fu, Y., J. D. Sander, et al., (2014). "Improving CRISPR-Cas nuclease specificity using truncated guide RNAs." Nat Biotechnol 32(3): 279-284.

Gabriel, R., A. Lombardo, et al., (2011). "An unbiased genome-wide analysis of zinc-finger nuclease specificity." Nat Biotechnol 29(9): 816-823.

Galgano, L. and Hutt, D. (2018) "HSCT: How Does It Work?". in The European Blood and Marrow Transplantation Textbook for Nurses. doi: 10.1007/978-3-319-50026-3_2.

Galgano, L. and Hutt, D. (2018). "HSCT: How Does It Work?." in The European Blood and Marrow Transplantation Textbook for Nurses. doi: 10.1007/978-3-319-50026-3_2.

Galy, A. et al., (1995). "Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset." Immunity. doi: 10.1016/1074-7613(95).90175-2.

Gammaitoni, L. et al., (2004). "Elevated telomerase activity and minimal telomere loss in cord blood long-term cultures with extensive stem cell replication." Blood. doi: 10.1182/blood-2003-09-3079.

Gardner, R. A. et al., (2017). "Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults." Blood. doi: 10.1182/blood-2017-02-769208.

Garrido, F., Ruiz-Cabello, F. and Aptsiauri, N. (2017). "Rejection versus escape: the tumor MHC dilemma." Cancer Immunology, Immunotherapy. doi: 10.1007/s00262-016-1947-x.

Garside, P., E. Ingulli, et al., (1998). "Visualization of specific B and T lymphocyte interactions in the lymph node." Science 281(5373): 96-99.

Gasiunas, G., R. Barrangou, et al., (2012). "Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria." Proc Natl Acad Sci U S A 109(39): E2579-2586.

Gattinoni, L., C. A. Klebanoff, et al., (2005). "Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells." J Clin Invest 115(6): 1616-1626.

Gattinoni, L., E. Lugli, et al., (2011). "A human memory T cell subset with stem cell-like properties." Nat Med 17(10): 1290-1297.

Gennery, A. R., K. Khawaja, et al., (2004). "Treatment of CD40 ligand deficiency by hematopoietic stem cell transplantation: a survey of the European experience, 1993-2002." Blood 103(3): 1152-1157.

Genovese, P. et al., (2014). “Targeted genome editing in human repopulating haematopoietic stem cells.” Nature. doi: 10.1038/nature13420.

Genovese, P., G. Schiroli, et al., (2014). "Targeted genome editing in human repopulating haematopoietic stem cells." Nature 510(7504): 235-240.

Gentner, B., I. Visigalli, et al., (2010). "Identification of hematopoietic stem cell-specific miRNAs enables gene therapy of globoid cell leukodystrophy." Sci Transl Med 2(58): 58ra84.

Germain, R. N. (2002). "T-cell development and the CD4-CD8 lineage decision." Nat Rev Immunol 2(5): 309-322.

Gigliotti, F. et al., (2002). “Passive intranasal monoclonal antibody prophylaxis against murine Pneumocystis carinii pneumonia.” Infection and Immunity. doi: 10.1128/IAI.70.3.1069-1074.2002.

Gluckman, E. and Rocha, V. (2005) “History of the clinical use of umbilical cord blood hematopoietic cells.” *Cytotherapy*. England, 7(3), pp. 219–227. doi: 10.1080/14653240510027136.

Gluckman, E. and Rocha, V. (2005). “History of the clinical use of umbilical cord blood hematopoietic cells..” Cytotherapy. England, 7(3)., pp. 219–227. doi: 10.1080/14653240510027136.

Goessling, W., R. S. Allen, et al., (2011). "Prostaglandin E2 enhances human cord blood stem cell xenotransplants and shows long-term safety in preclinical nonhuman primate transplant models." Cell Stem Cell 8(4): 445-458.

Grada, Z. et al., (2013). “TanCAR: A novel bispecific chimeric antigen receptor for cancer immunotherapy.” Molecular Therapy - Nucleic Acids. doi: 10.1038/mtna.2013.32.

Graf, D., S. Muller, et al., (1995). "A soluble form of TRAP (CD40 ligand) is rapidly released after T cell activation." Eur J Immunol 25(6): 1749-1754.

Greenbaum, A. M., L. D. Revollo, et al., (2012). "N-cadherin in osteolineage cells is not required for maintenance of hematopoietic stem cells." Blood 120(2): 295-302.

Grissa, I., Vergnaud, G. and Pourcel, C. (2007). “The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats.” BMC Bioinformatics. doi: 10.1186/1471-2105-8-172.

Groenen, P. M. A. et al., (1993). “Nature of DNA polymorphism in the direct repeat cluster of Mycobacterium tuberculosis; application for strain differentiation by a novel typing method.” Molecular Microbiology. doi: 10.1111/j.1365-2958.1993.tb00976.x.

Gross, G., Waks, T. and Eshhar, Z. (1989). “Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity.” Proceedings of the National Academy of Sciences of the United States of America. doi: 10.1073/pnas.86.24.10024.

Guilinger, J. P., D. B. Thompson, et al., (2014). "Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification." Nat Biotechnol 32(6): 577- 582.

Guo, J., T. Gaj, et al., (2010). "Directed evolution of an enhanced and highly efficient FokI cleavage domain for zinc finger nucleases." J Mol Biol 400(1): 96-

Guo, S., J. Lu, et al., (2010). "MicroRNA miR-125a controls hematopoietic stem cell number." Proc Natl Acad Sci U S A 107(32): 14229-14234.

Hacein-Bey-Abina, S. et al., (2003). “LMO2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1.” Science. doi: 10.1126/science.1088547.

Hacein-Bey-Abina, S. et al., (2008). “Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1.” Journal of Clinical Investigation. doi: 10.1172/JCI35700.

Hacein-Bey-Abina, S., A. Garrigue, et al., (2008). "Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1." J Clin Invest 118(9): 3132-3142.

Hacein-Bey-Abina, S., C. Von Kalle, et al., (2003). "LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1." Science 302(5644): 415-419.

Hadzic, N., A. Pagliuca, et al., (2000). "Correction of the hyper-IgM syndrome after liver and bone marrow transplantation." N Engl J Med 342(5): 320-324.

Hao, Q. L. et al., (1996). “Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population.” Blood. doi: 10.1182/blood.v88.9.3306.bloodjournal8893306.

Hao, Q. L., F. T. Thiemann, et al., (1996). "Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population." Blood 88(9): 3306- 3313.

Hauswirth, W. W., T. S. Aleman, et al., (2008). "Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial." Hum Gene Ther 19(10): 979-990.

Hayward, A. R., J. Levy, et al., (1997). "Cholangiopathy and tumors of the pancreas, liver, and biliary tree in boys with X-linked immunodeficiency with hyper-IgM." J Immunol 158(2): 977-983.

Heler, R., P. Samai, et al., (2015). "Cas9 specifies functional viral targets during CRISPR- Cas adaptation." Nature 519(7542): 199-202.

Hendel, A., R. O. Bak, et al., (2015). "Chemically modified guide RNAs enhance CRISPR- Cas genome editing in human primary cells." Nat Biotechnol 33(9): 985-989.

Hennino, A., M. Berard, et al., (2001). "FLICE-inhibitory protein is a key regulator of germinal center B cell apoptosis." J Exp Med 193(4): 447-458.

Hernandez-Novoa, B. et al., (2008). “ Immune responses to Pneumocystis murina are robust in healthy mice but largely absent in CD40 ligand-deficient mice .” Journal of Leukocyte Biology. doi: 10.1189/jlb.1207816.

Hoggatt, J., P. Singh, et al., (2009). "Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation." Blood 113(22): 5444-5455.

Hsu, P. D., D. A. Scott, et al., (2013). "DNA targeting specificity of RNA-guided Cas9 nucleases." Nat Biotechnol 31(9): 827-832.
Hubbard, N., D. Hagin, et al., (2016). "Targeted gene editing restores regulated CD40L function in X-linked hyper-IgM syndrome." Blood 127(21): 2513-2522.

Hutter, G., D. Nowak, et al., (2009). "Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation." N Engl J Med 360(7): 692-698.

Hwang, W. Y., Y. Fu, et al., (2013). "Heritable and precise zebrafish genome editing using a CRISPR-Cas system." PLoS One 8(7): e68708.

Ikuta, K. and Weissman, I. L. (1992). “Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation”, Proceedings of the National Academy of Sciences of the United States of America. doi: 10.1073/pnas.89.4.1502.

Ikuta, K. and Weissman, I. L. (1992). “Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation.” Proceedings of the National Academy of Sciences of the United States of America. doi: 10.1073/pnas.89.4.1502.

Ishino, Y., H. Shinagawa, et al., (1987). "Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product." J Bacteriol 169(12): 5429-5433.

Jabara, H. H., S. M. Fu, et al., (1990). "CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells." J Exp Med 172(6): 1861-1864.

Jacob, J. and G. Kelsoe (1992). "In situ studies of the primary immune response to (4- hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath- associated foci and germinal centers." J Exp Med 176(3): 679-687.

Jacobson, S. G., A. V. Cideciyan, et al., (2012). "Gene therapy for leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years." Arch Ophthalmol 130(1): 9-24.

Jansen, R., J. D. Embden, et al., (2002). "Identification of genes that are associated with DNA repeats in prokaryotes." Mol Microbiol 43(6): 1565-1575.

Jinek, M., F. Jiang, et al., (2014). "Structures of Cas9 endonucleases reveal RNA- mediated conformational activation." Science 343(6176): 1247997.

Jinek, M., K. Chylinski, et al., (2012). "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." Science 337(6096): 816-821.

Johnson, L. A., R. A. Morgan, et al., (2009). "Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen." Blood 114(3): 535-546.

June, C. H. and Levine, B. L. (2015). “T cell engineering as therapy for cancer and HIV: Our synthetic future.” Philosophical Transactions of the Royal Society B: Biological Sciences. doi: 10.1098/rstb.2014.0374.

Kaech, S. M., J. T. Tan, et al., (2003). "Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells." Nat Immunol 4(12): 1191-1198.

Kalos, M., B. L. Levine, et al., (2011). "T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia." Sci Transl Med 3(95): 95ra73.

Kang, E. M. et al., (2010). “Retrovirus gene therapy for X-linked chronic granulomatous disease can achieve stable long-term correction of oxidase activity in peripheral blood neutrophils.” Blood. doi: 10.1182/blood-2009-05-222760.

Kang, E. M., U. Choi, et al., (2010). "Retrovirus gene therapy for X-linked chronic granulomatous disease can achieve stable long-term correction of oxidase activity in peripheral blood neutrophils." Blood 115(4): 783-791.

Kawalekar, O. U. et al., (2016). “Distinct Signaling of Coreceptors Regulates Specific Metabolism Pathways and Impacts Memory Development in CAR T Cells.” Immunity. doi: 10.1016/j.immuni.2016.01.021.

Kay, M. A., Glorioso, J. C. and Naldini, L. (2001). “Viral vectors for gene therapy: The art of turning infectious agents into vehicles of therapeutics.” Nature Medicine. doi: 10.1038/83324.

Kay, M. A., J. C. Glorioso, et al., (2001). "Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics." Nat Med 7(1): 33-40.

Kennedy, E. M. et al., (2014). “Inactivation of the Human Papillomavirus E6 or E7 Gene in Cervical Carcinoma Cells by Using a Bacterial CRISPR/Cas RNA-Guided Endonuclease.” Journal of Virology. doi: 10.1128/jvi.01879-14.

Kershaw, M. H. et al., (2005). “Supernatural T cells: Genetic modification of T cells for cancer therapy.” Nature Reviews Immunology. doi: 10.1038/nri1729.

Kiel, M. J. et al., (2005). “SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells.” Cell. doi: 10.1016/j.cell.2005.05.026.

Kiel, M. J., O. H. Yilmaz, et al., (2005). "SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells." Cell 121(7): 1109- 1121.

Kim, S. W., K. Ramasamy, et al., (2012). "MicroRNAs miR-125a and miR-125b constitutively activate the NF-kappaB pathway by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20)." Proc Natl Acad Sci U S A 109(20): 7865-7870.

King, C., S. G. Tangye, et al., (2008). "T follicular helper (TFH) cells in normal and dysregulated immune responses." Annu Rev Immunol 26: 741-766.

Klebanoff, C. A., H. T. Khong, et al., (2005). "Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy." Trends Immunol 26(2): 111-117.

Kleinstiver, B. P., V. Pattanayak, et al., (2016). "High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects." Nature 529(7587): 490-495.

Klenk, H. P. et al., (1997). “The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon Archaeoglobus fulgidus.” Nature. doi: 10.1038/37052.

Kochenderfer, J. N. et al., (2012). “B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells.” Blood. doi: 10.1182/blood-2011-10-384388.

Kolls, J. K. (2017). “An emerging role of B Cell immunity in susceptibility to pneumocystis pneumonia.” American Journal of Respiratory Cell and Molecular Biology. doi: 10.1165/rcmb.2016-0360ED.

Kondrack, R. M., J. Harbertson, et al., (2003). "Interleukin 7 regulates the survival and generation of memory CD4 cells." J Exp Med 198(12): 1797-1806.

Korthauer, U., D. Graf, et al., (1993). "Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM." Nature 361(6412): 539-541.
Kotnis, A., L. Du, et al., (2009). "Non-homologous end joining in class switch recombination: the beginning of the end." Philos Trans R Soc Lond B Biol Sci 364(1517): 653-665.

Kotterman, M. A. and D. V. Schaffer (2014). "Engineering adeno-associated viruses for clinical gene therapy." Nat Rev Genet 15(7): 445-451.
Krause, A., H. F. Guo, et al., (1998). "Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes." J Exp Med 188(4): 619-626.

Kottom, T. J. et al., (2015). “Evidence for proinflammatory β-1,6 glucans in the pneumocystis carinii cell wall.” Infection and Immunity. doi: 10.1128/IAI.00196-15.

Kuo, C. Y. et al., (2018). “Site-Specific Gene Editing of Human Hematopoietic Stem Cells for X-Linked Hyper-IgM Syndrome.” Cell Reports. doi: 10.1016/j.celrep.2018.04.103.

Lander, E. S. (2016). "The Heroes of CRISPR." Cell 164(1-2): 18-28.

Lanzavecchia, A. (2007). "Pillars article: Antigen-specific interaction between T and B cells. 1985." J Immunol 179(11): 7206-7208.

Lee, D. W., J. N. Kochenderfer, et al., (2015). "T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose- escalation trial." Lancet 385(9967): 517-528.

Levy, J. et al., (1997). “Clinical spectrum of X-linked hyper-IgM syndrome.” Journal of Pediatrics. doi: 10.1016/S0022-3476(97).70123-9.

Levy, J., T. Espanol-Boren, et al., (1997). "Clinical spectrum of X-linked hyper-IgM syndrome." J Pediatr 131(1 Pt 1): 47-54.

Lewinski, M. K. and F. D. Bushman (2005). "Retroviral DNA integration--mechanism and consequences." Adv Genet 55: 147-181.

Lewinski, M. K. et al., (2006). “Retroviral DNA integration: Viral and cellular determinants of target-site selection.” PLoS Pathogens. doi: 10.1371/journal.ppat.0020060.

Liu, J., Y. M. Guo, et al., (2012). "A synthetic double-stranded RNA, poly I:C, induces a rapid apoptosis of human CD34(+) cells." Exp Hematol 40(4): 330-341.

Liu, R., W. A. Paxton, et al., (1996). "Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection." Cell 86(3): 367-377.

Liu, Y. J., D. E. Joshua, et al., (1989). "Mechanism of antigen-driven selection in germinal centres." Nature 342(6252): 929-931.

Löfqvist, T. et al., (1997). “Haemophilia prophylaxis in young patients - A long-term follow-up.” Journal of Internal Medicine. doi: 10.1046/j.1365-2796.1997.130135000.x.

Lombardo, A. et al., (2007). “Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery.” Nature Biotechnology. doi: 10.1038/nbt1353.

Lombardo, A., D. Cesana, et al., (2011). "Site-specific integration and tailoring of cassette design for sustainable gene transfer." Nat Methods 8(10): 861-869.
Lord, B. I., N. G.

Louis, C. U., B. Savoldo, et al., (2011). "Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma." Blood 118(23): 6050- 6056.

Lund, F. E. et al., (2006). “ B Cells Are Required for Generation of Protective Effector and Memory CD4 Cells in Response to Pneumocystis Lung Infection .” The Journal of Immunology. doi: 10.4049/jimmunol.176.10.6147.

Mackey, M. F., J. R. Gunn, et al., (1997). "Protective immunity induced by tumor vaccines requires interaction between CD40 and its ligand, CD154." Cancer Res 57(13): 2569- 2574.

MacLaren, R. E., M. Groppe, et al., (2014). "Retinal gene therapy in patients with choroideremia: initial findings from a phase 1/2 clinical trial." Lancet

383(9923): 1129- 1137.

MacLennan, I. C. (1994). "Germinal centers." Annu Rev Immunol 12: 117-139.

Maguire, A. M., F. Simonelli, et al., (2008). "Safety and efficacy of gene transfer for Leber's congenital amaurosis." N Engl J Med 358(21): 2240-2248.

Maguire, A. M., K. A. High, et al., (2009). "Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial." Lancet 374(9701): 1597-1605.

Maher, J., R. J. Brentjens, et al., (2002). "Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta /CD28 receptor." Nat Biotechnol 20(1): 70-75.

Mahnke, Y. D. et al., (2013). “The who”s who of T-cell differentiation: Human memory T-cell subsets.” European Journal of Immunology. doi: 10.1002/eji.201343751.

Mahnke, Y. D., T. M. Brodie, et al., (2013). "The who's who of T-cell differentiation: human memory T-cell subsets." Eur J Immunol 43(11): 2797-2809.

Maier, P., C. von Kalle, et al., (2010). "Retroviral vectors for gene therapy." Future Microbiol 5(10): 1507-1523.

Maier, P., Von Kalle, C. and Laufs, S. (2010). “Retroviral vectors for gene therapy.” Future Microbiology. doi: 10.2217/fmb.10.100.

Makarova, K. S., L. Aravind, et al., (2011). "Unification of Cas protein families and a simple scenario for the origin and evolution of CRISPR-Cas systems." Biol Direct 6: 38.

Mali, P., J. Aach, et al., (2013). "CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering." Nat Biotechnol 31(9): 833-838.

Mali, P., L. Yang, et al., (2013). "RNA-guided human genome engineering via Cas9." Science 339(6121): 823-826.

Manz, M. G., T. Miyamoto, et al., (2002). "Prospective isolation of human clonogenic common myeloid progenitors." Proc Natl Acad Sci U S A 99(18): 11872-11877.

Marraffini, L. A. and E. J. Sontheimer (2008). "CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA." Science 322(5909): 1843-1845.

Marraffini, L. A. and Sontheimer, E. J. (2008). “CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA.” Science. doi: 10.1126/science.1165771.

Matsumoto, A. and K. I. Nakayama (2013). "Role of key regulators of the cell cycle in maintenance of hematopoietic stem cells." Biochim Biophys Acta 1830(2): 2335-2344.

Matsumoto, A. and Nakayama, K. I. (2013). “Role of key regulators of the cell cycle in maintenance of hematopoietic stem cells.” Biochimica et Biophysica Acta - General Subjects. doi: 10.1016/j.bbagen.2012.07.004.

Maude, S. L., N. Frey, et al., (2014). "Chimeric antigen receptor T cells for sustained remissions in leukemia." N Engl J Med 371(16): 1507-1517.

May, C., S. Rivella, et al., (2000). "Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin." Nature 406(6791): 82-86.

McCarty, D. M., S. M. Young, Jr., et al., (2004). "Integration of adeno-associated virus (AAV) and recombinant AAV vectors." Annu Rev Genet 38: 819-845.

McCarty, D. M., Young, S. M. and Samulski, R. J. (2004). “Integration of Adeno-Associated Virus (AAV). and Recombinant AAV Vectors.” Annual Review of Genetics. doi: 10.1146/annurev.genet.37.110801.143717.

McLauchlin, J., C. F. Amar, et al., (2003). "Polymerase chain reaction-based diagnosis of infection with Cryptosporidium in children with primary immunodeficiencies." Pediatr Infect Dis J 22(4): 329-335.

Merten, O. W., Gény-Fiamma, C. and Douar, A. M. (2005). “Current issues in adeno-associated viral vector production.” Gene Therapy. doi: 10.1038/sj.gt.3302615.

Milyavsky, M., O. I. Gan, et al., (2010). "A distinctive DNA damage response in human hematopoietic stem cells reveals an apoptosis-independent role for p53 in self-renewal." Cell Stem Cell 7(2): 186-197.

Mingozzi, F. et al., (2003). “Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer.” Journal of Clinical Investigation. doi: 10.1172/JCI200316887.

Modlich, U., J. Bohne, et al., (2006). "Cell-culture assays reveal the importance of retroviral vector design for insertional genotoxicity." Blood 108(8): 2545-2553.

Modlich, U., S. Navarro, et al., (2009). "Insertional transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors." Mol Ther 17(11): 1919-1928.

Mohrin, M. et al., (2010). “Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis.” Cell Stem Cell. doi: 10.1016/j.stem.2010.06.014.

Moiani, A., Y. Paleari, et al., (2012). "Lentiviral vector integration in the human genome induces alternative splicing and generates aberrant transcripts." J Clin Invest 122(5): 1653- 1666.

Mojica, F. J. M. et al., (1995). “Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferax volcanii and could be involved in replicon partitioning.” Molecular Microbiology. doi: 10.1111/j.1365-2958.1995.mmi_17010085.x.

Mojica, F. J., C. Diez-Villasenor, et al., (2000). "Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria." Mol Microbiol 36(1): 244-246.

Mojica, F. J., C. Diez-Villasenor, et al., (2005). "Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements." J Mol Evol 60(2): 174-182.

Montini, E., D. Cesana, et al., (2006). "Hematopoietic stem cell gene transfer in a tumor- prone mouse model uncovers low genotoxicity of lentiviral vector integration." Nat Biotechnol 24(6): 687-696.

Montini, E., D. Cesana, et al., (2009). "The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy." J Clin Invest 119(4): 964-975.

Morgan, R. A., N. Chinnasamy, et al., (2013). "Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy." J Immunother 36(2): 133-

Morrison, S. J. and D. T. Scadden (2014). "The bone marrow niche for haematopoietic stem cells." Nature 505(7483): 327-334.

Nakamura-Ishizu, A. and Suda, T. (2014). “Not merely quiescent: Telomeres in quiescent HSCs.” Blood. doi: 10.1182/blood-2014-10-603258.

Nakamura-Ishizu, A., H. Takizawa, et al., (2014). "The analysis, roles and regulation of quiescence in hematopoietic stem cells." Development 141(24): 4656-4666.

Naldini, L. (1998). "Lentiviruses as gene transfer agents for delivery to non-dividing cells." Curr Opin Biotechnol 9(5): 457-463.

Naldini, L. (2011). "Ex vivo gene transfer and correction for cell-based therapies." Nat Rev Genet 12(5): 301-315.

Naldini, L. (2011). “Ex vivo gene transfer and correction for cell-based therapies.” Nature Reviews Genetics. doi: 10.1038/nrg2985.

Naldini, L. (2015). "Gene therapy returns to centre stage." Nature 526(7573): 351-360.

Naldini, L. (2015). “Gene therapy returns to centre stage.” Nature. doi: 10.1038/nature15818.

Naldini, L. et al., (1996). “Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector.” Proceedings of the National Academy of Sciences of the United States of America. doi: 10.1073/pnas.93.21.11382.

Naldini, L., U. Blomer, et al., (1996). "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector." Science 272(5259): 263-267.

Nandakumar, V. et al., (2017). “Differential macrophage polarization from Pneumocystis in immunocompetent and immunosuppressed hosts: Potential adjunctive therapy during pneumonia.” Infection and Immunity. doi: 10.1128/IAI.00939-16.

Nash, K., W. Chen, et al., (2008). "Complete in vitro reconstitution of adeno-associated virus DNA replication requires the minichromosome maintenance complex proteins." J Virol 82(3): 1458-1464.

Nathwani, A. C., E. G. Tuddenham, et al., (2011). "Adenovirus-associated virus vector- mediated gene transfer in hemophilia B." N Engl J Med 365(25): 2357-2365.

Neelapu, S. S. et al., (2017). “Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-Cell lymphoma.” New England Journal of Medicine. doi: 10.1056/NEJMoa1707447.

Nishimasu, H., F. A. Ran, et al., (2014). "Crystal structure of Cas9 in complex with guide RNA and target DNA." Cell 156(5): 935-949.

Notta, F. et al., (2011). “Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment.” Science. doi: 10.1126/science.1201219.

Notta, F., S. Doulatov, et al., (2011). "Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment." Science 333(6039): 218-221.

O'Connor, B. P., L. A. Vogel, et al., (2006). "Imprinting the fate of antigen-reactive B cells through the affinity of the B cell receptor." J Immunol 177(11): 7723-7732.

Okita, K., Ichisaka, T. and Yamanaka, S. (2007). “Generation of germline-competent induced pluripotent stem cells.” Nature. doi: 10.1038/nature05934.

Okita, K., T. Ichisaka, et al., (2007). "Generation of germline-competent induced pluripotent stem cells." Nature 448(7151): 313-317.

Otieno-Odhiambo, P., Wasserman, S. and Hoving, J. C. (2019). “The contribution of host cells to pneumocystis immunity: An update.” Pathogens. doi: 10.3390/pathogens8020052.

Ott, M. G. et al., (2006). “Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1.” Nature Medicine. doi: 10.1038/nm1393.

Ott, M. G., M. Schmidt, et al., (2006). "Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1." Nat Med 12(4): 401-409.

Palmer, T. D. et al., (1991). “Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes.” Proceedings of

the National Academy of Sciences of the United States of America. doi: 10.1073/pnas.88.4.1330.

Palmer, T. D., G. J. Rosman, et al., (1991). "Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes." Proc Natl Acad Sci U S A 88(4): 1330-1334.

Panch, S. R. et al. (2017). “Sources of Hematopoietic Stem and Progenitor Cells and Methods to Optimize Yields for Clinical Cell Therapy.” Biology of Blood and Marrow Transplantation. doi: 10.1016/j.bbmt.2017.05.003.

Panch, S. R. et al., (2017). “Sources of Hematopoietic Stem and Progenitor Cells and Methods to Optimize Yields for Clinical Cell Therapy.” Biology of Blood and Marrow Transplantation. doi: 10.1016/j.bbmt.2017.05.003.

Pape, K. A., D. M. Catron, et al., (2007). "The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles." Immunity 26(4): 491-502.

Passegue, E. and P. Ernst (2009). "IFN-alpha wakes up sleeping hematopoietic stem cells." Nat Med 15(6): 612-613.

Pattanayak, V., S. Lin, et al., (2013). "High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity." Nat Biotechnol 31(9): 839-843.

Paulos, C. M., C. Wrzesinski, et al., (2007). "Microbial translocation augments the function of adoptively transferred self/tumor-specific CD8+ T cells via TLR4 signaling." J Clin Invest 117(8): 2197-2204.

Paus, D., T. G. Phan, et al., (2006). "Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation." J Exp Med 203(4): 1081-1091.

Perez-Nazario, N. et al., (2013). “ Selective Ablation of Lung Epithelial IKK2 Impairs Pulmonary Th17 Responses and Delays the Clearance of Pneumocystis .” The Journal of Immunology. doi: 10.4049/jimmunol.1301679.

Phan, T. G., D. Paus, et al., (2006). "High affinity germinal center B cells are actively selected into the plasma cell compartment." J Exp Med 203(11): 2419-2424.

Pietrzyk, M. E., G. V. Priestley, et al., (1985). "Normal cycling patterns of hematopoietic stem cell subpopulations: an assay using long-term in vivo BrdU infusion." Blood 66(6): 1460-1462.

Pietrzyk, M. E., Priestley, G. V. and Wolf, N. S. (1985). "Normal cycling patterns of hematopoietic stem cell subpopulations: An assay using long-term in vivo BrdU infusion." Blood. doi: 10.1182/blood.v66.6.1460.bloodjournal6661460.

Porter, D. L., B. L. Levine, et al., (2011). "Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia." N Engl J Med 365(8): 725-733.

Pourcel, C., G. Salvignol, et al., (2005). "CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies." Microbiology 151(Pt 3): 653-663.

Principles of Immunopharmacology (2011). Principles of Immunopharmacology. doi: 10.1007/978-3-0346-0136-8.

Prlic, M., L. Lefrancois, et al., (2002). "Multiple choices: regulation of memory CD8 T cell generation and homeostasis by interleukin (IL)-7 and IL-15." J Exp Med 195(12): F49-52. Pule, M. A., B.

Qasim, W., Gaspar, H. B. and Thrasher, A. J. (2009). "Progress and prospects: Gene therapy for inherited immunodeficiencies." Gene Therapy. doi: 10.1038/gt.2009.127.

Qasim, W., H. B. Gaspar, et al., (2009). "Progress and prospects: gene therapy for inherited immunodeficiencies." Gene Ther 16(11): 1285-1291.

Ramamoorth, M. and A. Narvekar (2015). "Non viral vectors in gene therapy-an overview." J Clin Diagn Res 9(1): GE01-06.

Ramamoorth, M. and Narvekar, A. (2015). "Non viral vectors in gene therapy - An overview." Journal of Clinical and Diagnostic Research. doi: 10.7860/JCDR/2015/10443.5394.

Ran, F. A., P. D. Hsu, et al., (2013). "Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity." Cell 154(6): 1380-1389.

Randall, T. D. and Weissman, I. L. (1998). “Characterization of a population of cells in the bone marrow that phenotypically mimics hematopoietic stem cells: Resting stem cells or mystery population?.” Stem Cells. doi: 10.1002/stem.160038.

Randall, T. D. and Weissman, I. L. (1998). “Characterization of a population of cells in the bone marrow that phenotypically mimics hematopoietic stem cells: Resting stem cells or mystery population?.” Stem Cells. doi: 10.1002/stem.160038.

Rapaka, R. R. et al., (2010). “Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus Pneumocystis murina.” Journal of Experimental Medicine. doi: 10.1084/jem.20100034.

Rath, D., L. Amlinger, et al., (2015). "The CRISPR-Cas immune system: biology, mechanisms and applications." Biochimie 117: 119-128.

Recchia, A., C. Bonini, et al., (2006). "Retroviral vector integration deregulates gene expression but has no consequence on the biology and function of transplanted T cells." Proc Natl Acad Sci U S A 103(5): 1457-1462.

Rees, H. A. and Liu, D. R. (2018). “Base editing: precision chemistry on the genome and transcriptome of living cells.” Nature Reviews Genetics. doi: 10.1038/s41576-018-0059-1.

Renshaw, B. R. et al., (1994). “Humoral Immune Responses in CD40 Ligand-deficient Mice.” Journal of Experimental Medicine. doi: 10.1084/jem.180.5.1889.

Rezaei, N. and Notarangelo, L. D. (2013). “Hematopoietic stem cell transplantation for hyper-IgM syndromes.” Pediatric Transplantation. doi: 10.1111/petr.12012.

Ripamonti, C., Bishop, L. R. and Kovacs, J. A. (2017). “Pulmonary interleukin-17-positive lymphocytes increase during Pneumocystis murina infection but are not required for clearance of pneumocystis.” Infection and Immunity. doi: 10.1128/IAI.00434-16.

Robbins, P. F., R. A. Morgan, et al., (2011). "Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1." J Clin Oncol 29(7): 917-924.

Rock, K. L., B. Benacerraf, et al., (1984). "Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors." J Exp Med 160(4): 1102-1113.

Rodrigues, F., E. G. Davies, et al., (2004). "Liver disease in children with primary immunodeficiencies." J Pediatr 145(3): 333-339.

Rogerson

Rosenberg, S. A. et al., (2011). “Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy.” Clinical Cancer Research. doi: 10.1158/1078-0432.CCR-11-0116.

Rosenblum, M. D., S. S. Way, et al., (2016). "Regulatory T cell memory." Nat Rev Immunol 16(2): 90-101.

Rossi, L. et al., (2012). “Less is more: Unveiling the functional core of hematopoietic stem cells through knockout mice.” Cell Stem Cell. doi: 10.1016/j.stem.2012.08.006.

Rossi, L., K. K. Lin, et al., (2012). "Less is more: unveiling the functional core of hematopoietic stem cells through knockout mice." Cell Stem Cell 11(3): 302-317.

Roth, S. L., Malani, N. and Bushman, F. D. (2011). “Gammaretroviral Integration into Nucleosomal Target DNA In Vivo.” Journal of Virology. doi: 10.1128/jvi.00635-11.

Roth, S. L., N. Malani, et al., (2011). "Gammaretroviral integration into nucleosomal target DNA in vivo." J Virol 85(14): 7393-7401.

Sacco, M. G., M. Ungari, et al., (2000). "Lymphoid abnormalities in CD40 ligand transgenic mice suggest the need for tight regulation in gene therapy approaches to hyper immunoglobulin M (IgM) syndrome." Cancer Gene Ther 7(10): 1299-1306.

Sadler, A. J. and Williams, B. R. G. (2008). “Interferon-inducible antiviral effectors.” Nature Reviews Immunology. doi: 10.1038/nri2314.

Sallusto, F., Geginat, J. and Lanzavecchia, A. (2004). “ C entral M emory and E ffector M emory T C ell S ubsets : Function, Generation, and Maintenance .” Annual Review of Immunology. doi: 10.1146/annurev.immunol.22.012703.104702.

Sallusto, F., J. Geginat, et al., (2004). "Central memory and effector memory T cell subsets: function, generation, and maintenance." Annu Rev Immunol 22: 745-763.

Samson, M., F. Libert, et al., (1996). "Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene." Nature 382(6593): 722-725.

Samulski, R. J. and N. Muzyczka (2014). "AAV-Mediated Gene Therapy for Research and Therapeutic Purposes." Annu Rev Virol 1(1): 427-451.

Sander, J. D. and J. K. Joung (2014). "CRISPR-Cas systems for editing, regulating and targeting genomes." Nat Biotechnol 32(4): 347-355.

Sapranauskas, R., G. Gasiunas, et al., (2011). "The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli." Nucleic Acids Res 39(21): 9275-9282.

Sauvageau, G., N. N. Iscove, et al., (2004). "In vitro and in vivo expansion of hematopoietic stem cells." Oncogene 23(43): 7223-7232.

Schaerli, P., K. Willimann, et al., (2000). "CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function." J Exp Med 192(11): 1553-1562. Schatz, D. G. and P. C. Swanson (2011). "V(D)J recombination: mechanisms of initiation." Annu Rev Genet 45: 167-202.

Schiroli, G. et al., (2017). “Preclinical modeling highlights the therapeutic potential of hematopoietic stem cell gene editing for correction of SCID-X1.” Science Translational Medicine. doi: 10.1126/scitranslmed.aan0820.

Schiroli, G. et al., (2019). “Precise Gene Editing Preserves Hematopoietic Stem Cell Function following Transient p53-Mediated DNA Damage Response.” Cell Stem Cell. doi: 10.1016/j.stem.2019.02.019.

Schlesinger, S. et al., (2013). “Proviral Silencing in Embryonic Cells Is Regulated by Yin Yang 1.” Cell Reports. doi: 10.1016/j.celrep.2013.06.003.

Schluns, K. S., W. C. Kieper, et al., (2000). "Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo." Nat Immunol 1(5): 426-432.

Schonbeck, U. and P. Libby (2001). "The CD40/CD154 receptor/ligand dyad." Cell Mol Life Sci 58(1): 4-43.

Schuster, A., E. Apfelstedt-Sylla, et al., (2005). "Autoimmune retinopathy with RPE hypersensitivity and 'negative ERG' in X-linked hyper-IgM syndrome." Ocul Immunol Inflamm 13(2-3): 235-243.

Seita, J. and I. L. Weissman (2010). "Hematopoietic stem cell: self-renewal versus differentiation." Wiley Interdiscip Rev Syst Biol Med 2(6): 640-653.

Seita, J. and Weissman, I. L. (2010). “Hematopoietic stem cell: Self-renewal versus differentiation.” Wiley Interdisciplinary Reviews: Systems Biology and Medicine. doi: 10.1002/wsbm.86.

Seyama, K., S. Nonoyama, et al., (1998). "Mutations of the CD40 ligand gene and its effect on CD40 ligand expression in patients with X-linked hyper IgM syndrome." Blood 92(7): 2421-2434.
Sharma, R., X. M. Anguela, et al., (2015). "In vivo genome editing of the albumin locus as a platform for protein replacement therapy." Blood 126(15): 1777-1784.

Shi, Y., E. Seto, et al., (1991). "Transcriptional repression by YY1, a human GLI-Kruppel- related protein, and relief of repression by adenovirus E1A protein." Cell 67(2): 377-388. Shimizu, Y., C. Sollu, et al., (2011). "Adding fingers to an engineered zinc finger nuclease can reduce activity." Biochemistry 50(22): 5033-5041.

Shin, H. and Iwasaki, A. (2013). “Tissue-resident memory T cells.” Immunological Reviews. doi: 10.1111/imr.12087.

Skov, S., M. Bonyhadi, et al., (2000). "IL-2 and IL-15 regulate CD154 expression on activated CD4 T cells." J Immunol 164(7): 3500-3505.

Slaymaker, I. M., L. Gao, et al., (2016). "Rationally engineered Cas9 nucleases with improved specificity." Science 351(6268): 84-88.

Soong, R. S., L. Song, et al., (2014). "Direct T cell activation via CD40 ligand generates high avidity CD8+ T cells capable of breaking immunological

tolerance for the control of tumors." PLoS One 9(3): e93162.

Sotillo, E. et al., (2015). “Convergence of acquired mutations and alternative splicing of CD19 enables resistance to CART-19 immunotherapy.” Cancer Discovery. doi: 10.1158/2159-8290.CD-15-1020.

Sotomayor, E. M., I. Borrello, et al., (1999). "Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through in vivo ligation of CD40." Nat Med 5(7): 780-787.

Srivastava, A., E. W. Lusby, et al., (1983). "Nucleotide sequence and organization of the adeno-associated virus 2 genome." J Virol 45(2): 555-564.

Srivastava, S. and Riddell, S. R. (2015). “Engineering CAR-T cells: Design concepts.” Trends in Immunology. doi: 10.1016/j.it.2015.06.004.

Stanevsky, A., Goldstein, G. and Nagler, A. (2009). “Umbilical cord blood transplantation: Pros, cons and beyond.” Blood Reviews. doi: 10.1016/j.blre.2009.02.001.

Stanevsky, A., Goldstein, G. and Nagler, A. (2009). “Umbilical cord blood transplantation: Pros, cons and beyond.” Blood Reviews. doi: 10.1016/j.blre.2009.02.001.

Stroes, E. S., M. C. Nierman, et al., (2008). "Intramuscular administration of AAV1- lipoprotein lipase S447X lowers triglycerides in lipoprotein lipase-deficient patients." Arterioscler Thromb Vasc Biol 28(12): 2303-2304.

Surh, C. D., O. Boyman, et al., (2006). "Homeostasis of memory T cells." Immunol Rev 211: 154-163.
Swanson, P. C. (2004). "The bounty of RAGs: recombination signal complexes and reaction outcomes." Immunol Rev 200: 90-114.

Tahara, M., R. G. Pergolizzi, et al., (2004). "Trans-splicing repair of CD40 ligand deficiency results in naturally regulated correction of a mouse model of hyper-IgM X-linked immunodeficiency." Nat Med 10(8): 835-841.

Tomás, A. L. et al., (2016). “Serological diagnosis of pneumocystosis: Production of a synthetic recombinant antigen for immunodetection of Pneumocystis jirovecii.” Scientific Reports. doi: 10.1038/srep36287.

Turtle, C. J. et al., (2016). “CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients.” Journal of Clinical Investigation. doi: 10.1172/JCI85309.

Tuscano, J. M., K. M. Druey, et al., (1996). "Bcl-x rather than Bcl-2 mediates CD40- dependent centrocyte survival in the germinal center." Blood 88(4): 1359-1364.

Uren, A. G., J. Kool, et al., (2005). "Retroviral insertional mutagenesis: past, present and future." Oncogene 24(52): 7656-7672.

Urnov, F. D. et al., (2005). “Highly efficient endogenous human gene correction using designed zinc-finger nucleases.” Nature. doi: 10.1038/nature03556.

Uttenthal, B. J., I. Chua, et al., (2012). "Challenges in T cell receptor gene therapy." J Gene Med 14(6): 386-399.

van Kooten, C. and J. Banchereau (1997). "Functions of CD40 on B cells, dendritic cells and other cells." Curr Opin Immunol 9(3): 330-337.

van Kooten, C. and J. Banchereau (2000). "CD40-CD40 ligand." J Leukoc Biol 67(1): 2-17. Vera, J., B.

Vavassori, S. et al., (2009). “In vivo post-transcriptional regulation of CD154 in mouse CD4+ T cells.” European Journal of Immunology. doi: 10.1002/eji.200839163.

Verma, I. M. and M. D. Weitzman (2005). "Gene therapy: twenty-first century medicine." Annu Rev Biochem 74: 711-738.

Verma, I. M. and Weitzman, M. D. (2005). “GENE THERAPY: Twenty-First Century Medicine.” Annual Review of Biochemistry. doi: 10.1146/annurev.biochem.74.050304.091637.

Vigna, E. and L. Naldini (2000). "Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy." J Gene Med 2(5): 308-316.

Vigna, E. and Naldini, L. (2000). “Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy.” The

Journal of Gene Medicine. doi: 10.1002/1521-2254(200009/10).2:5<308::aid-jgm131>3.3.co;2-v.

Wagner, J. E., J. N. Barker, et al., (2002). "Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival." Blood 100(5): 1611-1618.

Walasek, M. A., van Os, R. and de Haan, G. (2012). "Hematopoietic stem cell expansion: Challenges and opportunities." Annals of the New York Academy of Sciences. doi: 10.1111/j.1749-6632.2012.06549.x.

Wang, J., C. M. Exline, et al., (2015). "Homology-driven genome editing in hematopoietic stem and progenitor cells using ZFN mRNA and AAV6 donors." Nat Biotechnol 33(12): 1256-1263.

Weissman, I. L. and J. A. Shizuru (2008). "The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases." Blood 112(9): 3543-3553.

Weissman, I. L. and Shizuru, J. A. (2008). "The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases." Blood. doi: 10.1182/blood-2008-08-078220.

Wilen, C. B., J. Wang, et al., (2011). "Engineering HIV-resistant human CD4+ T cells with CXCR4-specific zinc-finger nucleases." PLoS Pathog 7(4): e1002020.

Williams, D. A. et al., (1984). "Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse." Nature. doi: 10.1038/310476a0.

Wilson, A. et al., (2008). "Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair." Cell. doi: 10.1016/j.cell.2008.10.048.

Wilson, A., E. Laurenti, et al., (2008). "Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair." Cell 135(6): 1118-1129.

Winkelstein, J. A., M. C. Marino, et al., (2003). "The X-linked hyper-IgM syndrome: clinical and immunologic features of 79 patients." Medicine (Baltimore) 82(6): 373-384.

Wold, W. and Toth, K. (2014). “Adenovirus Vectors for Gene Therapy, Vaccination and Cancer Gene Therapy.” Current Gene Therapy. doi: 10.2174/1566523213666131125095046.

Wold, W. S. and K. Toth (2013). "Adenovirus vectors for gene therapy, vaccination and cancer gene therapy." Curr Gene Ther 13(6): 421-433.

Wolf, D. and Goff, S. P. (2009). “Embryonic stem cells use ZFP809 to silence retroviral DNAs.” Nature. doi: 10.1038/nature07844.

Wolf, D. and S. P. Goff (2009). "Embryonic stem cells use ZFP809 to silence retroviral DNAs." Nature 458(7242): 1201-1204.

Wright, A. V., J. K. Nunez, et al., (2016). "Biology and Applications of CRISPR Systems: Harnessing Nature's Toolbox for Genome Engineering." Cell 164(1-2): 29-44.

Wu, X., D. A. Scott, et al., (2014). "Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells." Nat Biotechnol 32(7): 670-676.

Wykes, M., A. Pombo, et al., (1998). "Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response." J Immunol 161(3): 1313-1319.

Yin, A. H. et al., (1997). “AC133, a novel marker for human hematopoietic stem and progenitor cells.” Blood. doi: 10.1182/blood.v90.12.5002.5002_5002_5012.

Yin, A. H., S. Miraglia, et al., (1997). "AC133, a novel marker for human hematopoietic stem and progenitor cells." Blood 90(12): 5002-5012.

Zetsche, B., J. S. Gootenberg, et al., (2015). "Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system." Cell 163(3): 759-771.

Zychlinski, D., A. Schambach, et al., (2008). "Physiological promoters reduce the genotoxic risk of integrating gene vectors." Mol Ther 16(4).: 718-725.