PROJECT FINAL REPORT

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1.1 Final publishable summary report

1.1.1 Executive summary

For many disabling or fatal diseases, there is pre-clinical or clinical evidence of the therapeutic potential of gene therapy. Yet, current gene transfer technologies limit wide application of gene therapy because of partial efficacy and/or safety concerns. Within the field of immunohematology in particular, the use of genetically modified hematopoietic stem cells (HSC) for the treatment of primary immune-deficiencies and the use of receptor-modified T cells for the treatment of tumours have both shown considerable promise in early phase clinical trials. Yet, clinical testing of these two approaches has also uncovered a number of important roadblocks (mentioned below). The goal of SUPERSIST was to exploit the innovative potential of emerging key technologies based on *ex vivo* gene targeting by artificial nucleases and gene delivery by lentiviral vectors to address these issues and advance HSC and T-cell gene therapy towards effective and safe application to a range of human diseases. The research and development activities were focused on two diseases chosen as paradigmatic for testing the therapeutic potential of gene editing: X-linked Severe Combined ImmunoDeficiency (SCID-X1), which is a severe rare inherited disease of children, and acute leukaemia, a disease affecting adults and children, as summarized below.

HSC-based gene therapy of SCID-X1. For inherited disorders, stem cells are the ideal cell target for gene transfer, because they retain the ability to renew themselves through mitotic cell division (self-renewal) and can differentiate into a wide range of specialized cell types (multilineage differentiation). However, to fully exploit the therapeutic potential of HSC gene therapy and broaden its application to other diseases, the adverse consequences of insertional mutagenesis and unregulated or ectopic transgene expression associated with the use of semi randomly integrating vectors must be abrogated. SUPERSIST aimed at overcoming these hurdles by exploiting targeted gene editing strategies for *in situ* correction of the disease-causing mutations. The results of this project have been: 1) the development of a robust gene editing protocol targeting human long-term repopulating HSC; 2) the generation of suitable preclinical models to determine the efficacy and safety of the new procedure; 3) the establishment of clinical-grade processes for manufacturing the therapeutic cell product; 4) the definition of an ethically compliant and scientifically sound rationale for what may become the first clinical translation of HSC gene editing as treatment for inherited diseases.

T-cell based gene therapy of acute leukemia. Cancer immunotherapy aims at harnessing the exquisite power and specificity of the immune system for the treatment of malignancies. However, this approach has been limited, for several years, by a combination of biological and technological hurdles, including immune escape mechanisms adopted by tumors, the immunosuppressive tumor microenvironment, immune defects secondary to patient treatments, etc. SUPERSIST aimed at exploiting site-specific genome editing technology to permanently edit T cell specificity and generate safe and efficient engineered T cells able to recognize specific tumor antigens. TCR edited T cells will provide a new "off-the-shelf" personalized cellular product for the treatment of leukemia patients harbouring the ability to provide long term protection from disease recurrence.

Both HSC gene therapy and cancer adoptive immunotherapy are facing an accelerated phase of development, largely due to the first therapeutic successes obtained with genetically modified cells in clinical trials of inherited diseases and lymphoid malignancies, respectively. We can envisage that adoptive T cell therapy will soon become a major pillar in cancer treatment. The outcome of SUPERSIST is paradigmatic of the next generation of HSC- and T cell-based gene therapies, in which conventional gene addition is replaced by the more precise and powerful approach of targeted gene editing, achieved through a combination of state of the art gene transfer vectors and artificial nucleases. While two diseases have been selected for investigation within the project for their paradigmatic features, the strategies developed are expected to be potentially applicable to several additional human diseases. Thus, the outcome of SUPERSIST should benefit the EU population by

providing a long-sought perspective of effective treatment for a number of severe diseases, leading to improvement in population health and reduction in health care costs, while at the same time increasing the competitiveness of EU biomedical and pharmaceutical industry through the generation of new scientific knowledge, processes and technologies.

1.1.2 Summary description of project context and objectives

1.1.2.1 Context (State of Art at the project start, Key challenges)

Substantial evidence supports the therapeutic potential of *ex vivo* gene therapy based on Hematopoietic Stem Cell (HSC) or T lymphocytes to treat inherited diseases or cancer. Yet, the intrinsic limitations of current gene replacement approaches based on semi-randomly integrating vectors, and the occurrence in some clinical trials of severe adverse events related to gene transfer, prevent safe deployment and broad application of gene therapy.
This project aimed to overcome these limits by exploiting the results of an earlier highly successful integrated project (PERSIST), which demonstrated the potential of emerging gene targeting technologies based on engineered nucleases to provide radical new solutions to these hurdles.

The recent development of engineered endonucleases, such as Zinc Finger Nucleases (ZFNs) (Urnov, *Nat Rev Genet* 2010) and Transcription Activator-Like Effector Nucleases (TALENs) (Mussolino, *Curr Opin Biotechnol* 2012), has brought the possibility of gene targeting within the reach of cell and gene therapy. These artificial proteins comprise a nuclease domain derived from the *Fok*I endonuclease and a DNA binding domain whose sequence specificity can be engineered as required. They are used to target a DNA double strand break (DSB) with high efficiency and specificity to a pre-selected sequence within the genome (Gabriel, *Nat Biotech* 2012). According to the repair process that seals the break (Ciccia, *Mol Cell* 2010), the outcome can be:

1) Mutagenesis at the nucleases target sites introduced by the error-prone Non-Homologous End Joining (NHEJ) pathway, giving rise to a somatic gene knock-out if essential coding sequences are targeted.
2) Faithful reconstitution of the original sequence by Homology Driven Repair (HDR). If an exogenous donor template is provided that contains sequence homology to each side of the DSB, the targeted sequence can be edited and novel sequences introduced at the site according to the design of the donor.

USR first showed that gene targeting can be used to correct genetic mutations by inserting a functional cDNA copy of the gene downstream its own promoter (Lombardo, *Nat Biotech* 2007). This approach has the advantage that most disease-causing mutations affecting the locus, including deletions, can be treated with the same engineered nuclease(s). Importantly, gene correction, as opposed to gene replacement, not only restores the function of the gene but also its physiological expression control, coming close to the long-sought holy grail of gene therapy. Despite the great promise of these technologies, some challenges remained to be addressed to fully exploit their potential for cell and gene therapy. Delivery of nucleases and cognate donor template DNA had been challenging in primary cells. USR was also the first to report a delivery strategy based on integrase–defective LV (IDLV) to achieve significant levels of gene targeting in several human primary cell types (Lombardo, *Nat Biotech* 2007; Lombardo, *Nat Methods* 2011). Despite this strategy allows reaching clinically relevant levels of gene targeting in T-cells (Lombardo, *Nat Methods* 2011; Provasi, *Nat Med* 2012), gene targeting in the more primitive human hematopoietic stem/progenitor cells (HSPC) remained for long time rate-limiting for clinical translation. The poor compliance of HSC to HDR is likely due to poor permissiveness to transduction of multiple factors, the requirement for transit through the S/G2 phase of the cell cycle during treatment, and induction of apoptosis and growth arrest upon excess DNA damage.

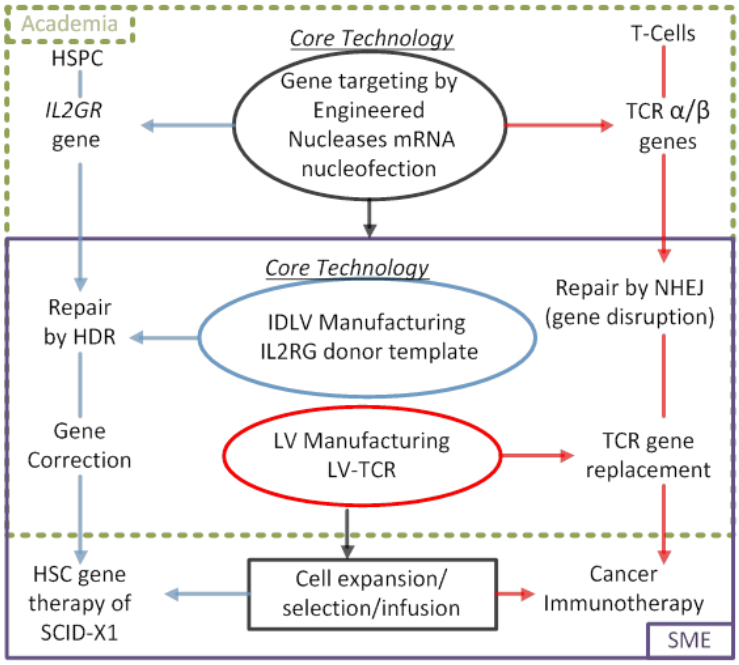

Figure 1. SUPERSIST scientific approach

In SUPERSIST, as depicted in Figure 1, these hurdles were overcome by optimizing nucleases specificity and delivery, and devising clinically grade culture conditions that support robust *ex vivo* manipulation of the targeted HSPC.

The SUPERSIST consortium was uniquely placed to meet this goal, thanks to the successful track record of USR and UCL in the clinical development of HSC gene therapy, and to the unique expertise of DKFZ, which has pioneered comprehensive clonality and integration site analyses in preclinical and clinical gene therapy. USR and UCL have now reached more than 10 years follow-up of ADA-SCID and SCID-X1 patients treated with HSC gene therapy in a set of seminal studies providing evidence both of the substantial clinical benefit of gene therapy and its associated risks (Aiuti, *N Engl J Med* 2009; Gaspar, *Sci Transl Med* 2011a & 2011b). Moreover, USR has recently obtained near-complete gene marking of hematopoiesis in human Leukodystrophy patients by using lentiviral vectors (LV), with up to 80% of cells in all hematopoietic lineages being vector-marked at the latest follow-up of 6 year post gene therapy (Sessa, Lancet, 2016). This unprecedented level of HSC gene marking was achieved by establishing together with the SME partner MOLMED a manufacturing process that yields high titer and quality LV and ensures efficient transduction while limiting detrimental effects on HSPCs. These results provide a long-sought proof that *ex vivo* culture of HSC in state-of-the-art conditions is compatible with maintenance of engraftment and long-term repopulation potential in humans, and thus should be amenable to adaptation to the requirements of nuclease delivery and gene correction.

Although gene therapy was initially designed to correct genetic defects, it was readily adapted for treating cancer. The possibility to tame toxicity, increase effector activity and ultimately redirect T-cells to kill cancer cells by genetic engineering, allows deploying all the firing power of immunity against cancer. C. Bonini (USR) and C. Bordignon (MOLMED) pioneered suicide gene therapy applied to allogeneic HSC transplant, to exploit the antileukemic potential of alloreactive lymphocytes while controlling detrimental GvHD (Bonini, *Science* 1997, *Nat Med* 2003; Ciceri, *Lancet Oncol* 2009). More than 120 patients have been safely treated worldwide and the approach, with a cumulative follow-up >223 person/year and the longest follow-up at 17 years, represents one of the largest clinical experience of cell-based gene therapy, and one of the first to be tested in a randomized phase III clinical trial. As described above for HSC gene therapy, this partnership has allowed building protocols, infrastructure and the expertise required for clinical translation of T-cell based gene therapy through a harmonized and productive academic/industrial effort. On this topic, USR collaborated with DKFZ and NKI. Indeed, DKFZ has set up a next generation sequencing platform to study immune repertoires (T cell receptor diversity) in healthy and diseased conditions followed by intensive

bioinformatic data analysis. NKI is investigating TCR gene therapy for the treatment of solid tumors, and has recently opened a phase I trial in patients with metastatic melanoma. Taking into account that a substantial fraction of the antigens that are expressed in haematological malignancies are also expressed in solid tumours, and considering the large patient group with diverse solid tumours monitored by NKI, this offers a clear avenue for the broader assessment and exploitation of the results that have emerged from SUPERSIST.

1.1.2.2 Objectives of the project

By combining gene targeting technologies by engineered nucleases with gene delivery by viral vectors, as investigated initially by USR in the PERSIST project, the objective of SUPERSIST was to achieve genetic correction of disease-causing mutant genes in HSC, such as a defective *IL2RG* gene in SCID-X1, and to genetically edit T cell specificity for generating tumor-specific T lymphocytes with enhanced efficacy and safety for adoptive cell therapy of leukemia.

In order to achieve these objectives, the project was organized in 3 scientific work packages (WP) with the following targets:

(WP1) Establishment of robust protocols for correction of the *IL2RG* gene in SCID-X1, by:

1) Selecting the best performing and most specific nucleases for functional correction of the *IL2RG* gene;
2) Developing a robust and scalable protocol for targeted integration of the corrective transgene cDNA in human HSPC, assayed for long-term repopulation of NSG mice;
3) Providing proof-of-efficacy of disease correction in SCID-X1 HSC.

(WP2) Establishment of robust protocols for TCR gene editing in T lymphocytes for the treatment of leukemias, by:

1) Developing a library of LV expressing TCR against leukemia-associated antigens;
2) Identifying the optimal T cell subset for TCR gene editing;
3) Developing a robust and scalable protocol for TCR genes disruption and replacement;
4) Providing proof of anti-tumor efficacy of TCR gene edited T cells in NSG mice challenged with human cancers.

(WP3) Development and scale-up of manufacturing of HSPC and T-cell products, by:

1. Developing processes for production of suitable quality mRNA and LV to support pre-clinical studies;
2. Scale up and optimization of gene targeting protocols in HSPC and T-cells for the two proposed gene therapy applications;
3. Preparation of one full-scale lot of modified cells for each proposed gene therapy application;
4. Performing preclinical GLP studies of toxicity and bio-distribution of both types of cell product – gene corrected HSPC and TCR gene edited T-cells in NSG mice.

1.1.3 Description of the main S&T results/foregrounds

1.1.3.1 Establishment of robust protocols for correction of the IL2RG gene in SCID-X1 (WP1)

WP leader: USR

Task 1 – Selection of the best performing nucleases for functional correction of the IL2RG gene (M1-M24)

By using ZFNs specific for a mutational hotspot in the exon 5 of the *IL2RG* gene, USR previously reported a gene targeting strategy which can be used to correct genetic mutations by inserting a functional cDNA copy of the gene downstream its own promoter (Lombardo et al., Nat Biotech 2007; Genovese et al., Nature 2014). Gene correction, as opposed to gene replacement, not only restores the function of the gene but also its physiological expression control, coming close to the long-sought holy

grail of gene therapy. Moreover, this approach has the advantage that most disease-causing mutations affecting the locus, including deletions, can be treated with the same engineered nuclease(s). Thus, in order to develop a nuclease set that could be used to correct most of the disease-causing mutations in the *IL2RG* gene, we here created and tested a panel of nucleases targeting an upstream region of the *IL2RG* gene. By targeting such upstream region of the gene, we used a donor template containing a splice acceptor site upstream of the corrective cDNA, which is transcribed and spliced to the upstream endogenous exon 1 of the gene. By this strategy any intervening sequence can be spliced out from the chimeric transcript leading to reconstitution of a functional *IL2RG* reading frame.
USR has developed a panel of artificial nucleases -based on either TALE or ZFP architecture- targeting upstream regions of the *IL2RG* gene. Specifically, we tested two ZFNs pairs targeting exon 1 and 3 ZFNs pairs targeting intron 1(all ZFNs are developed by our collaborators Sangamo Biosciences), 1 TALENs pair targeting exon 2 and one targeting intron 2. Potential off-target activity of the nucleases was initially evaluated by bioinformatics analysis of the intended binding site and in vitro SELEX approach, to screen for the highest specificity. We transiently expressed these nucleases in K562 by plasmid or mRNA electroporation and then measured NHEJ-induced mutations at the expected nucleases target sites using a mismatch selective endonucleases assay (Cel1). These analyses showed that all nucleases pairs induced significant levels (from 12% up to 74%) of on-target gene modification. Among these nucleases, we selected as best candidate lead reagent one pair of ZFNs targeting intron 1 of the gene because: i) these nucleases yielded high levels of on-target gene modification and ii) selection of an intron rather than an exon as landing path for the corrective cDNA is more advantageous for the design of the donor vector. The rationale behind the choice of the target site is multi-fold. Indeed, in parallel experiments performed with the IL2RG-ZFNs targeting exon 5, we found that recoding of the ZFNs binding site in the donor vector was mandatory in order to avoid cut of these sequences by the ZFNs, which in turns decreases donor availability for HDR or inactivates the corrected gene. Furthermore, as opposed to targeting the first exon of the gene, selecting the first intron is favorable as it allows constructing homology arms for HDR in which minimal -if none- transcription regulatory sequences of the endogenous gene are present, thus avoiding the potential unregulated expression of the corrective cDNA in case of its random genomic integration. Additional benefits of this design would be that NHEJ-mediated integration of the donor DNA into the ZFNs target site would also lead to gene correction, and that exons are spared from NHEJ-induced mutations (although this is of minor concern when dealing with already defective alleles). To obtain targeted transgene integration with this new set of IL2RG-ZFNs, we constructed a donor DNA template containing within the *IL2RG* homology arms a corrective cassette composed of a splice acceptor site followed by an in-frame fusion of all the *IL2RG* exons located downstream of the nuclease target site (corrective cDNA). In order to efficiently deliver this donor DNA into CD34+ cells we and UCL generated both IDLV and different serotypes (natural or engineered) of AAV vectors. Using both delivery strategies USR achieved high levels of and targeted insertion of the corrective cDNA into intron 1 of *IL2RG* in bulk treated HSPC. Importantly (see Task 2 below), these edited cells were able to engraft after transplantation into conditioned NSG mice and to normally differentiate into hematopoietic lineages (Schiroli et al., manuscript in prep).

As parallel strategy, UCL tested a site specific integration approach aimed to insert an *IL2RG* expression cassette into safe harbour loci, such as *AAVS1*, in order to safely achieve expression of the replaced therapeutic transgene. To construct an expression cassette that sustain efficient *IL2RG* protein expression, UCL constructed a lentiviral vector containing a codon optimized *IL2RG* gene. By performing gene replacement experiments with this recoded vector, UCL demonstrated superior gene expression levels per transgene copy compared to wild type sequence (and current SIN gammaretroviral platforms) in T cell lines deficient in common cytokine receptor gamma chain expression (ED7R), and in primary CD34+ cells. UCL also demonstrated effective and complete correction of a murine model of SCID-X1 in vivo. This vector is currently being evaluated for clinical translation in a conventional gene replacement setting (in collaboration with Genethon, FR), and will thus provide a reference standard for comparing the gene editing approaches.

In parallel with these experiments, in collaboration with Partner DKFZ, USR assessed the specificity of the newly generated nucleases. To this aim, DKFZ used the genome-wide IDLV-bait trapping

approach, previously developed with USR (Gabriel et al, Nat Biotech 2011). Briefly, retrieval of recurrent IDLV-bait integration sites in ZFNs treated cells by LAM-PCR and bioinformatics analysis were used to map the location of on- and off-target DNA double strand breaks (DSBs) induced by the nucleases. This approach was initially used to validate the specificity of the exon 5 *IL2RG*-ZFNs, and showing that they are able to yielded a 100-fold ratio between on target vs off-target ZFNs activity in treated CD34+ cells (Genovese et al., Nature 2014). DKFZ analysed with this method also the off-target activity of the two *IL2RG*-TALENs mentioned above, resulting in the identification of two off-target positions for the TALENs pair targeting exon 2 and none for the TALENs pair targeting intron 2. In collaboration with Sangamo BioSciences, USR assessed the specificity of the final ZFN leads targeting the intron 1 region of the *IL2RG* gene by using a similar but more sensitive GuideSeq approach (Tsai, et al., Nat. Biotech 2015). By this strategy, we were able to screen the specificity of different intron 1 *IL2RG*-ZFN leads and successfully identify a combination of *IL2RG*-ZFN pair and dose that allows to maximize both activity and specificity (**Deliverable 1.1**; Schiroli et al., manuscript in prep).

Concerning the development of assays to directly measure the extent of HDR, USR developed a quantitative PCR strategy based on the use of digital droplets PCR (ddPCR) system. This approach allows to quantify proper HDR integration of the corrective cDNA into the ZFN target site. The advantage of this strategy is that gene correction can be measured without the use of any reporter gene, thus it will be instrumental to verify targeted insertion of the corrective donor during pre-clinical development and to release the final cell product upon manufacturing (**Deliverable 1.2;** Schiroli et al., manuscript in prep).

In parallel, DKFZ generated a deep-sequencing assay to measure genome modification at the nuclease target site, using the T cell receptor (TCR) locus as suitable reporter (see also WP2). Repair of DSB can lead to a multitude of possible outcomes. In the presence of a donor sequence, at least five possible types of product can be foreseen after cutting and repair of the target locus: A) HDR-mediated integration of donor encoded sequences; B) NHEJ-derived indel mutations encompassing the nuclease target site, including large deletions; C) capture of the entire or truncated donor DNA by NHEJ; D) chromosomal translocation joining the target locus to an off-target site on another chromosome; E) seamless repair without any change relative to the original sequence leaving the wild-type allele. Depending on the protocol design, only outcomes A) and/or B) represent desired products. To characterize these events, DKFZ constructed a set of TALEN as well as the corresponding donor vectors necessary for targeted integration of a GFP cassette into the human genome (See WP2, task 5). The TALEN were expressed in K562 cells along with different donor sequences to generate samples that have different combinations of the above described possible outcomes. DKFZ used these samples to develop an assay that allows targeted deep sequencing of the loci distal to the nuclease target site. DKFZ made use of an asymmetric sequencing strategy, where the 450bp sequencing read initiates from the unknown distal side and extends through the nuclease target site into the known genomic sequence. An intermediate amplification step using primers annealing outside of the homology region at sites absent in the donor sequence is included in the assay to avoid mis-interpretations due to inclusion of the donor sequences. After sequencing, the frequencies of different genome modifications at the target locus can be analyzed bioinformatically (Knipping et al, unpublished).

Task 2 - Develop a robust and scalable protocol for targeted integration in NSG-mouse long-term repopulating human HSC (M12-M30).

Targeted genome editing by artificial nucleases has brought the goal of site-specific transgene integration and gene correction within the reach of gene therapy. Whereas we and others already reported high levels of targeted gene modification in some clinically relevant primary cells, its application to long-term repopulating Hematopoietic Stem Cells (HSCs) has remained elusive for long time.

USR has shown that gene targeting in the most primitive HSPC is constrained by poor gene transfer efficiency and by a bias of the DNA repair pathway toward the use of NHEJ rather than HDR. By combining IDLV transduction and mRNA transfection for donor template delivery and ZFNs expression, respectively, with the use of culture conditions tailored to expand HSPC, we have overcome in part these barriers and provided stringent evidence of targeted integration in human HSPC. Using the already available ZFNs targeting exon 5 of the *IL2RG* gene, we demonstrated that gene correction can be effectively used to restore both expression and function of the gamma chain coupled receptors in the lymphoid progeny of edited HSPCs. These results represent a milestone achievement for the project (**Milestone 1**), were published in a top scientific journal and received as a break-thru from the scientific community, and have opened the way to the exploitation of targeted genome editing strategies in HSC-based gene therapy (**Deliverable 1.3**; Genovese et al, Nature 2014).

Whereas these results represent the first proof-of-concept of targeted genome editing in human HSC, several challenges remained to be addressed in order to improve the efficiency and robustness of the process and to establish its safety for clinical translation. Thus, accordingly to the strategies originally described in the WP1 work plan, we refined our gene targeting protocol in order to further improve gene targeting efficiency in the primitive HSPC and achieve functional correction of the *IL2RG* gene using the intron 1 specific ZFNs selected in Task 1. To improve nuclease expression while decreasing cellular innate response to mRNA transfection we included modified nucleotides during mRNA production and performed HPLC purification after in-vitro transcription. The use of this optimized mRNA and clinical grade purification of IDLV (based on plasmid DNA removal followed by anion exchange chromatography) allowed decreasing type-1 interferon activation and electroporation toxicity, respectively (see also Deliverable 3.4). To further optimize ex-vivo HSPC manipulation we tested pyrimidoindole derivatives added to the culture and found a combination promoting HSPC expansion in conditions that preserve their primitive phenotype, increasing the yield of edited cells that are able to repopulate NSG mice (**Deliverable 1.4**). We then compared the gene targeting efficiency obtained with IDLV vs AAV vectors as delivery vehicles for the donor DNA template. By optimizing dose and timing, we found that AAV6 vector outperforms IDLV for delivering the HDR template, reaching up to 40% targeted integration in bulk HSPC cultures. After xenotransplantation, HSPC edited with highly purified IDLV or AAV6 sustained normal hematopoiesis contributing to a mean of 5% or 10%, respectively, of the total human graft in all the transplanted NSG mice, thus reaching clinically relevant levels of editing in long-term engrafting human HSC (**Milestone 2**). Finally, we demonstrated the therapeutic potential of our strategy by correcting the *IL2RG* gene in HSPC from a genotyped SCID-X1 patient (Schiroli et al., manuscript in prep).

In parallel, UCL has tested both TALEN or CRISPR-Cas9 based nucleases on several endogenous genomic loci (including *IL2RG*, *RAG1*, *BTK*, and *AAVS1*) of human CD34+ cells to design site-specific integration strategies either at natural gene loci (RAG1/BTK, in order to harness native regulatory sequences) or in safe harbors.

Task 3 - Proof-of-efficacy of disease correction in SCID-X1 HSPCs.

UCL has an on-going clinical trial, external to the SUPERSIST project, of SIN gamma-retroviral gene therapy for SCID-X1 performed with an expression cassette based on a human elongation factor-1 alpha regulatory element and an *IL2RG* cDNA. Data from this study were recently published and demonstrate effective reconstitution of T cell immunity, but limited reconstitution of humoral immunity (Hacein-Bey-Abina et al, NEJM 2014). This reflects an absence of HSC engraftment without pre-conditioning of patients, indicating that while this treatment is effective in restoring the T cell lineage compartment, some myeloablation is required in order to allow engraftment of HSC modified by standard gene addition or corrective gene editing. Data from this and previous studies were also used to estimate a threshold level of gene correction in CD34+ cells needed for successful gene correction in unconditioned patients (>10^6 cells per Kg).

To support the scientific rationale and explore the safety of the new proposed treatment based on HSC gene editing, we took advantage of suitable preclinical models of disease. USR and UCL previously developed a mouse model carrying the *IL2RG* human gene harboring a common disease-causing mutation (G691A) in place of the murine *Il2rg* gene, allowing to use the same nuclease and donor set utilized for gene correction of human cells. USR characterized these mice and found impairment in lymphoid development (no B, NK cells and few non-functional T cells) which is identical to the observations reported for *Il2rg-/-* mice. To evaluate efficacy and safety of the hematopoietic reconstitution from a limited number of corrected HSPC, USR performed competitive transplants with wild-type (WT) and *Il2rg-/-* HSPC and found that 1% of WT cells are sufficient to partially reconstitute and 10% of WT cells fully reconstitute the functionality of T and B cell compartments. Unexpectedly, USR found that administration of a conditioning regimen before HSPC infusion is required to avoid the risk of thymic lymphoma development in the treated mice, likely because of replicative stress-induced transformation of transplanted T-cell progenitors in the absence of sustained replenishment from engrafted functional HSC (**Deliverable 1.5**).

In order to obtain proof-of-correction of the mutated *IL2RG* gene by gene editing, UCL exploited an in vitro differentiation protocol to accurately recapitulate the cellular disease phenotype and thus demonstrate restoration of stage-specific T cell development in combination with full maturation of T cells. The collaboration with Toni Cathomen (Freiburg) and colleagues, which have established a T cell differentiation protocol, enabled UCL to generated mature T cells from murine ESCs in a stage-specific manner. UCL verified the potential of this protocol for disease modelling by utilizing ESCs that were produced to generate the humanised mouse model for X-SCID (USR and UCL). After correction of the underlying mutation with the TALEN technology, a newly developed protocol was applied to differentiate ESCs to mature single-positive CD8+ cells that showed restored IL-2 dependent activation and proliferation, hence confirming functional correction of the cellular X-SCID phenotype *in vitro* (Alzubi et al., manuscript submitted).
To obtain proof-of-correction of the disease phenotype *in vivo* by gene editing, USR decided to develop a protocol for ex vivo gene editing of mouse HSPC. USR found that these cells are highly sensitive to electroporation and nuclease treatment, and thus require less harsh vector-mediated delivery of nuclease. Because ZFN are not amenable to such vector based delivery, we turned to CRISPR/Cas9 and used guide RNAs targeting the same intron of *IL2RG* and achieved substantial levels of targeted DNA repair by NHEJ (70%) and HDR (~25%) on murine *IL2RG*-/- HSPC. The surviving cells remained capable of expansion in culture and maintained their clonogenic potential. Importantly, upon transplant into lethally irradiated mice, only the gene corrected cells were able to generate lymphoid lineages (B and T cells), showing a clear selective advantage over the un-corrected SCID cells. The corrected lymphoid cells persisted long-term in the mice and generated a functional T cell response upon challenging the mice with a murine pathogen, thus indicating that successful *IL2RG* edited HSPC cells are able to sustain lymphopoiesis and partially correct the disease phenotype, as shown for a limiting fraction of WT cells (**Deliverable 1.6**). Overall, the studies conducted in the SCID-X1 mouse model allowed establishing the functionality of the edited HSPC progeny at rescuing the disease phenotype in vivo, and defining the threshold level of gene editing in the therapeutic cell product that, together with a proper choice of conditioning regimen, should support a safe and potentially effective first clinical testing of our gene editing strategy (Schiroli et al., manuscript in prep).

To compare gene correction to an alternative gene replacement approach, UCL have developed a third-generation lentiviral vector carrying a codon-optimized human *IL2RG* cDNA under the control of the human EF1α-S promoter. The vector was VSV-G-pseudotyped and produced by transient transfection of 293T cells, followed by ion-exchange chromatography, tangential-flow filtration and formulation in HSC growth medium (Genethon, Evry). Replacement of the native *IL2RG* open reading frame by a codon-optimized sequence resulted in a 3-fold increase in mRNA expression and a 1.5-fold increase in IL2RG protein expression per integrated vector copy. The performance of the vector was demonstrated *in vitro* by the restoration of a normal level of IL2RG mRNA or protein in a human IL2RG-deficient T-cell line at a VCN of 1 to 3, and by high efficiency (>80%) transduction of human mobilized $CD34^+$ HSPCs and transgene expression with no impact on viability or clonogenic capacity.

An *in vitro* immortalization assay (IVIM) showed a safe genotoxic profile, while the *in vivo* safety and efficacy of the vector was tested it in a preclinical model of SCID-X1 gene therapy based on transplantation of genetically corrected Lin^- cells from *IL2rg*$^{-/-}$ donor mice into lethally-irradiated *IL2rg*$^{-/-}$*-Rag2*$^{-/-}$ recipients. The study showed biodistribution of the transgene in hematopoietic organs only, restoration of T, B and NK cell counts in bone marrow and peripheral blood, normalization of lymphoid organs (thymus and spleen) and a low frequency of hematopoietic malignancies, comparable to that of untreated animals, six months after transplantation. An extensive insertion site analysis was carried out in bone marrow, thymus and peripheral blood of individual or groups of animals to detect the presence of any clonal abnormality or skewing, and any deviation from a normal lentiviral integration pattern. The study showed the expected genomic integration profile and no signs of clonal dominance of transduced cells. Interestingly, analysis of >100,000 integration sites in pre- and post-transplant murine cells showed that lentiviral vectors target at high frequency a substantially different set of genes compared to human $CD34^+$ cells, uncovering the need for appropriate controls and at the same time the limits in the predictive power of mouse-based genotoxicity studies, particularly those addressing proto-oncogene activation and clonal dominance. These studies will enable a multicenter phase-I/II clinical trial aimed at establishing the safety and clinical efficacy of lentiviral vector-mediated gene therapy for SCID-X1. The clinical protocol is based on transplantation of autologous $CD34^+$ HSPCs transduced with the EF1a-IL2RGco lentiviral vector in infant patients after a non-myeloablative conditioning regimen, and aims at stable and sustained restoration of both T- and B-cell immunity. Studies in US and Europe are planned to commence in mid-2017, and regulatory submissions are underway.

1.1.3.2 Establishment of robust protocols for TCR gene editing in T lymphocytes for the treatment of leukemias (WP2)

WP leader: USR

Task 1 - Develop a library of LV expressing TCR targeting tumor antigens (M1-M24).

One of the main barriers limiting the exploitation of TCR gene therapy for clinical treatment of cancers is the lack of tumour-specific T-cells and corresponding TCRs. In this Task we addressed the low availability of tumour-specific TCRs, and attempted to widen the number of tumor-specific TCR to be used in TCR-based immunotherapeutic approaches.

Within the first project period, NKI exploited its already established 'TCR capture' strategy for TCR alpha beta cloning to obtain antigen-specific TCRs in a number of projects (see below). In addition, we have developed and validated a technology that can be used to obtain TCR alpha beta sequences from single cells with high efficiency (Kelderman et al. unpublished). This technology has also been utilized in the projects described below, and makes it feasible to obtain TCR sequences from T cells with very limited in vitro expansion potential such as some intratumoral T cells. With respect to the use of these TCR cloning strategies:

- In a first project, NKI has employed these technologies to analyze tumor-specific T cell reactivity in the CD4+ T cell compartment in melanoma patients. Using cancer exome data as input, we showed that:

  a) CD4+ T cell recognition of neo-antigens that are formed as a consequence of mutations is common;

  b) The CD4+ T cells display (the expected) preferential reactivity towards the mutant antigen relative to its wild-type counterpart;

  c) These CD4+ T cells respond to patient-specific antigens (Linneman et al, Nat Med 2015).

  Collectively, these data indicate that when T cell editing is used with the aim of creating neo-antigen-specific CD4+ T cells in diseases such as melanoma, this will require the development of approaches that can 'tap' the tumor-specific TCR repertoire of individual patients.

- With the aim of testing the feasibility of such 'tapping' of the patient-specific TCR repertoire, NKI cloned TCRs from tumor-reactive T cells from tumor lesions of 3 melanoma patients for whom they have also developed patient-derived tumor xenograft models. In ongoing work we are introducing these TCRs into PBMC by retroviral gene transfer. This will allow USR and NKI to compare the anti-tumor activity of T cells modified with autologous tumor-reactive TCRs with tumor-infiltrating T cells from the same patient. These TCRs are now ready to be exploited for the TCR gene editing approach (**Milestone 3**).
- Finally, in collaboration with F. Sallusto, NKI has employed such technology to assess the kinship of human CD4 helper cells that emerge *in vivo* (Becattini et al., Science 2015).

The majority of tumour-associated antigens are self-antigens, thus most T-cells specific for such antigens are eliminated or anergized due to central and peripheral tolerance. Despite this, naturally occurring tumour-specific T-cells have been observed in healthy donors and patients, particularly in patients affected by hematological malignancies, after allogeneic hematopoietic stem cell transplantation (allo-HSCT) where frequencies of tumor-specific lymphocytes have been correlated with disease regression (Kapp, M. et al. Bone Marrow Transplantation 43,399–410 (2009); and Tyler, E.M. et al. Blood 121,308–317 (2013)). USR has optimized a protocol that allows rapid identification of leukemia specific T cells from leukemic patients based on the isolation of BM infiltrating T cells in expressing inhibitory receptors.

Task 2 - Identify the optimal T cell subset for TCR gene editing (M1-M12)

The ability to remember and respond more vigorously in a second encounter with a pathogen is a critical property of the adaptive immune system. This process has been proposed to involve a stem cell-like memory T-cell subset, able to rapidly differentiate to effectors and self-renew upon antigen re-encounter (Gattinoni et al., Nat. Med. 2011). The ability to exploit such a T-cell subset is of high clinical relevance for the development of T-cell based strategies able to promote long-term clinical remissions in cancer patients. Indeed, the majority of adoptive cellular therapy approaches have relied so far on effector T cells, able to rapidly clear the tumor, but often unable to expand and persist long-term in treated patients. This has been largely attributed to a progressive loss of proliferative potential associated to the reciprocal acquisition of effector functions, which correlates with T-cell differentiation. USR demonstrated that activation of Naïve T cells (TN) through beads conjugated to anti-CD3 and anti-CD28 antibodies (bCD3/CD28) prior to genetic modification of human T cells allows to enrich for gene-modified T cells with a memory stem T cells (TSCM) (Cieri et al., Blood 2013). Gene-modified TSCM, defined as postmitotic CD45RA+ CD62L+ CCR7+ IL-7R+ CD95+ T lymphocytes, are endowed with exceptional persistence and functional capacity *in vitro* and *in vivo*, documented also by serial transplantation experiments.

In parallel, UCL tested similar stimulation condition to genetically engineering peripheral or cord blood T cells using lentiviral transfer of antigen specific receptors. For these experiments, cells were activated with either CD3/CD28 antibodies and IL-2 or a combinations of IL-2, IL-7 and IL-15 then exposed to a single round of lentiviral transduction with a vector expressing a T cell receptor (directed against WT1) or a second generation chimeric antigen receptor (CAR) directed against CD19. Flow cytometric analysis revealed a mean of 70% (49-95%) gene transfer in bead-activated cells and 38% (26–47%) in T cell populations stimulated with cytokines alone. Modified cells exhibited antigen specific target cell lysis, with cytokine stimulated populations retaining markers of naive phenotype.

To verify the possibility of producing TCR gene edited TSCM, USR applied the TCR complete editing and single editing procedure (see below) on total CD3+ cells and/or CD45RA+CD62L+ sorted naive T cells, using the culture protocol that we developed to enrich for gene modified TSCM cells. No differences in the transduction efficiencies were detected, suggesting that the initial selection of naive T cells does not affect the transduction/gene editing procedures. The gene edited T cells derived from modified TN displayed a functional phenotype typical of the TSCM phenotype, while CD3+ derived edited T cells displayed a more variable phenotype.

To analyze and compare the persistence capacity of different memory T cell subsets USR took advantage of suicide gene therapy clinical model. This unique setting of gene therapy offers us the opportunity to exploit genetic marking to study the fate of infused memory T cells directly in humans. Suicide gene therapy represents one of the first gene therapy approaches clinically tested, today in phase III clinical experimentation (Bonini et al., Science 1997; Ciceri, Bonini et al., Lancet Oncol. 2009; Vago et al., Blood 2012). Genetically engineered memory T lymphocytes infused to patients with leukemia can be distinguished from other T cells thanks to the expression of a surface marker. The genetic integrations associated to retroviral mediated gene transfer and the TCR genes represent unique clonal markers for each T lymphocyte infused. USR characterized and traced, over several years, genetically modified T lymphocytes infused in 10 patients after haploidentical hematopoietic stem cell transplantation. At 2-14 years after infusion patients were in complete remission, free from transplant complications, and in the presence of a broad and resting immune system. USR could then still detect genetically engineered cells, circulating at low but stable levels in all patients. Characterization of engineered cell products infused to patients showed that the amount of infused T_{SCM} cells positively correlates with early expansion and long-term persistence of gene-marked cells. By combining sorting of memory T-cell subsets with sequencing of integration sites, TCRα and TCRβ clonal markers performed by DKZF, we longitudinally traced T-cell clones from infused products to late follow-up time-points. We showed that although T cells retrieved long-term are enriched in clones originally shared in different memory T-cell subsets, dominant long-term clonotypes preferentially originate from infused T_{SCM} clones, followed by T_{CM} cells. Altogether, these results indicate that long-term persistence of gene-modified memory T cells after haploidentical transplantation is affected by antigen exposure and by the original phenotype of infused cells. Adoptive immunotherapy might thus benefit of cellular products enriched in T_{SCM} lymphocytes (Oliveira et al, Science Translational Medicine 2015 Dec 23;7(319):319er9)

Task 3 - Development of robust TCR editing protocols (M1-M18)

Although TCR gene transfer is a promising approach of adoptive immunotherapy for cancer patients, T lymphocytes transduced with an exogenous TCR have proved less effective than naturally occurring tumor specific T cells, and led to suboptimal clinical results. This could be due to the dilution of the tumor specific TCR that competes with endogenous TCRs for its expression on the T cell surface. Moreover, random mispairing between endogenous and exogenous TCR α and β chains could generate new TCRs with unpredictable and potentially harmful specificities. To overcome these issues, our group developed the TCR gene editing procedure, based on the knockout of the endogenous TCR genes by transient exposure to α and β chain specific Zinc Finger Nucleases (ZFNs), followed by the introduction of tumor-specific TCR genes by lentiviral vectors (Provasi, et al, Nature Medicine 2102).
The complete editing (CE) strategy requires multiple manipulation steps involving repeated cell activation cycles and four different transductions: such laborious procedure is at the moment barely applicable to a clinical setting. In the first year of SUPERSIST, USR has developed the 'single TCR editing' (SE), based on the disruption of the single endogenous TCR α chain, followed by transfer of the tumor specific TCR, to generate redirected T cells fully devoid of their natural TCR repertoire, in a single round of cell activation. The SE protocol was validated exploiting an HLA-A2 restricted TCR specific for the cancer testis antigen NY-ESO-1-derived peptide. NY-ESO-1 is highly expressed by a large number of solid tumors and by the majority of monoclonal plasmacells of patients affected by high risk multiple myeloma, while its expression in healthy tissues is restricted to testicular germ cells. T cells harvested from healthy donors were activated with beads conjugated to antibodies specific for CD3 and CD28, and cultured with low doses of IL7 and IL15. 2 days after activation ZFNs specific for the constant region of the TCR α chain were transiently delivered into T cells either by RNA transfection or by infection with adenoviral vectors, thus obtaining CD3neg T cells at similar efficacy. Sorted CD3neg T cells were transduced with a lentiviral vector encoding for α and β chains of the NY-ESO-1-specific TCR. The SE strategy allowed the generation of high numbers of extremely fit tumor specific T cells, enriched for cells with an early differentiated phenotype

(CD45RA+/CD62L+/CD95+, namely stem memory T cells) (Mastaglio et al., manuscript under revision; **Milestone 4**).

In parallel, UCL is testing the possibility of producing non-alloreactive, TCR depleted, T cells that express a Chimeric Antigen Receptor (CAR) instead of a TCR. Thus they generate CAR19 expressing T cells with the SE protocol by using transcription activator-like effector nucleases (TALENs) targeting the constant domain of the TCR α chain. They found that electroporation of TALEN mRNA into cord blood T cells transduced with CAR19 yielded populations of antigen specific T cells with substantially reduced TCR expression and consequently reduced alloreactive potential.

Task 4 - Preclinical validation of TCR gene edited cells (M18-M30).

We evaluated the specificity, safety and efficacy of single edited (SE) T cells in vitro and in vivo. NY-ESO-1 TCR expression, investigated through dextramer specific binding, was significantly higher in SE than in T cells undergoing conventional TCR gene transfer (TR). To test the efficacy of NY-ESO-1 redirected T cells we took advantage of the NY-ESO-1^{+}HLA-A2^{+} U266 myeloma cell line. When tested against U266 cells in different *in vitro* assays (co-cultures, g-IFN release and ^{51}Cr release), all NY-ESO-1 redirected T cells showed a strikingly high killing potential. However, comparing the alloreactive potential of the different NY-ESO-1 redirected T cell populations in mixed lymphocyte reactions against completely mismatched allogeneic targets, USR interestingly observed that lysis of the allogeneic target by TR cells was significantly higher than that of SE T cells ($p=0.05$). This suggests that the residual endogenous polyclonal TCRs and possibly mispaired TCRs expressed on the cell surface of TCR-transferred T cells can lead to off-target recognition, while SE T cells are devoid of such reactivity. Using the single gene editing protocol USR could generate within a 15 days manipulation procedure and upon a single step of T cell activation, large numbers of highly performing tumor specific T cells, completely devoid of their endogenous TCR repertoire, and thus particularly appealing for a future clinical translation.
To verify the efficacy and safety profile of TCR SE lymphocytes, USR developed an experimental humanized-mouse preclinical model of multiple myeloma. Briefly NY-ESO-1 TCR transferred (TR) and SE T cells and untransduced lymphocytes were intravenously infused in NSG mice 4 weeks after irradiation and i.v. injection of U266 cells. One week after infusion, TR and SE cells expanded, reaching a peak around 7 days after injection. The initial T cell expansion was associated with high amounts of human IFNg in animal sera. While untransduced and TR T cells displayed a second wave of expansion and IFNg secretion, timely correlated with body weight loss, ruffled fur and hunchback, all animals infused with SE cells displayed a stable and low chimerism, low serum IFNg levels and a body weight similar to untreated littermates. Animal organs were analyzed at sacrifice to reveal the presence of residual myeloma cells and/or pathological signs of GvHD. While mice receiving untransduced T cells displayed variable levels of myeloma infiltration in the bone marrow (BM), all mice treated with NY-ESO-1 redirected T cells were completely tumor-free. When we repeated the experiment with luciferase labeled U266, we observed that NY-ESO-1 redirected T cells act in a very short timeframe. While one week after lymphocyte infusion, untreated and mock-transduced treated animals showed variable levels of luminescence, indicative of the persistence of malignant cells, TR and SE infused mice were completely signal-free. Thus, early waves of IFNg secretion and T cell expansion observed within the first week after adoptive transfer of TR and SE cells correlate with multiple myeloma debulking. At the end of the experiment, while no differences were observed between TR and SE treated mice in terms of CD3^{pos} marrow infiltration, splenic infiltration by TR cells was higher than that of SE cells, and similar to that of untransduced lymphocytes. Such different behavior is consistent with a difference in the ability of TCR redirected cells to induce off-target toxicity in this experimental model. Accordingly, TR cells, despite efficiently clearing multiple myeloma, induced a high rate of xenogeneic GvHD, observed in 75% of treated animals, resulting in lower overall and event free survival than those obtained with SE cells. At the end of the experiments, all mice treated with SE cells were alive and well (overall survival: 26,7% vs 100% in TR and SE infused animals, respectively; $p<0.005$). All untreated mice had to be euthanized before the end of the experiment because of disease progression. All animals infused with mock-transduced T cells required premature sacrifice, due to either multiple myeloma progression (3/15 animals presented extra-

medullary localizations), or xenogeneic GvHD, with 27% of animals showing signs of both MM and GvHD.
At sacrifice, several organs (skin, tongue, heart, lung, kidney, liver, gut) were collected, fixed in formalin and blindly analyzed by immunohistochemistry for human T cell infiltration. A pathological grading was conventionally generated, ranging from 0 to 3. The number of organs showing T cell infiltration was significantly increased in TR (mean 4) compared to SE (mean 1) treated mice. Most importantly, the global pathological score, corresponding to the degree of T cell infiltration for each organ, was also significantly higher in animals infused with non-edited T cells than in animals receiving SE cells (means of 6,2 and 1,9 for TR and SE, respectively.
Overall these results indicate that the potent anti-myeloma activity of NY-ESO-1-TCR transferred lymphocytes is limited by a high alloreactive potential *in vivo*. Conversely, NY-ESO-1 SE T cells exhibited high tumor specificity, resulting in optimal anti-tumor activity, in the absence of GvHD (Mastaglio et al., manuscript under revision; **Milestone 5).**

Task 5 - TCR repertoire deep sequencing (M1-M36).

DKFZ analyzed off-target (OT)-activity of ZFN targeting TRAC and TRBC in K562 cells. The cells were treated either with a single nuclease pair or a combination of both ZFN. To ovoid cross -ZFN heterodimerization when the two ZFNs sets (TRAC and TRBC) were used together, a TRAC specific ZFN with an orthologous FokI variant was used.
IDLV capture assay in K562 cells revealed a high number of clustered integration sites in all samples tested, indicative of OT activity at both ZFN target sites. By deep-sequencing DKFZ confirmed OT cleavage at a subset of these clustered integration sites.
These findings prompted DKFZ to evaluate alternate designer nucleases for TRAC/TRBC modification. DKFZ assembled 13 TALEN and five guide RNAs for the CRISPR/Cas9 system to knockout TCR expression. We established an mRNA electroporation assay for efficient delivery of the TALEN to cell lines and primary cells to achieve high-levels of gene modification. Using TALEN mRNA, TCR knockout was successful in up to 79% of T cells. Additionally, DKFZ were able to verify targeted gene addition using a donor suitable for replacing the comprised transgene with therapeutic TCR chains. In up to 4.7% of treated T cells, TALEN-mediated DNA DSB were repaired by HDR at the target site. Remarkably, analysis of TALEN and CRISPR/Cas9 specificity using integrase-defective lentiviral vector capture revealed only one OT site for one of the gRNAs and three OT sites for both of the TALEN, indicating a high level of specificity (Knipping et al., unpublished).

DKFZ have applied our RNA-based T cell receptor (TCR) sequencing approach (a variant of LAM-PCR to dissect rearranged TCRs) (Ruggiero et al. 2015) on genetically modified T lymphocytes that were infused in 10 patients after haploidentical hematopoietic stem cell transplantation (HSCT). Combining T cell sorting with sequencing of integration (IS) and TCR repertoire sequencing enabled to study dynamics and long-term persistence of gene-modified memory T cells after haploidentical HSCT (Oliveira et al, 2015; **Milestone 6**).

1.1.3.3 Development and scale-up of manufacturing of HSPC and T-cell products (WP3)

WP leader: MOLMED

Task 1 - Develop processes for production of suitable quality mRNA and LV to support pre-clinical studies

The objective of Task 1 was to produce suitable quality mRNA and LV to support the preclinical studies for the indications described in details in WP1 (severe combined immunodeficiency X-linked, SCID-X1) and WP2 (treatment of leukemias).

Although hematopoietic stem cell (HSC)-based gene addiction therapy has provided therapeutic benefit for several genetic diseases (Aiuti et al., 2013; Biffi et al., 2013; Cartier et al., 2009; Cavazzana-Calvo et al., 2010) with low associated risks, semi-random integration of viral vectors used in these clinical trials still remains a potential cause of adverse events *in vivo*. Thus targeted gene correction therapy is the new avenue for safer and, likely, more effective gene therapy approaches for the future. In this context, WP1 describes the novel strategy to ultimately achieve targeted gene correction of the *IL2RG* gene in SCID-X1 patients. This approach has been illustrated in a recent publication by USR, (Genovese et al., Nature 2014). The manuscript describes that ZFN-mediated insertion of a corrective cDNA into a mutational hotspot of the *IL2RG* gene of HSCs from healthy donors gave rise to functional lymphoid cells with a selective growth advantage over those carrying disruptive *IL2RG* mutations when these cells were xenotransplanted in NSG mice (Genovese et al., 2014) (Fischer, 2014).
The transient expression of ZFNs pair targeting *IL2RG* gene will be obtained by the electroporation of mRNA encoding the specific ZFNs. The mRNA will be produced by *in vitro* transcription starting from plasmid DNA as template.

For the SCID-X1 study, we have developed two processes: a) the production of two ZFNs mRNAs targeting exon 5 of the *IL2RG* gene and b) the production and purification of the integrase defective lentiviral vector, IDLV-IL2RG, containing the corrective *IL2RG* cDNA (part of the IL2GR gene) as template for the homology-directed recombination induced by the exon 5 specific ZFNs.

USR has recently developed a novel experimental strategy to reach high levels of endogenous TCR disruption by gene editing of both α and β TCR genes (Provasi et al., Nat Med 2012). In SUPERSIST project, one (in the single editing approach) or two (in the double editing approach) ZFNs pairs targeting the α and/or β TCR genes will be delivered to T stem cell memory (TSCM) cells to permanently avoiding the expression of the endogenous TCR. Site specific integration of the exogenous expression cassette for the tumor-specific TCR into the endogenous TCR α or β gene will be then achieved by lentiviral vector delivery.

ZFNs expression will be obtained by transient transfection of specific ZFN mRNA in primary T cells, which tolerate well nucleofection and allow efficient levels of targeted gene modification. The ZFN pairs mRNAs will be produced by *in vitro* transcription starting from plasmid DNA as template.

For the leukemia treatment study, so far we have developed the process for the production of two pairs of ZFN mRNAs targeting the human TRAC and TRBC loci.
To reach the objectives described above these sequential activities have been carried out:

1. Production of large stocks of plasmids encoding the specific ZFN pairs (D3.1 and D3.2)
2. Construction and production of the TRAC bicistronic plasmid
3. *In vitro* transcription of small scale ZFN mRNAs (D3.3)
4. Purification of small scale ZFN mRNAs by dHPLC (D3.3)
5. Production of IL2RG IDLV in small scale (D3.4)

Task 2 - Scale up and optimization of gene targeting protocols in HSPC and T-cells for the two proposed gene therapy applications

Ex-vivo modification of T cells is currently limited to a small number of centres with the required infrastructure and expertise. The process requires isolation, activation, transduction, expansion and cryopreservation steps. To simplify procedures and widen applicability for clinical therapies, automation of these procedures is being developed. The CliniMACS Prodigy (Miltenyi Biotec) has recently been adapted for lentiviral transduction of T cells. UCL has tested the feasibility of a clinically compliant T-cell engineering process for the manufacture of T cells encoding chimeric antigen receptors (CAR) for CD19 (CAR19), a widely targeted antigen in B-cell malignancies (see Task3 of WP2). Using a closed, single-use tubing set we processed mononuclear cells from fresh or frozen leukapheresis harvests collected from healthy volunteer donors. Cells were phenotyped and subjected to automated processing and activation using TransAct, a polymeric nanomatrix activation

reagent incorporating CD3/CD28-specific antibodies. Cells were then transduced and expanded in the CentriCult-Unit of the tubing set, under stabilised culture conditions with automated feeding and media exchange. The process was continuously monitored to determine kinetics of expansion, transduction efficiency and the phenotype of the engineered cells in comparison to small-scale transductions run in parallel. We found that transduction efficiencies, phenotype and function of CAR19 T cells were comparable to existing procedures and overall T-cell yields sufficient for anticipated therapeutic dosing. The automation of closed-system T-cell engineering should widen dissemination of emerging immunotherapies and greatly widen applicability. Similar strategies are currently being evaluated for HSC manipulation. In collaboration with Cellectis (UCL PI Qasim), we have recently demonstrated the clinical use of gene edited T cells to treat paediatric leukaemia. T-cells engineered to express chimeric antigen receptors against CD19 (CAR19) can mediate leukemic remission, but manufacturing bespoke cell products can be challenging and yields from heavily treated subjects may be suboptimal. Universal healthy donor CAR19 T-cells (UCART19) were generated by lentiviral transduction followed by simultaneous gene-editing of T cell receptor and CD52 gene loci with Transcription activator-like effector nucleases (TALENs). Two infants with post-transplant relapse of CD19+ B-cell acute lymphoblastic leukemia received lymphodepleting chemotherapy and anti-CD52 serotherapy, followed by a single dose of UCAR19T-cells. Molecular remissions were achieved in both infants, providing a novel bridging strategy to successful allogeneic stem cell transplantation. An industry sponsored clinical trial to follow up on these findings has been initiated.
UCL have also explored automated transduction of CD34+ HSPCs. In collaboration with Miltenyi Biotec we first tested the CD34+ enrichment protocol and found that yield and purity of CD34+ cells were comparable with the existing clinically approved protocols. We then tested the feasibility of a clinical compliant engineering process for the manufacture of lentivirally transduced CD34+ cells. Briefly, we cultured and lentivirally transduced CD34+ cells using the CentriCult unit of the TST30 tubing set (automated process). In parallel we performed small scale transduction runs in the laboratory (manual process). After three days of culture we transplanted the transduced CD34+ cells into immune deficient mice in order to evaluate the fitness of HSPCs. We found that the phenotype, viability and engraftment potential of transduced CD34+ cells were similar between the CliniMACS Prodigy and the manual process while the manual process outperformed the CliniMACS Prodigy in transduction efficiency. The experience of our collaborators in USR/Molmed has corroborated the above observations. Then, we evaluated the use of a T cell protocol/tubing set TS520 for the transduction of CD34+ cells. The extremely user friendly T cell programme allowed us to make crucial adjustments in the automated feeding and media exchange process in order to accommodate two rounds of transduction. Transduction efficiency and phenotype of the CD34+ cells product were comparable with the existing clinical procedure (average vector copy number was 2.48 for the automated and 1.62 for the manual process). Following these encouraging results, we are now optimising the T cell protocol for the manipulation of CD34+ cells as we believe that it can be developed for clinical application.

During the second part of the project, the development of gene targeting protocols in T cells was temporarily put on hold by MolMed secondary to the fact that the retrieval of an array of novel tumor-specific TCRs ended up to be more difficult than initially expected (WP2). MolMed thus focused exclusively on the optimization of gene-targeting protocols in HSC for SCID-X1 therapy application. Based on the encouraging results of the production/purification of zinc finger nuclease (ZFN) mRNA and IDLV for the interleukin-2 receptor common gamma chain (*IL2RG*), obtained during the first eighteen months of SUPERSIST project, we proceeded with the a) production of high-grade reagents and b) the scale-up of the cell product for the HSPC biodistribution studies to be conducted under GLP.

To reach the objectives of Task 2 of WP3, the following activities have been carried out by MolMed:

- 3.3.2.1 Production of plasmids encoding the specific ZFN pairs
- 3.3.2.2 Production and purification by dHPLC of medium-large scale ZFN mRNAs targeting human IL2RG gene
- 3.3.2.3 Production and purification of research- and GMP-grade plasmids and IL2RG IDLV at medium-large scale

3.3.2.1 Production of plasmids encoding the specific ZFN pairs

USR previously reported a successful gene targeting strategy to correct genetic mutations of the IL2RG gene by using ZFN pair specific for a mutational hotspot in exon 5.
During the first 18 months of the project, MolMed manufactured mRNA of exon 5-targeting ZFNs using a panel of different pairs of plasmids, i.e. from S009/S010 to S024/S025 pair. More recently, in collaboration with Sangamo Biosciences, USR tested the efficacy of a couple of new pairs of nucleases (i.e. S018/S019 and S027/S028) targeting a specific region of the intron 1 of human IL2RG gene. The switch between exon 5 to intron 1 targeting region was driven by several reasons, as discussed in WP1 above.
During the second half of the project, MolMed produced plasmids for both types of ZFN pairs specific for targeting either exon 5 or intron 1 of the human IL2RG gene. MolMed received the seven plasmids from USR that, in turn, obtained them from Sangamo Bioscience. On 16/03/2015, S018, S019 and S023 plasmids were shipped to Plasmid Factory GmbH & Co. KG (Bielefeld, Germany), which provides high-quality grade, medium-scale plasmid lots. The plasmids, spotted on filter paper, arrived at destination on 17/03/2015. Fifty vials for each plasmid preparation were sent back to MolMed on dry ice on 29/04/2015, at the concentration of 1 mg/mL in TE buffer.

3.3.2.2 Production and purification by dHPLC of medium-large scale ZFN mRNAs targeting IL2RG

During the first year and half of the project, we developed protocols for the small-scale production of mRNA encoding the ZFN pairs targeting the human IL2RG gene. In comparison to the protocol for small-scale lots, two modifications were applied to the medium-large scale protocols:

- the *in vitro* transcription reaction was performed in the presence of the nucleotide analogs pseudouridine
- ΨU- and 5-methylcytidine -m5C- in place of the corresponding U and C canonical nucleotides, respectively;
- the polyA tailing reaction was skipped since the new ZFN-coding plasmids include a polyA tail downstream the ZFN coding sequence.

Experiments conducted by USR confirmed the improvements achieved in mRNA production efficiency thanks to these modifications, as previously demonstrated by Karikò et al. and Anderson et al. (Anderson et al., 2011; Kariko et al., 2008). Over M13-M42, we performed several mRNA production runs ranging from a medium (6X) to large scale (200X) in order to test the scalability of the production and purification protocols and to produce suitable high quality mRNA to support GLP studies. The 1X production run consists in a single 20-µl *in vitro* reaction in which 1 µg of DNA was used as template. The mean yield was 43.2 ± 3.8 µg of raw mRNA (n=9). The scale up was carried out by proportionally increasing the volume of the reagents in a single test tube (50X, 1 ml) or in 4 independent test tubes (200X, 4 × 1 ml). We carried out only a single large-scale production (200X) which resulted in 10 mg of each ZFN raw mRNA. The purification of the *in vitro* transcribed mRNA was performed by dHPLC with a reverse phase column made of non-porous matrix consisting of polystyrene-divinylbenzene copolymer beads alkylated with C-18 chains (Transgenomic, LTD). The large-scale mRNA lot (10 mg) was then purified by dHPLC, loading up to 600 µg per purification run. The maximum amount of mRNA recovered after dHPLC multiple purification runs was 5-6 mg. The average yield of the recovered mRNA for all scales applied was about 50-60% of the starting loaded material.
During the last step of mRNA purification by dHPLC, the elution buffer contains about 12.5% of acetonitrile, which is a toxic substance. Usually, mRNA product is washed three times with water to fully eliminate the residual solvent. Despite this accurate washing procedure was applied, we decided to test the organic solvent residues possibly present in mRNA samples to make sure that the product was save for future clinical applications. Eurofins Biolab subcontractor provided acetonitrile quantification on the three serial washes at their GMP Facility (Milan, Italy). The subcontractor

applied a customized optimization of the headspace gas chromatography with flame-ionization detection (HS-GC/FID) analytical method of the §2.4.24 of the European Pharmacopoeia 8.0.
According to the Pharmacopoeia 8.0, the permitted daily exposure (PDE) for acetonitrile is 410 parts per million (ppm) per day = 4.1 mg/day (infusion). If we assume to treat an adult patient of 70 kg with 7×10^8 cells (max cell dose = 1×10^7 cells/kg) nucleofected with 12 mg of purified mRNA in a single infusion, acetonitrile concentration limit would be 341 ppm/infusion. As reported in Table ***3.3.1.2.3***, the acetonitrile quantification in the tested samples is 33 ppm for a 6X preparation. Assuming that 12 mg of purified mRNA derives from 60 6X reactions (mean value of one 6X is about 200 µg see Table ***3.3.1.2.1***) the final amount would be 0.51 mg/day (infusion), a value way below the permitted limit.

3.3.2.3 Production and purification of research- and GMP-grade plasmids and IL2RG IDLV in medium-large scale

Based on the modifications described in the Amendment 1 to GA 601958 – Project "SUPERSIST", MolMed was responsible of the production of high-grade reagents, which include: a) the production of two GMP-grade plasmid stocks in place of the originally planned research grade plasmids described in the original Task 3 of WP3. These reagents were necessary in preparation of a future clinical trial to support the production of the GMP grade vector stocks as required for filing an IMPD. To this aim, we selected Eurogentec S.A. (EGT, Liège, BE) that provided the best quotation among three CMOs contacted. b) The production of one research-grade and two GMP-grade large-scale IDLV-IL2RG vector lots.
Large-scale production of the IDLV IL2RG by transient transfection in cell factories and vector purification were performed following the Master Batch record LVV/SUP/01 rev14 developed in MolMed for clinical grade LV production. Seven large-scale lots were produced from M18 to M42 as follows:

- n=3 lots at half-scale (6 10-tray CF) by NTC (New Technologies Unit), using research-grade plasmids
- n=1 lot at full-scale (12 10-tray CF) by PDE (Process and Development Unit), using research-grade plasmids
- n=2 lots at full-scale by PRU (Production Unit), using GMP-grade plasmids

Before proceeding with the GMP productions, PDE unit performed a preliminary engineering run at full scale (12 10-trays CF; 24 L bulk viral vector) in the presence of 1 mM Na Butyrate using research-grade plasmids (NTC/Plasmid Factory). Vector purification was performed with MolMed standard downstream purification process, consisting in sequential DEAE chromatography, tangential flow filtration (TFF), gel filtration and a final sterilizing filtration.
In this contest, MolMed optimized the Real-Time qPCR method for the titration of IDLV vector initially designed by USR partner. The optimization included the amplification of the standard curve to determine the number of cells (human telomerase system) with the Sybr Green technology. Standard curves and positive control of the Real-Time qPCR were obtained by the genomic DNA extracted from cell clones of CEM A3.01 cells transduced with a SIN LVV vector containing the Green Fluorescent Protein (GFP) gene and characterized for the number of copies of provirus integrated. CEM A3.01 clone #41 was used as control for PCR standard curve construction (1 copy integrated), while CEM A3.01 clone#37 was chosen as PCR positive control (5 copies integrated). The assay is specific and present a total variability of 100 ± 42 % including the evaluation of accuracy of qPCR, repeatability and intermediate precision. The qPCR quantification ranges are the following: $25 \leq X \leq 3000$ copies for IDLV qPCR. $50 \leq X \leq 6000$ copies for hTERT qPCR. In these ranges of quantification, a vector that presents an infectious viral titer between 2.7E+05 TU/ml and 3.3E+10 TU/ml can be quantified with precision and accuracy, analyzing dilutions between not diluted and 4^{-9} dilution. Vector with higher titer, can be analysed with further 1:4 dilution. The lower limit of detection for IDLV system is 35.6 Ct while the lower limit of detection for hTERT system is 34.9 Ct. The result of the method are the number of IDLV unit/ml, expressed as TU/ml.

3.3.2.4 Nucleofection of T-lymphocytes with ZFN mRNAs

In contrast to what initially planned, MolMed did not develop the large-scale nucleofection process by using the MaxCyte instrument, but a large-scale beta-test nucleofector provided by Lonza. MolMed

received the Lonza 4D-Nucleofector™ β-test LV Unit system on 12/01/2016 after signing a loan agreement establishing the commitment of MolMed to evaluate the performance of this device on human T and $CD34^+$ cells.
First, ZFN activity was tested by transient transfection of specific ZFN mRNAs in the T lymphoblastic leukemia CEM A3.01 cells and primary T lymphocytes obtained from normal healthy donors. Primary T lymphocytes were activated with Dynabeads® Human T-Activator CD3/CD28, and cultured with low doses of IL7 and IL15 (5 ng/ml each). Two days after activation, GFP- or ZFN-coding mRNAs were nucleofected into T cells. The optimal mRNA concentration during nucleofection previously established by USR corresponded to 12.5 µg/ml. To establish the optimal amount of mRNA to use in medium-large scale experiments, we tested two parameters: constant mRNA concentration (12.5 µg/ml) and constant mRNA/cell ratio (0.25 µg/1×10^6 cells). We handle up to 1 × 10^7 cells of both CEM A3.01 and primary T cells with the GFP-coding mRNA. We obtained either higher percentage of GFP^+ cells (CEM A3.01), or similar percentage of GFP^+ cells and higher MFI (primary T cells) by keeping constant the mRNA/cell ratio. Both CEM A3.01 and primary T cells tolerate very well nucleofection. To evaluate whether nucleofection efficiency and toxicity was different between DNA and mRNA, we nucleofected 1 × 10^7 primary T-cells with GFP-coding DNA plasmid (pmaxGFP™ Control Vector, provided by Lonza) and GFP-coding purified mRNA (lot#12 GFP S023). The results reported in Figure ***3.3.1.4.1*** show that mRNA is expressed more efficiently and its toxicity is lower respect to that of DNA. As expected, mRNA stability overtime (after 120 hours) is lower as compared to that of plasmid DNA because the drop of $GFP^+/CD3^+$ cells from 48 hours to 120 hours is in proportion greater (from 93.8 to 56.7 vs from 78.1 to 65.2). Finally, we scaled-up nucleofection to large scale (1 × 10^8 T cells) using ZFN-coding mRNA (production #13 IL2RG Intron1 ZFNs S028/S029). ZFN activity was evaluated by measuring the Non-Homologous End Joining (NHEJ)-induced mutations at the expected nuclease target sites using a mismatch selective endonuclease assay (Cel-I assay). The ZFN performance was consistently higher in the large-scale sample compared to the medium-scale one (1 × 10^7 cells), while no toxicity was observed in both conditions.

3.3.2.5 Development of large-scale $CD34^+$ IL2RG Intron1 gene-targeting

To maximize gene targeting and minimize toxicity after transduction of IL2RG IDLV and nucleofection of ZFN mRNA, we performed two small-scale experiments on bone marrow derived $CD34^+$ cells provided by Lonza. As $CD34^+$ cells are very expensive, to reach the number of cells needed to perform these preliminary experiments, cells were expanded for 9 days before starting gene targeting processes (IDLV transduction followed by ZFN mRNA nucleofection). Although we were aware that the expansion protocol leads to a more differentiated cell population, we decided to proceed to limit the cost of the initial experiments.
For each small-scale experiment (5 × 10^5 cells), we evaluated the following parameters: i) cellular phenotype and viability at different time points (post-thawing, pre-transduction, 24 hours post-transduction, 24 and 120 hours post-nucleofection) by FACS analysis; ii) ZFN activity and iii) gene-targeting efficiency. Representative results of viability and phenotype post-transduction from one experiment indicate that IDLV transduction did not affect cellular viability or phenotype.

Task 3 - One lot of gene-modified cells prepared at full scale for the proposed gene therapy application

To prepare one lot of gene-modified cells at full scale for HSC therapy of SCID-X1, USR provided us with 3.5 × 10^7 cord blood-derived $CD34^+$ frozen cells purchased from Lonza. Cells were stimulated at the concentration of 1 × 10^6 cells/ml in CellGro medium supplemented with penicillin, streptomycin and human early-acting cytokines (stem cell factor, SCF 100 ng/ml, Flt3 ligand, Flt3-L, 100 ng/ml, thrombopoietin, TPO 20 ng/ml and interleukin 6, IL-6 20 ng/ml, CellGenix) for 48 hours in Vuelife Cell Culture Bags (CellGenix). StemRegenin-1 (SR-1, Stem Cell Technologies) and UM171 (Stem Cell Technologies) were supplemented to the culture medium. Cells were transduced by 1 hit of IL2RG Intron 1 Codon Optimized IDLV (PDE lot#B16067) at multiplicity of infection (MOI) 100, or with the IL2RG Intron 1 WT IDLV (NTC lot #4) at MOI 30 following the Master Batch record LVV/TRA/02 rev11, developed in MolMed for clinical $CD34^+$ lentiviral vector transduction. Few modifications were implemented to adapt the transduction protocol to the current developing process. The following day, cells were electroporated with 140 µg/10^6 cells ZFNs encoding mRNA (mRNA

lot#21 and #22) with the 4D-NucleofectorTM β-test LV Unit system (P3 primary cell nucleofector kit, program EO 100, Lonza). Twenty-four hours post-nucleofection, the cells were collected and counted. Unexpectedly, cell recovery was very low (<3%) and the number of alive cells was not sufficient for the planned GLP study. The transduction/nucleofection process includes many different steps that need precise optimization. This unexpected failure prompted us to reconsider testing some parameters that might have influenced cellular response to the process and caused cell death post-nucleofection. The possible parameter might be: 1) high MOI during transduction; 2) high cellular concentration during nucleofection (3×10^7 cells/ml); 3) high RNA concentration during nucleofection (4,200 µg/ml); 4) mRNA viscosity, secondary to speed-vac concentration carried out to keep the mRNA volume <15% of nucleofection volume, as recommended by nucleofector's manufacturer. It is likely that the high mortality observed in this test is due to the combination of some or all the above.

Task 4 - Perform preclinical GLP studies of toxicity and biodistribution of gene corrected HSPC in NSG mice

As explained in the Amendment 1 of the project (M32), some logistic changes recently occurred as, for example, the fact that in 2014 the Coordinator established a GLP Test Facility which was then certified by the Italian Ministry of Health to perform toxicity and bio-distribution studies of cell and gene therapy products according to GLP OECD principles. This makes possible to perform the preclinical studies of engraftment, toxicity and biodistribution of the genetically edited human CD34+ cells in the newly certified facility of the Coordinator (USR). After the unexpected failure of Task 3 reported by MOLMED, USR better tailored the parameters of the gene targeting protocols by systematically assessing the optimal cell concentration and ZFNs mRNA dose at small scale, maximizing gene targeting efficiency while alleviating cellular toxicity. The best performing condition was then used to produce a large-scale lot of gene-modified HSPC cells (**Deliverable 3.6**), which was then used for the preclinical bio-distribution study. Considering the intrinsic explorative nature of these studies, USR performed within the GLP Test Facility a pilot study in GLP-like conditions in which scalability and choice of donor template delivery were the two parameters of the HSPC gene editing protocol compared for efficiency of editing and levels of engraftment of the edited cells in NSG mice. In agreement with the GLP Test Facility Management, a study plan was produced before the beginning of the experimental activity and all the study was conducted following principles of data integrity (MHRA GxP Data Integrity Definitions and Guidance for Industry) and OECD Good Laboratory Practice Principles. Briefly, the purpose of this study was to monitor the biodistribution of human HSPCs treated for *IL2RG* gene editing and of their progeny following transplantation into NSG mice after sub-lethal conditioning, in comparison with un-transduced, equally manipulated HSPCs. For this study, human umbilical cord blood (UCB)-derived CD34+ cells, edited at large scale or small scale with AAV6 as donor template were used as Test Items, and compared to a Reference Item (small scale treatment with IDLV donor template) and an untransduced Control Item. Main objectives of the study were: i) to determine that after the treatment, human CD34+ cells engraft, differentiate into mature progeny and distribute to lymphohematopoietic murine organs similarly to control un-transduced, equally manipulated CD34+ cells; ii) to compare efficiency and tolerability of the treatment performed at small scale or large scale; iii) to compare efficiency and tolerability of the treatment performed with an IDLV or an AAV6 as donor template vector. The treated mice will be monitored for survival, clinical signs and body weight weekly up to 20 weeks after transplantation. At the end of the study, the engraftment, the differentiation to specific lineages and the persistence of edited human cells in hematopoietic organs (blood, bone marrow, spleen, thymus) will be assessed. By performing molecular analyses on the fraction of treated cells not transplanted, we found that AAV6 vector outperforms IDLV for delivering the HDR template, reaching 30% vs. 15% targeted integration in bulk HSPC cultures, respectively (**Deliverable 3.7**). After xenotransplantation, HSPC edited with IDLV or AAV6 donors have sustained normal haematopoiesis contributing to a mean of 5% or 10%, respectively, of the total human graft in all the transplanted NSG mice until the latest time of analysis (Schiroli et al., manuscript in preparation). These findings gratifyingly confirm at large scale the results of the editing protocol optimization performed under WP1 above.

1.1.4 Potential impact, main dissemination activities and exploitation of results

1.1.4.1 Potential impact

HSC gene therapy and cancer adoptive immunotherapy are facing an accelerated phase of development, largely due to the first therapeutic successes obtained with genetically modified cells in clinical trials of inherited diseases and lymphoid malignancies. We can envisage that adoptive T cell therapy will soon become a major pillar in cancer treatment. The outcome of SUPERSIST is paradigmatic of the next generation of HSC- and T cell-based gene therapy, in which conventional gene addition is replaced by the more precise and powerful approach of targeted gene editing, achieved through a combination of state of the art gene transfer vectors and artificial nucleases. While two diseases have been selected for investigation within the project for their paradigmatic features, the strategies developed are expected to be potentially applicable to several additional human diseases. Thus, the outcome of SUPERSIST should benefit the EU population by providing a long-sought perspective of effective treatment for a number of severe diseases, leading to improvement in population health and reduction in health care costs, while at the same time increasing the competitiveness of EU biomedical and pharmaceutical industry through the generation of new scientific knowledge, processes and technologies.

1.1.4.2 Main dissemination activities

During the project, all the partners contributed to perform different activities aimed to disseminate SUPERSIST activities and results to the scientific community as well as to external actors potentially interested to the field such as pharmaceutical industries or patients' associations.

The dissemination activities performed in line with the tasks of WP4 were the following:

- project website for external communication, with publications intended for specialist and non-specialist audiences;
- presentation of the most important results obtained at relevant international conferences and workshops such as the annual meetings of the European Society of Gene and Cell Therapy, the European Society of Blood and Marrow Transplantation, the American Society for Gene and Cell Therapy and the American Society of Hematology;
- publication by each partner of their research results in international, peer-reviewed journals;
- sponsorship by SUPERSIST of international conferences dedicated to cell and gene therapy, including the sponsorship of scientific sessions at the ESGCT Annual Conferences and of an European workshop in the last year of the project;
- active engagement through publications in scientific journals, public debates held at conference venues, interviews and articles on lay journals on the medical and societal perspectives, and ethical issues raised by genome editing.

The Consortium created a website dedicated to the SUPERSIST project that can be found at the following address: www.supersist-project.eu.

As part of the dissemination and promotion of SUPERSIST results, Consortium partners have achieved a significant number of scientific publications in relevant magazines and a large number of presentations at scientific conferences and workshops, and also a large number of lectures were given. These materials or the publication references can be found on the project website.

1.1.4.3 Exploitation of results

The main exploitation actions undertaken by SUPERSIST partners have consisted in filing International patent application:

- USR, as part of its achievements in WP1, has filed an International Patent application on delivery methods and compositions for nuclease-mediated genome engineering in hematopoietic stem cells (PCT/US2014/060918 filed on October 16, 2014; priority: US provisional dated October 17, 2013). The patented technology can be applied to the clinical development of the gene editing approach for the treatment of X-SCID-1 and several additional disease conditions.
- MOLMED, as part of its achievements in WP3, has developed new mRNA encoding ZFN purification protocols and a large scale manufacturing process that includes as main steps nucleofection of mRNA and transduction of cells with IDLV.
  - The protocols, the list of relevant materials, devices and the technological knowledge developed in respect to the manufacturing methods and including the mRNA purification and nucleofection was protected as know-how and trade secret.
  - In addition, manufacturing and release of the following GMP material was performed: 1) The packaging plasmid; 2) The transfer vector encoding the corrective IL2RG gene; 3) Two batches of GMP grade IDLV carrying the corrective IL2RG for future use in clinical applications.

1.1.5 Address of the project public website and relevant contact details

The address of the project public website is: **www.supersist-project.eu**

Project logo:

SUPERSIST

Consortium contacts:

Partner	Location	Contact person	Email
Universita Vita-Salute San Raffaele	Milan, Italy	Prof Luigi Naldini	naldini.luigi@hsr.it
Stichting Het Nederlands Kanker Instituut	Amsterdam, Netherlands	Prof Ton Schumacher	t.schumacher@nki.nl
Deutsches Krebsforschungszentrum	Heidelberg, Germany	Dr Manfred Schmidt	Manfred.schmidt@nct-heidelberg.de
University College London	London, UK	Prof Adrian Thrasher	a.thrasher@ucl.ac.uk
Molecular Medicine Spa	Milan, Italy	Dr Chiara Boloventa	Chiara.Bovolenta@molmed.com

1.2 Use and dissemination of foreground

The material below is made available on-line on ECAS participant portal.

List of scientific publications

Articles	
Partner	**References**
USR	Gattinoni L, Speiser DE, Lichterfeld M, Bonini C. **T memory stem cells in health and disease.** Nat Med. 2017 Jan 6;23(1):18-27. doi: 10.1038/nm.4241. Cartier-Lacave N, Ali R, Ylä-Herttuala S, Kato K, Baetschi B, Lovell-Badge R, Naldini L, Thrasher A. **Debate on Germline Gene Editing.** Hum Gene Ther Methods. 2016 Aug;27(4):135-42. doi: 10.1089/hgtb.2016.28999.deb. Cossu G, Previtali SC, Napolitano S, Cicalese MP, Tedesco FS, Nicastro F, Noviello M, Roostalu U, Natali Sora MG, Scarlato M, De Pellegrin M, Godi C, Giuliani S, Ciotti F, Tonlorenzi R, Lorenzetti I, Rivellini C, Benedetti S, Gatti R, Marktel S, Mazzi B, Tettamanti A, Ragazzi M, Imro MA, Marano G, Ambrosi A, Fiori R, Sormani MP, Bonini C, Venturini M, Politi LS, Torrente Y, Ciceri F. **Intra-arterial transplantation of HLA-matched donor mesoangioblasts in Duchenne muscular dystrophy.** EMBO Mol Med. 2016 Dec 1;8(12):1470-1471. doi: 10.15252/emmm.201607129. Norelli M, Casucci M, Bonini C, Bondanza A. **Clinical pharmacology of CAR-T cells: Linking cellular pharmacodynamics to pharmacokinetics and antitumor effects.** Biochim Biophys Acta. 2016 Jan;1865(1):90-100. Naldini L. **Gene therapy returns to centre stage.** Nature. 2015 Oct 15;526(7573):351-60. doi: 10.1038/nature15818. Bonini C, Mondino A. **Adoptive T-cell therapy for cancer: The era of engineered T cells.** Eur J Immunol. 2015 Sep;45(9):2457-69. doi: 10.1002/eji.201545552. Bosley KS, Botchan M, Bredenoord AL, Carroll D, Charo RA, Charpentier E, Cohen R, Corn J, Doudna J, Feng G, Greely HT, Isasi R, Ji W, Kim JS, Knoppers B, Lanphier E, Li J, Lovell-Badge R, Martin GS, Moreno J, Naldini L, Pera M, Perry AC, Venter JC, Zhang F, Zhou Q. **CRISPR germline engineering--the community speaks.** Nat Biotechnol. 2015 May;33(5):478-86. Oliveira G, Ruggiero E, Stanghellini MT, Cieri N, D'Agostino M, Fronza R, Lulay C, Dionisio F, Mastaglio S, Greco R, Peccatori J, Aiuti A, Ambrosi A, Biasco L, Bondanza A, Lambiase A, Traversari C, Vago L, von Kalle C, Schmidt M, Bordignon C, Ciceri F, Bonini C. **Tracking genetically engineered lymphocytes long-term reveals the dynamics of T cell immunological memory.** Sci Transl Med. 2015 Dec 9;7(317):317ra198. Greco R, Oliveira G, Stanghellini MT, Vago L, Bondanza A, Peccatori J, Cieri N, Marktel S, Mastaglio S, Bordignon C, Bonini C, Ciceri F. **Improving the safety of cell therapy with the TK-suicide gene.** Front Pharmacol. 2015 May 5;6:95. doi: 10.3389/fphar.2015.00095. Cieri N, Oliveira G, Greco R, Forcato M, Taccioli C, Cianciotti B, Valtolina V, Noviello M, Vago L,

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DKFZ	Knipping F, Petri K, Osborn MJ, Tolar J, Glimm H, von Kalle C, Schmidt M, Gabriel R. **Genome-wide specificity of highly efficient TALEN and CRISPR/Cas9 for T cell receptor modification.** Submitted to Molecular Therapy Nucleic Acids Ruggiero E, Nicolay JP, Fronza R, Arens A, Paruzynski A, Nowrouzi A, Ürenden G, Lulay C, Schneider S, Goerdt S, Glimm H, Krammer PH, Schmidt M, von Kalle C. **High-resolution analysis of the human T-cell receptor repertoire.** Nat Commun. 2015 Sep 1;6:8081. Gabriel R, von Kalle C, Schmidt M. **Mapping the precision of genome editing.** Nat Biotechnol. 2015 Feb;33(2):150-2. Gabriel R, Kutschera I, Bartholomae CC, von Kalle C, Schmidt M. **Linear amplification mediated PCR--localization of genetic elements and characterization of unknown flanking DNA.** J Vis Exp. 2014 Jun 25;(88):e51543
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Posters	
USR	• ESGCT / ISSCR / ABCD COLLABORATIVE CONGRESS - October 2016, Florence, Italy • ASGCT 18th Annual Meeting (May 2015, New Orleans, USA
DKFZ	• Genome Editing for Gene and Cell Therapy Symposium, Hannover, Germany, 11/2016 • Annual Meeting of ESGCT and ISSCR, Florence, Italy, 10/2016 • 19th Annual Meeting of ASGCT, Washington, DC, USA, 5/2016 • Annual Meeting of ESGCT and FSGT, Helsinki, Finland, 9/2015 • 18th Annual Meeting of ASGCT, New Orleans, USA, 5/2015 • Annual Meeting of ESGCT and NVGCT, The Hague, Netherlands, 10/2014 • 17th Annual Meeting of ASGCT, Washington, DC, USA, 5/2014 • XX Annual Meeting of DG-GT, Ulm, Germany, 3/2014 • Annual Meeting of ESGCT and SETGyC, Madrid, Spain, 10/2013 ASGCT 17th Annual Meeting (May 2014, Washington D.C.): • Disrupting the Endogenous TCR expression by TALEN and RNA-Guided Nucleases • Discovering the Human T Cell Receptor Repertoire BY Deep Sequencing
MolMed	ASGCT 19th Annual Meeting Washington DC, 4-7 May 2016, Giuliani E. et al. "**Purification of Large Scale mRNA Encoding ZFN Nucleases by dHPLC Technology**" ESGCT/ISSCR/ABCD Collaborative Congress, Florence, 18-21 October 2016, Giuliani E. et al., " **Production, purification and nucleofection of large scale mRNA encoding ZFN nucleases into human primary T cells and CD34+ cells**"
Lectures	
USR	**AAAS 2016 Annual Meeting**, Washington, USA, 11-15/02/2016 "TCR Gene Editing to Treat Hematological Malignancies", Bonini C. **Young Investigator Award Lecture at the ESGCT / ISSCR / ABCD COLLABORATIVE CONGRESS** - October 2016, Florence, Italy • Towards clinical translation of gene editing technologies for empowering adoptive immunotherapy or correcting inherited mutations **Presentations at the ESGCT / ISSCR / ABCD COLLABORATIVE CONGRESS** - Oct 2016, Florence, Italy • Advanced genetic engineering of hematopoiesis to treat human diseases • TCR gene edited memory stem T cells for cancer immunotherapy • Multiple inhibitory receptors are expressed on central memory and memory stem T cells infiltrating the bone marrow of AML patients relapsing after allo-HSCT Invited Lecture: **Future Medicine / Innovation in Health Sciences** – Nov. 2016, Berlin, Germany. **BIOGEN IDEC**, Boston, USA, 13/10/16 Seminar "Advancing applications of hematopoietic stem cells and liver-directed gene therapy by

lentiviral gene transfer and targeted gene editing "

Georgetown University Italian Research Institute, Washington, USA, 14/10/16
Lecture "Turning Foes into Friends: Exploiting HIV for the Gene Therapy of Inherited Diseases and Cancer"

ESGCT-ISSCR Joint Meeting Florence 2016 October 18/21 :
Lecture: Advanced genetic engineering of hematopoieisis to treat human diseases.

The **58th Annual Meeting of the Japanese Society for Inherited Metabolic Dise**ases, 27-29 October 2016, Tokyo, Japan
Special Lecture "Genetic Engineering of Human Hematopoiesis for the Treatment of Inherited Diseases and Cancer"

Invited Lecture: **5th International Conference of the Cyprus Society of Human Genetics** – October 2016, Cyprus.

Highlights From EHA – Firenze 17/09/16
Lettura Speciale "Terapia genica con cellule staminali ematopoietiche: dal gene transfer all'editing genico mirato"

Invited Lecture: **1st Annual Symposium on Cell and Gene Therapy** - Christian Medical College Campus, August 2016, Vellore, India.

ASH Workshop on Genome Editing (Washington DC), July 14-15, 2016
"Gene Editing of HSC for the therapy of inherited diseases"

Invited Lecture: **Genome Editing Science Club - Giannina Gaslini Institute**, June 2016, Genoa, Italy.

Festival Della Scienza Medica. Le Eta' Della Vita - 19-22 Maggio 2016
Tavola Rotonda su "il futuro delle biotecnologie: opportunità di Innovazione e crescita sostenibile"

Retreat TIGEM-DTI , 26-28 Maggio 2016, Roma

FASEB Science Research Conference Genome Engineering – Cutting-Edge Research and Applications, Lisbon, Portugal, June 05-10, 2016
"HSC-mediated gene therapy of inherited diseases: from gene replacement to gene editing"

European Hematology Conference: Copenhagen, Denmark, June 09-12 2016
Targeted genome editing in hematopoietic stem cells

SIBBM 2016, June 17 2015, Naples, Italy
"Genetic Engineering of Hematopoiesis to treat Inherited Diseases and Cancer"

Invited Lecture: **Annual Meeting of the French society of cellular and gene therapy (SFTCG)** - March 2016, Marseilles, France.

1st Symposium of the Vienna Center for Rare and Undiagnosed Diseases, which will take place 19-20 February 2016 in Vienna
"Genetic Engineering of Hematopoiesis to treat inherited diseases and cancer"

Federation of European Academies of Medicine "European workshop on Human Genome Editing: Opportunities and Challenges for Europe", 28 April 2016

Chair in the Session 3: Clinical research and applications in human somatic cells–current state, opportunities, and regulation

ASGCT 19th Annual Meeting
"HSC-based gene therapy of inherited diseases and cancer"

XLVIII Jiménez Díaz Memorial Lecture. Madrid, 17 May 2016
"Turning Foes into Friends: Exploiting HIV for the Gene Therapy of Inherited Diseases and Cancer"

ENCALS Meeting (network of ALS centres in Europe): Milan, Italy , May 19th, 2016
Opening lecture: "La Terapia Genica con cellule Staminali Ematopoietiche: dal bancone di laboratorio al letto del paziente"

Invited Lecture: **18th Annual Meeting of the American Society of Gene and Cell Therapy (ASGCT)** - May 2015, New Orleans, USA.

Presentations at the ASGCT 19th Annual Meeting (May 2016, Washington D.C., USA):

- Correction of SCID-X1 by Targeted Genome Editing of Hematopoietic Stem/Progenitor Cells (HSPC) in the Mouse Model
- Towards Clinical Translation of Hematopoietic Stem Cell Gene Editing for the Correction of SCID-X1 Mutations

Hearing at the Council of Europe_Secretariat of the Committee on Social Affairs, Health and Sustainable Development of the Parliamentary Assembly of the Council of Europe: Thursday 1st of October 2015, the European Palace in Strasbourg.
"Manufacturing the new human species".

2015 Biomedical Symposium on Stem Cell Biology, St. Jude Children's Research Hospital October 23, 2015
"Genetic engineering of hematopoiesis to treat inherited disease and cancer"

Workshop organized by the ESPGHAN (European Society for Paediatric Gastroenterology Hepatology and Nutrition) and Genethon entitled: Targeting liver disease at DNA level-Novel Diagnostic Tools and Gene Transfer Technologies in Paediatric Hepatology. Venice, Italy the 29-30 October 2015.
"Lentiviral gene transfer to the liver"

EMBO/EMBL Science & Society Conference, Heidelberg
"Blood Stem Cell Gene Therapy to fight inherited diseases and cancer"

The **EBMT Cellular Therapy and Immunobiology Symposium** From Transplantation to Gene Therapy: cellular therapy in evolution, San Raffaele Scientific Institute, 11-13 November 2015
"Lentiviral Vectors and Nucleases"

ASH 2015. Presidential Symposium:
"Gene Replacement and Targeted Genome Editing in Hematopoietic Stem Cells for the Treatment of Human Diseases"

Presentations at the ESGCT 22th Annual Meeting (October 2015, Helsinki, Finland):

- Targeted Genome Editing in Human Long-Term Repopulating Hematopoietic Stem Cells for the Correction of SCID-X1
- Targeted Genome Editing in Mouse Hematopoietic Stem/Progenitor Cells (HSPC) to model Gene Correction of SCID-X1

Presentations at the ASGCT 18th Annual Meeting (May 2015, New Orleans, USA):

- Targeted Genome Editing in Mouse Hematopoietic Stem/Progenitor Cells (HSPC) to model Gene Correction of SCID-X1

Keynote Lecture at the EMBO Conference on "Cell therapy today: achievements, hopes and hypes". Manchester 09th and 10th September 2015
"Genetic engineering of hematopoietic stem cells for the treatment of inherited diseases and cancer"

The **Annual Meeting of the European Society of Gene and Cell Therapy (ESGCT)**, which this year is organized in collaboration with the Finnish Society of Gene Therapy (FSGT) to take place September 17-20, 2015 in Helsinki, Finland.
"Gene transfer by lentiviral vectors and gene editing from bench to bedside"

Keynote Lecture at the 13th European Congress of Toxicological Pathology in collaboration with the British Society of Toxicological Pathology (UK) , University of Surrey in Guildford, United Kingdom, September 22nd to 25th , 2015
Gene Therapy Overview.

"Doctor Honoris Causa - Distinguished Lecture" at the Faculty of Medicine and Pharmacy, The Vrije Universiteit Brussel, 28 May 2015
"Genetic engineering of human hematopoietic stem cells for the treatment of inherited diseases and cancer"

Lettura magistrale presso la Scuola Superiore Sant'Anna nell'ambito del ciclo seminariale dal titolo "Orizzonti in Biologia e Medicina" Venerdì 05 Giugno 2015
"La terapia genica con cellule staminali ematopoietiche: dal laboratorio al letto del paziente".

Lecture at the Venetian Institute of Molecular Medicine - June 9th, 2015
Genetic Engineering of Human Hematopoiesis for Treating Inherited Diseases and Cancer"

"Giornata dei Giovani Ricercatori", Policlinico San Donato, 16 giugno 2015 - La terapia genica con cellule staminali.

Annual Meeting for the International Society for Stem Cell Research, Stockholm, June 24-27 2015

The **3rd International Michelangelo Conference** on "Promises and challenges of developing new drugs in oncology", Milan, Italy 2nd and 3rd July 2015 at the Museo della Scienza e della Tecnologia,
Engineered cells for cancer therapy.

8th Stem Cell Clonality and Genome Stability Retreat, New Orleans, USA

Lettura Accademia Medica Roma, 22 Gennaio 2015
" La Terapia Genica con Cellule Staminali Ematopoietiche: dal laboratorio al letto del paziente "

Gordon Conference: Lysosomal Diseases, 03/15/2015 - 03/20/2015, Location: Hotel Galvez in Galveston TX United States

TEDxRoma, evento della grande community TED, 21 marzo 2015 al Teatro Olimpico, Roma
"HIV, un cavallo di Troia per la terapia genica"

Symposium on "Novel therapies for monogenic diseases" at the Collège de France in Paris on April 16th -17th, 2015.

Key note lecture - Meeting of the Austrian Society of Hematology and Medical Oncology - 23rd

April 2015
"Targeted Genome Editing - Possibilities of Clinical Application"

International Sympsium on Hematopoietic Stem Cells (Tubingen); April 22nd to April 25th 2015; "Targeted genome engineering of HSC for treating genetic diseases and cancer"

First CRG course in "Somatic Cell Reprogramming"; 7-12th November 2014, Barcelona.
"Genetic Engineering of Hematopoietic Stem Cells for treating Human Disease".

Premio Agostinelli (Accademia delle Scienze Torino); 10 Novembre 2014, Torino.

IV Edition of the International Conference "Bioeconomy Rome 2014", 13 November 2014.

ETH Zurich/D-BSSE departmental seminar, 25 November 2014, Zurich, CH.

Presentations at the ASGCT 17th Annual Meeting (May 2014, Washington D.C.):
- Single Edited T Cells Redirected Towards NY-ESO-1 Ensure Tumor Rejection Without Inducing Xenogeneic GvHD
- Site-Specific Genome Editing in Human Long-Term Repopulating Hematopoietic Stem Cells for Correction of SCID-X1

Outstanding Achievement Award Lecture at the ASGCT 17th Annual Meeting (May 2014, Washington D.C.):
- Lentiviral Vectors from Bench to Bedside: A Lifetime Journey

Invited Lecture: "Use of genetically modified T lymphocytes in HSCT." **7th International Symposium Haploidentical Stem Cell Transplantation**, Weizmann Institute of Science, Israel, Feb 9-10, 2014.

Invited Lecture: "Memory stem T cells." **GvH/GvL Symposium**, Regensburg, Germany, March 26-28, 2014.

Invited Lecture: "Use of genetically modified Lymphocytes in allogeneic stem cell transplantation" **EBMT Annual Meeting** Milano March 31- April 2, 2014.

Invited Lecture: "Editing T cell Specificity Towards Leukemia by Zinc Finger Nucleases and Lentiviral Gene Transfer." Educational Program. **ASGCT Annual Meeting**, Washington DC. May 20-24th 2014.

Presentations at the ASGCT 16th Annual Meeting (May 2013, Salt Lake City):
- TCR Gene Editing to Treat Acute Leukemia and Multiple Myeloma
- Efficient Site-Specific Integration and In Situ Gene Correction of Human Long-Term Repopulating Hematopoietic Stem Cells

Keynote Lecture at FASEB Meeting: Genome Engineering: Cutting-Edge Research and Application (June 2013; Nassau, Bahamas):
- Targeted Genome Editing in Human Repopulating Hematopoietic Stem Cells

Invited Lecture: Immunotherapy of Leukemia with TCR transduced T cells. **EBMT Immunobiology Working part Educational Event. Perugia**, IT September 2013.

Invited Lecture: CIK or central memory cells for adoptive immunotherapy? **The International Congress on Controversies in Stem Cell Transplantation and Cellular Therapies (COSTEM)** Berlin, Germany, October 10-13, 2013.

	Invited Lecture: Bases of Gene Therapy in Leukemias. **ESGCT Annual Meeting**. Madrid October 25-28 2013. Invited Lecture: TCR gene editing for the treatment of hematological malignancies. **ESGCT Annual Meeting**. Madrid October 25-28 2013. Invited Lecture: TCR editing, suicide gene e CARs come principali strategie di terapia genica in campo oncologico. **Immunoterapia Antitumorale e Terapia Genica: Regole e Sperimentazione,** Milano, IT 4 Novembre 2013. Invited Lecture: "Ex Vivo T-Cell depletion". **Scientific Symposium of the Acute Leukemia Working Party (ALWP)** European Group for Blood and Marrow Transplantation (EBMT). Marseille, Nov 22-23, 2013.
NKI	Invited lecture: **CRUK Clinical Research Centre Southampton**, February 12, 2014, Southampton, UK. What T cells see on human cancer? Invited lecture: **CIML** seminar series, March 12, 2014, Marseille, FR. Neo-antigen specific T cell reactivity in cancer immunotherapy. Invited lecture: **San Raffaele Institute**, March 18, 2014, Milan, IT. What T cells see on human cancer? Invited lecture: **MSKCC Immunology series** March 23, 2014, New York, USA. What T cells see on human cancer? Invited lecture: **MD Anderson**, March 24, 2014, Houston, USA. What T cells see on human cancer Invited lecture: **Max Planck Institute Freiburg** seminar series, April 3, 2014, Freiburg, DE. Dissection of antigen specific T cell responses in mice and men Acceptance speech: **San Salvatore Award**, May 17, 2014, Lugano, CH. What T cells see on human cancer? Invited lectures: **Weizmann Institute Immunology series**, May 19-20, 2014, Rehovot, Israel. What T cells see on human cancer? Invited lecture: **Oslo Cancer Symposium**, June 10-11, 2014, Oslo, Norway. Neo-antigen specific T cell reactivity in cancer immunotherapy. Invited lecture: **Harvard Medical School** Immunology series, Sept 10, 2014, Boston USA. What T cells see on human cancer? Invited lecture: **MGH**, Sept 11, 2014, Cambridge, USA. What T cells see on human cancer? Invited lecture: **DC2014 Symposium**, Sept 15-17, 2014, Tours, Fr. What T cells see on human cancer? Keynote lecture: **ISGCT symposium**, Sept 25-26, 2014, Amsterdam, The Netherlands. What T cells see on human cancer?
DKFZ	Invited Lecture: **ESGCT and ISSCR Congress** (17 - 22 October 2016, Florence)

	Invited Lecture: **ASGCT 18th Annual Meeting** (13 – 16 May, New Orleans) Invited Lecture: **ESGCT and FSGT Congress** (17 - 20 September 2015, Helsinki) Invited Lecture: **Genome Editing Congress** (12 - 13 November 2015; London) - Genome-wide Analyses Of Gene Editing – On-and Off-target Modifications Invited Lecture: **5th International Conference on Retroviral Integration** (26 October 2014, Asilomar) - Integration of Retroviral Vectors in Gene Therapy - Understanding and Avoiding Severe Side Effects Invited Lecture: **NIH Genome Editing workshop** (10 June 2014, Washington D.C.): - Unbiased Capture Approaches to Identify Double Strand DNA Breaks Invited Lecture: **ASGCT 17th Annual Meeting** (21-24 May 2014, Washington D.C.) - Integrating Vectors, Avoiding Oncogenesis Oral Presentation: **ASGCT 17th Annual Meeting** (21-24 May 2014, Washington D.C.) Oral Presentation: **XXI Annual Meeting of DG-GT**, Vienna, Austria, 2/2015 Oral Presentation: **XXII Annual Meeting of DG-GT**, Heidelberg, Germany, 9/2016 Oral Presentation at the **ASGCT 17th Annual Meeting** (21-24 May, Washington D.C.) - Integrating Vectors, Avoiding Oncogenesis Oral Presentation at **NIH Genome Editing workshop** (10 June, Washington D.C.): - Unbiased Capture Approaches to Identify Double Strand DNA Breaks
UCL	**ISCT**, Paris, 23-26 April 2014. “Gene correction of immune and metabolic defects” **ASGCT**, Washington, 21st-24th May 2014 “Refining clinical trials for inherited immunodeficiencies” **Frontiers in Immunology** - from molecules to disease, Stockholm 12-14 June 2014 “Maturing Gene Therapy for Primary Immunodeficiency” **PIDI course**, Hong Kong, 20-21st June 2014 Roland Levinsky Memorial Lecture: “The path to effective gene therapies in inherited immunodeficiency” **ESGCT**, The Hague 23rd-26th October 2014 “Progress in gene therapy for PIDs” **Third International Conference on Primary Immunodeficiency Diseases**, Chennai, India, 21-23 February 2015 “Update on gene therapies and other stem cell modalities” **Modern Concepts in DNA – EMBO**, Evry, 30th March-3rd April 2015 1. “LV in gene therapy of hematopoietic stem cells diseases” 2. “Update on current and future clinical trials for gene therapy”

Rare Diseases Conference, Oxford, 6-7th April 2015
"Maturing gene therapy for immunodeficiencies"

NIHR Rare Diseases TRC for Immune and Blood Rare Diseases
"Inherited immune disorders – An introduction"

BSGCT, Glasgow, 9-11th June 2015
"Evolving Gene Therapy for Primary Immunodeficiency"

IIC PID Course, Oxford 29th June – 1st July 2015
"Gene Therapy for Immunodeficiency – Job Done"

GENYO, Granada 7-9th July 2015
"Evolving safety and efficacy of gene therapy for immunodeficiencies"

2nd Biotest Immunology Forum, Royal College of Physicians, London, 30th September 2015
"Gene Therapy for PID"

Eurofancolen/Advances of Gene Therapy in monogenic diseases affecting the hematopoietic system, 8th-9th February 2016
"Evolving gene therapy inprimary immunodeficiencies"

Centre for Immunodeficiency Winter School, Windsor, 7th -9th March 2016
"Gene therapy for PID: update"

ESGCT, DG-GT and DZIF Spring School, 20th-22nd April 2016
"Developing successful gene therapies in immunological disorders"

Regulatory Science Symposium, **NIBSC** 40th Annivesary, London, 18th May 2016
"Gene therapy in severe immunodeficiency"

Annual Immunology Symposium with Pears Lecture, London, 21 June 2016
"Updates on clinical gene therapy for immunological disease"

XXII Annual Meeting of the German Society of Gene Therapy (DG-GT), Heidelberg, 14-16 September 2016
"Evolving Gene Therapy for Primary Immunodeficiency

Hematopoiesis: From basic biology to clinical medicine, Lund, 22-23 September 2016
"Evolving gene therapy for primary immunodeficiency"

World Life Science Conference, Beijing, 31 October– 4 November 2016
"Evolving gene therapy for human primary immunodeficiency"

Sponsorship of congresses

With the aim of promoting the knowledge of the project in the scientific community, SUPERSIST sponsored the following congresses as listed below:

1) ESGCT and NVGCT Collaborative Congress: The Hague - 23 to 26 October 2014 as gold sponsor

2) ESGCT and FSGT Collaborative Congress, Helsinki 17 to 20 September 2015 as gold sponsor
3) The EBMT cellular therapy and immunobiology working party scientific symposium Milano, November 11th – 13th, 2015
4) ESGCT/ISSCR/ABCD Collaborative Congress Florence, 18 to 21 October 2016

Citations in the press, media briefings, interviews

In addition, a significant number of national press articles that are citing the work performed by USR within the SUPERSIST project have been reported since 2014:

- Un bisturi molecolare per correggere i geni malati – Wired, maggio 2014
- Un passo avanti per la terapia genica – Le Scienze, maggio 2014
- Corriere della Sera, 29 maggio 2014
- Bisturi molecolari correggono i geni delle staminali del sangue – Tgcom24, 29 maggio 2014
- Ricerca Telethon, scienziati Tiget 'correggono' Dna staminali sangue – ASCA, maggio 2014
- Stampa, maggio 2014
- Svolta italiana in terapia genica, corretto errore in Dna malato – Adnkronos, maggio 2014
- Telethon 140529 RS Naldini Nature, Corriere della sera, maggio 2014
- Al via l'era dell'editing genomico, maggio 2014
- Biotechniques – The International Journal for Life Science Method, june 2014
- Telethon 140610 RS Naldini Nature, La Discussione, 07
- Il Sole 24 Ore_Nova, dicembre 2014
- Science World Focus, dicembre 2014
- Riscrivere il Dna, per correggere i geni malati – Galileo, Giornale di Scienza, 2015
- Mondo Salute_Lombardia, settembre 2015
- Corriere della Sera_Genenta, novembre 2015
- Tutto Scienze e Tcnologia, La Stampa, dicembre 2015
- Avvenire E'Vita, dicembre 2015
- http://www.smh.com.au/national/health/extraordinary-results-in-blood-cancer-therapy-for-terminally-ill-patients-20160217-gmwi46.html
- http://www.theaustralian.com.au/news/latest-news/cells-may-protect%E2%80%A6ainst-cancer-for-life/news-story/0a890bcc911ec86af783f7da28e8f254
- http://www.mirror.co.uk/news/technology-science/science/doctors-battling-stop-cancer-hail-7377474
- http://english.aawsat.com/2016/02/article55347654/promising-cancer-cure-discovered
- http://www.irishtimes.com/news/world/us/extraordinary-94-success-rate-reported-in-cancer-trial-1.2536701
- Gazzetta di Mantova, 16 febbraio 2016, Leucemia: “costruite” le cellule killer contro il cancro
- http://www.admolombardia.org/intervista-a-chiara-bonini/
- http://www.askanews.it/top-10/cellule-modificate-killer-delle-leucemie-funzionano-come-un-vaccino_711736484.htm
- http://www.news.com.au/lifestyle/health/health-problems/scientists-hail-breakthrough-in-cancer-research-using-tcells/news-story/4785f6911fa0ce40c5e311b59c859740
- http://www.nzherald.co.nz/lifestyle/news/article.cfm?c_id=6&objectid=11590976
- http://www.ilfattoquotidiano.it/2016/02/16/tumori-ricerca-italiana-identifica-cellule-killer-per-battere-il-cancro-ed-evitarne-il-ritorno/2469376/
- http://news.uschinapress.com/2016/0216/1053890.shtml
- Corriere innovazione, giugno 2016
- Il Sole 24 Ore_Nova, luglio 2016
- Agenzie ANSA, settembre 2016
- QN, ottobre 2016
- About pharma and Medical devices, ottobre 2016
- TuttoScienze e Tecnologia, La Stampa, ottobre 2016
- La Voce di Mantova, novembre 2016