



Introduction to SFTCG 2011

10th Annual Congress of the French Society of Cell and Gene Therapy
6–8 June 2011, Nantes, France

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SFCTG WELCOME NOTE

The French Society for Cell and Gene Therapy was created in 2001 to promote the development of Cell and Gene Therapy in France and to become the active interface between the people involved in the field of biotherapies at every level of translational research from preclinical laboratory work to clinical applications.

The past few years have witnessed considerable progress, with translation of numerous important new treatments demonstrating that patients with severe diseases can be treated efficiently and effectively by Cell Therapy and by Gene Therapy.

The emergence of this new field of medicine has underlined the importance of:

- Strong collaborations between many different partners
- The development of technological tools, medicinal cells and gene transfer vectors
- A responsive regulatory context
- Tools for evaluating efficiency and tolerance
- Sharing problems raised by the potential risks and working together to find solutions
- The cost of financing this new medicine, and
- The pivotal role played by patients' associations, which have created a large, dynamic network that our society aims to promote, support and further unify.

Our annual congress is the opportunity for all the people involved in this human network—researchers, physicians, industry, and government representatives—to meet. For students in particular, this is a great chance to present their work, create exchanges, and form collaborations, which are very important tools in their training.

I would like the SFCTG to develop its unifying role in the field of biotherapies both for basic research and the different clinical stages. Recent progress: reprogramming of stem cells, targeted integration of therapeutic genes, use of miRNA, are a testimony to the pace at which we are moving and justify our ever growing enthusiasm.

More specifically, my wishes for the years to come are:

- Firstly, to promote the education of students through the organisation of congresses, the sharing of scientific information, and the facilitating of an exchange forum.
- Secondly, to set up a strong interaction with other societies involved in the field of biotherapies both nationally and internationally. Such interactions, including the co-organisation of meetings and the consolidation of training and exchange tools for students, are essential to the sustained advance of these fields.

We need your support and active participation. I would like to urge people involved in the many facets of this new and fascinating field of biotherapies and regenerative medicine to join and work with the SFCTG—researchers, physicians and patient associations, government and industry representatives. Help us fulfil our mission by contributing to new, ethically sound approaches in Cell and Gene Therapy, thus creating therapeutic hope for patients suffering from severe genetic or acquired diseases.

Nathalie Cartier
Président

SFTCG 2011 Invited Presentations*

Inv 1

Tailoring adeno-associated viral vectors for gene transfer and gene therapy

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Recombinant AAV vectors (rAAV vectors) have emerged as one of the leading gene transfer systems. Although various serotypes and variants have been developed as gene vectors, rAAV2 is still the dominant serotype used in clinical trials. Weaknesses of rAAV2 (and other AAV serotypes) include i) propensity to accumulate in liver tissue, ii) transduction of non-target cells and iii) pre-existing immunity due to prior exposure to wild-type AAV.

The AAV capsid determines viral/vector tropism and displays epitopes recognized by the host's adaptive immune system. Therefore, to address some of the shortcomings of rAAV vectors, capsid engineering has been exploited. In particular, by insertion of ligands that mediate binding to cell surface receptors, vectors were generated that are capable of transducing non-permissive cells even in the presence of anti-AAV2 antibodies. Similar capsid modifications have helped improve transduction efficiency for permissive cells and/or cell-type specificity. However, the knowledge on ligands suitable for targeting approaches is limited and therefore libraries of capsid insertion mutants have been subjected to high-throughput selection protocols allowing the selection of mutants with a desired phenotype. The same strategy has been applied to identify mutants capable of escaping immune recognition. Specifically, point mutations were introduced in the viral *cap* gene and mutants were screened for infectivity in the presence of neutralizing antibodies. Although initially applied to rAAV2 vectors, capsid engineering is now also being used to generate specifically tailored rAAV vectors based on alternative serotypes. Hence, powerful techniques are in place to optimize AAV for gene transfer and gene therapy.

Inv 2

Adenovirus tropism and engineering for gene therapy

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Adenoviral serotype 5 (Ad5) vectors are used frequently for local and systemic applications in gene therapy and vaccination. However, much of their potential for systemic delivery is limited by their strong tropism for liver and spleen, mediated through binding to coagulation factors, leading to effects on virion, sequestration, limited gene transfer to alternate tissue and toxicity. We, and others, recently elucidated the mechanism underlying liver and

splenic uptake. We have shown the role of coagulation factor X (FX) in mediating liver uptake of adenovirus in rodent models and in non-human primates. This occurs through a high affinity interaction between the FX Gla domain and the hypervariable regions of the Ad5 hexon trimer. We observed specific contact points within the hexon in hypervariable regions 5 and 7. Creation of novel vectors with deletions and amino acid mutations in these regions had dramatic effects on FX-mediated gene delivery *in vitro* and *in vivo*. Binding to the hepatocyte surface is mediated via specific amino acids in the serine protease domain. Mutagenesis of these sites eliminates surface binding. We have also assessed the impact of HSPG structure on adenovirus uptake and transport of Ad:FX complexes. These findings highlight the fundamental importance of the hexon:FX interaction dictating *in vivo* tropism as well as novel avenues for vector retargeting to alternate sites *in vivo*.

Inv 3

Stealthy lentiviral vectors targeted to hematopoietic stem cells

François-Loïc Cosset and Els Verhoeven

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Gene therapy proved to be promising for the cure of several inherited and acquired diseases as evidenced by the success in treatment of immunodeficiencies. Such gene therapies must target human hematopoietic stem cell (HSC), without modifying their properties. *Ex vivo* targeted HSC gene delivery implies the risk of cell differentiation and loss of homing/engraftment potential of these cells, which might be overcome by *in vivo* targeted gene delivery. A major barrier in lentiviral vector (LV) transduction of HSCs is that the majority of HSC are residing into the G₀ phase of the cell cycle, which is a state poorly permissive to classical VSV-G-pseudotyped LV transduction without cytokines stimulation. We previously engineered LVs displaying 'early-activating-cytokine' (TPO and SCF), that allowed a slight and transient stimulation of hCD34⁺-cells, resulting in efficient *ex vivo* gene transfer while preserving the 'stemness' of the targeted HSC with comparable engraftment abilities with respect to un-manipulated HSCs and while promoting selective transduction of long-term NOD/SCID repopulating cells.

Two major challenges for *in vivo* gene delivery by such vectors are the exposure to the host complement system and the off-target cell gene transfer upon systemic administration, owing to the sensitivity of the VSV-G glycoprotein (gp) co-incorporated to allow LV-cell membrane fusion and to the wide distribution of its receptor on all tissues. To surmount these hurdles, we have designed complement-resistant vectors capable of specifically targeting *in vivo* the very rare immature progenitor cells. To achieve this, the VSVG gp was exchanged for another fusion partner, the RD114 gp from a cat endogenous virus, which is attractive since

*Presentors are underlined for all abstracts.

RD114 gp is resistant to the degradation by human complement, RD114-pseudotyped LVs efficiently transduce CD34⁺ HSCs, and a RD114 gp mutant allowing efficient incorporation onto LVs was engineered. These novel vectors achieved a highly selective transduction of hCD34⁺-cells in unfractionated total cord blood and BM. Remarkably these new LVs targeted gene transfer to hCD34⁺-cells *in vivo* in the BM of humanized Balbc rag2^{-/-}, γ c^{-/-} mice. Thus, this vector opens improved alternatives for gene therapy of BM failure syndromes both *ex vivo* and *in vivo*.

Inv 4

Molecular imaging of gene expression using the Na/I symporter: applications in gene therapy

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Molecular imaging of gene expression *in vivo* requires a reporter gene, a tracer and a scanner capable of detecting the presence of the tracer in the live, anaesthetised animal. This methodology has been extensively used in gene therapy to determine the biodistribution of gene expression allowed by viral and non-viral gene delivery vectors and to study the specificity of promoter fragments driving the expression of reporter genes. In term of methodology, both optical and isotopic imaging modalities are available. In this presentation, I will focus on *in vivo* molecular imaging using radiotracers as probes. More specifically, I will present examples of imaging using the Na/I symporter (NIS) as a reporter gene. This membrane protein induces the cellular uptake of iodide and, through the utilisation of relevant radio-iodide isotope, Positron emission tomography (PET) or Single Photon Emission Tomography (SPECT) scanner can be used to detect the biodistribution of iodide in the experimental subject. In addition to its potential as a reporter gene, NIS can also be used as a therapeutic transgene in cancer gene therapy, promoting targeted radiotherapy. Examples of the combination of imaging and therapy mediated by NIS will be presented in the context of oncolytic adenovirus.

Inv 5

The application of targeted nanoparticles in tumour suppression

Andrew David Miller¹

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Inv 6

In vivo gene transfer and non-invasive imaging in rodent brain with viral vectors: progress and challenges

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Viral vectors are extremely efficient tools for gene transfer into living cells. We are using lentiviral vectors and adeno-associated viral (AAV) vectors for stable overexpression and suppression of gene expression in rodent brain. Stereotactic injection of these viral vectors into different brain regions of mouse and rat brain induces overexpression or inhibition of disease-related genes, which can be used for target validation, functional genomics and generation of disease models. We have invested over the last years into reporter gene technology and non-invasive imaging in rodent brain. We have optimized bioluminescence imaging to monitor gene expression in mouse brain and to track neuronal stem cell migration and differentiation. In order to specifically target certain neuronal cell populations we have recently developed conditional lentiviral and AAV viral vectors based on Cre-mediated recombination. Application of these viral vectors in specific cre-transgenic mouse strains allows to specifically label endogenous neural stem cells in the subventricular zone or dopaminergic cells in the substantia nigra. This approach holds promise to non-invasively follow up endogenous neurogenesis and neurodegeneration over time.

Inv 7

Use of transgenic and cloned animals in xenotransplantation

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Xenotransplantation is transplantation between individuals from different species. It is in fact an old procedure initiated in patients in the early XXth century, by pioneer surgeons that at least allowed showing the technical feasibility of a vascularized transplantation. Its interest regained in the 90th, because of a constant and worldwide organ shortage (in case of success, xenotransplantation could offer an unlimited supply) and because of the development of the new techniques such as transgenesis and gene therapy in large animals. In fact, the pig has been identified as the potential donor of organs and not primates because of important ethical issues and risks of virus transmission. Whereas pigs offer advantages, such as size and physiology compatibilities with humans, rapidity and easiness of reproduction, less ethical issues, they bring nevertheless drawbacks due to immunological discordance and also a viral risk. The immunological discordance is mainly due to the existence in humans of preformed antibodies (Ab) directed against an antigen (Ag) that is a disaccharide galactose 1-3 galactose (Gal) expressed on the majority of mammalian cells. Once anti Gal Ab bind their specific target, they activate EC and complement leading to a rejection so fast that it is called hyperacute rejection (HAR). Transgenic pigs expressing human complement regulatory molecules have been generated and have brought an advantage by being protected from HAR, but nevertheless are secondary rejected. Lately, Gal KO animals have been generated and are currently under investigation. The most promising trend of

ABSTRACT WITHDRAWN

xenotransplantation is currently concerning pancreatic islets and neurons that are obviously less rejected.

Inv 8

Jose V Castell¹

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ABSTRACT WITHDRAWN

Inv 9

Long-term muscle repair and clinical efficacy in Duchenne Muscular Dystrophy dogs after systemic delivery of allogenic Muscle Stem (MuStem) cells

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Duchenne Muscular Dystrophy (DMD) is a genetic progressive muscle disease resulting from the lack of dystrophin and without effective treatment. The characterization of adult stem cells has given new impetus to cell-based therapy of neuromuscular diseases. One of them, the Muscle-Derived Stem Cells isolated from mouse muscle by delayed adhesion has been shown to contribute to injured muscle repair. However, these data were collected in dystrophic mice that do not exhibit tissue phenotype and clinical features of DMD patients. Here, we isolated and characterized canine delayed adherent stem cells and investigated efficacy of their systemic delivery in clinically relevant DMD animal model to assess potential therapeutic application in Human.

Delayed adherent stem cells were isolated from healthy dog muscle with a pre-plating technique. These cells, that we named MuStem cells, were defined by a large expansion capacity with atypical division modalities and a multi-lineage differentiation potential in appropriate induction media. Phenotypically, they corresponded to early myogenic progenitors and uncommitted cells. When injected in Golden Retriever Muscular Dystrophy (GRMD) dog, they contributed to myofiber regeneration, satellite cell replenishment and dystrophin expression. Systemic delivery of allogenic MuStem cells in immunosuppressed GRMD dogs resulted in long-term dystrophin expression and muscle damage correction consisting in regeneration activity increase and reduced interstitial fibrosis. Importantly, the clinical follow-up revealed a striking improvement of the transplanted dogs with a major quantified impact on locomotion persisting for months.

The translational findings here demonstrate that MuStem cells provide an attractive therapeutic avenue for DMD patients.

Inv 10

Antisense delivery for mRNA-based therapeutics for neuromuscular diseases

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Many disease-associated mutations in humans affect the pre-mRNA splicing and maturation processes, because they inactivate splicing signals, create cryptic ones or modify binding sites for splicing regulators. They result in exon skipping, inclusion of cryptic exons or intron retention, and to the production of non-functional mRNAs or proteins. Nonetheless, the processing of pre-mRNA can be modulated with high specificity by antisense oligoribonucleotides (AON) that mask key determinants of splicing through Watson-Crick pairing. This approach is currently used to counteract the deleterious effect of mutations, and more broadly as a means to influence the cell phenotype by selectively manipulating alternative splicing. More generally, the AON technology emerges as a useful complement to RNA interference because it allows to selectively remove information in an mRNA rather than destroying it. Recently, we have adapted viral gene transfer vector for the delivery of antisense sequences and demonstrated their efficacy for mRNA based intervention in the context of Duchenne Muscular Dystrophy, Spinal Muscular Atrophy and Myotonic Dystrophy.

Inv 11

Innate immune response: Mechanisms and pathways

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Microbes and their products are sensed by cells of the innate immune system through Toll-Like receptors (TLR) and several other receptors including NLRs and lectin receptors. Pathogen induced activation of antigen presenting cells recruits lymphocytes to mount an innate and adaptive immune response with effective elimination of the pathogen. The role TLR mediated sensing and activation has been investigated for G+ and G- bacteria, fungi, parasites and viruses. Our team focussed on *Mycobacterium tuberculosis* (MTB) infection using mice deficient for single TLRs, as well as the TLR adaptor proteins, MyD88, Mal/TIRAP and TRIF. MyD88 deficient macrophages do not respond to mycobacteria, display reduced phagocytosis and defective killing of bacilli. Aerosol infection of MyD88 deficient mice with MTB (100CFU) is lethal within 4 weeks with 3 log higher CFU in the lung and acute, necrotic pneumonia despite the induction of an adaptive immunity. By contrast, TIRAP and TRIF deficient mice are resistant to mycobacterial infection. Similar data are obtained for another intracellular pathogen, *Listeria monocytogenes* and for *Citrobacter rodentium*. The recognition by live and dead pathogens involves different sensors and pathways as live microorganisms secrete proteins, glycolipids and microbial mRNA, which activates a robust innate immune response. This is of importance for vaccine development as live vaccine inducing stronger protective immunity.

Inv 12

Mechanisms of immunization by gene transfer

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Gene transfer vectors can immunize or not, depending upon specific interactions with the immune system. There are two key points in this context: one is the capacity to trigger innate reactions underlying the development of adaptive immunity, the other is the capacity to present antigenic epitopes derived from the vector or transgene. We have studied these two aspects in the context of recombinant adeno-associated virus (rAAV) vector gene transfer. These vectors are widely developed for gene therapy applications due to their efficacy and low inflammatory properties, but they trigger strong antibody and T cell responses against the viral capsid and transgene-encoded products, due to mechanisms that remain insufficiently understood. In murine models, rAAV is not inflammatory but triggers specific innate signals via the central adaptor MyD88, to induce adaptive CD4 T and B cell responses against the vector capsid. Blocking these innate signals is probably important to fully control the immunogenicity of the vector. We found that rAAV interacts with various types of antigen-presenting cells (APCs) *in vivo*. However, not necessarily the same APCs present antigens from the capsid or from the transgene, suggesting possibilities for complex interplays. Since rAAV can infect and transduce APCs *in vivo*, the transgene can be directly presented by APCs to T cells leading to strong cellular immune responses and rejection of gene-modified cells. This mechanism can be effectively blocked by regulating transgene expression to achieve long-term tolerance against gene-modified cells. Thus, non-replicative, highly purified and engineered gene transfer vectors like rAAV provide a useful system to understand and to manipulate specific mechanisms involved in viral or cellular immune responses.

Inv 13

Antigen encoded by vaccine vectors derived from human adenovirus serotype 5 is preferentially presented to CD8⁺ T lymphocytes by the CD8alpha⁺ dendritic cell subset

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Different subsets of dendritic cells (DC) elicit qualitatively different immune responses. In mice, two lymphoid tissue-resident subsets, CD8alpha⁺ and CD8alpha⁻, have been implicated in the induction of T helper 1 (Th1) or Th2 responses, respectively. Moreover, CD8alpha⁺ DC play a major role in priming CD8⁺ T lymphocyte responses to viral antigens during viral infections. These considerations have been less extensively explored for vaccine vectors derived from viruses. Despite inefficient *ex vivo* transduction of DC, vectored vaccines derived

from human adenoviruses of serotype 5 (Ad5) elicit robust immune responses, predominantly of the Th1 orientation, in humans and mice. At present it is unknown whether Ad5 interacts with DC subsets in a differential manner, thereby influencing the quality of the elicited IR. To address this issue, successive steps (attachment, transgene expression, MHC class I antigen presentation and activation of antigen-specific T lymphocytes) involved in induction of immune responses by Ad5-based vectors were examined in CD8alpha⁺ or CD8alpha⁻ murine DC subsets. Although in both *ex vivo* and *in vivo* experiments CD8a⁺ and CD8a⁻ DC subsets captured an Ad5-based vector to a similar extent, transgene expression and subsequent MHC class I display of a transgene-encoded antigen were more efficient in CD8alpha⁺ DC. Moreover, following *in vivo* and *ex vivo* transduction with an Ad5-based vaccine, antigen-specific CD8⁺ T lymphocytes were more efficiently activated by CD8alpha⁺ DC than by CD8alpha⁻ DC. Thus, superior antigen expression and MHC class I display in CD8alpha⁺ DC may contribute to preferred priming of antigen-specific CD8⁺ lymphocytes by Ad5-transduced CD8alpha⁺ DC.

Inv 14

The participation of Multipotent Mesenchymal stem/stromal cells (MSC) in the tumor microenvironment: tumor-associated fibroblasts and potent anti-cancer cellular vehicles

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To meet the requirements for rapid tumor growth, a complex array of non-neoplastic cells are recruited to the tumor microenvironment. These host-derived cells facilitate tumor development by providing extracellular matrices, cytokines and growth factors as well as vascular networks for nutrient and waste exchange.

Our group was the first to demonstrate [Cancer Res 62:3603, 2002; JNCI 96:1593, 2004.] that a progenitor cell type called Mesenchymal stem cells or Multipotent stromal cell (MSC) was recruited into the tumor microenvironment and responded to tumor-produced signals where they participate in tumor stroma formation. We hypothesized that MSC, after systemic injection, can preferentially engraft into numerous solid tumors and participate in tumor stroma formation. This suggests that MSC have potential as cellular delivery vehicles for intratumoral expression of anti-cancer agents. However, another strategy is to understanding the migratory mechanisms of MSC for tumors, and inhibit the migration and thus the incorporation of the MSC into the tumor microenvironment, preventing the generation of tumor-associated stroma.

Using the Skov-3 ovarian carcinoma we blocked chemoattractive inflammatory signals originating in the tumor, or by blocking the expression of the corresponding receptors on MSC we inhibited MSC migration both *in vitro* and *in vivo*. Based on our analysis, the hyaluronan receptor, CD44 was critical in eliciting a migratory response. First, blocking MSC-expressed CD44 with neutralizing antibody, soluble CD44 decoy receptor, or siRNA knockdown inhibited migration of MSC toward Skov-3 conditioned media *in vitro* by greater than 75%, 82% and 45%, respectively. *In vivo*, a 60% inhibition of systemically injected MSC towards engrafted Skov-3 tumors was achieved using a soluble CD44 decoy receptor. Finally, *in vivo* admixed MSC and tumor cells show high vascularization and structural stromal components identified by CD31, α -smooth muscle actin, fibroblast specific protein and fibroblast activation protein. However, compared

to their wild-type counterparts, CD44 knockout MSC admixed tumors displayed reduced stroma and reduced levels of all aforementioned proteins. Together, these results indicate that CD44 expressed on MSC is critical to tumor tropic migration and tumor vascular support by MSC and may be an important therapeutic target in the tumor microenvironment.

Inv 15

Recent progress in non viral gene delivery and therapy

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Clinical gene therapy most recent successes have been obtained by using viral vectors such as lenti- or adenovirus derived vectors. However, given the broad scope of gene therapy, it is considered that both viral and non viral vectors will find their own niche of excellence and application.

For instance, “naked DNA” vaccination is now widely used in the veterinary field for prophylactic purposes, such as salmon vaccination against infectious hematopoietic virus in Canada (Novartis), horse vaccination against the West Nile virus infection (Ft Dodge Animal Health), and for an immunotherapeutic treatment of melanoma in dogs in the USA (Meril). Another non viral gene therapy product enables the sustained expression of growth hormone-releasing hormone (GHRH) to avoid fetal loss in swine (VGX Animal Health).

The recent progresses obtained both in our laboratory and by others will be presented, including:

- new biosafe plasmids such as minicircles or plasmids free of antibiotic resistance markers (pFAR) for both genetic immunization and gene therapy
- improved physical techniques such as electrotransfer for passive and active immunotherapy and for vaccination
- the use of another physical technique, hydrodynamic liver delivery, for the treatment of both systemic and brain metabolic disease.
- the use of chemical vectors for SiRNA delivery.

These recent progresses give hope for a rapid extension of non viral gene delivery for both cognitive and translational science at the laboratory level, and also for human or animal clinical applications.

Inv 16

Oncolytic virotherapy of glioblastoma: an overview

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Viruses belonging to the genus Parvovirus, within the Parvoviridae family, are able to replicate autonomously in the absence of helper viruses, in contrast to the adeno-associated virus members. The experimental infectivity and excellent tolerance of some rodent autonomous parvoviruses in humans, together with their oncosuppressive effects in preclinical models, speak for the inclusion of these agents in the arsenal of oncolytic viruses under

consideration for cancer therapy. In particular, wild-type parvovirus H-1PV can achieve a complete cure of various tumors in animal models and kill tumor cells that resist conventional anticancer treatments. There is growing evidence that parvoviral oncosuppression involves an immune component in addition to the direct viral oncolytic effect. This talk will summarize the recent assessment of H-1PV antineoplastic activity in preclinical models, laying the foundation for the present launch of a first phase I/IIa clinical trial on glioma patients.

Inv 17

Ten years of gene therapy for SCID-X1

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The long-term follow-up of the first gene therapy trial for X-linked severe combined immunodeficiency (SCID-X1, characterized by a lack of the cytokine receptor common γ chain (γ_c)) demonstrates that gene therapy based on the development of T cell immunity provided a clear therapeutic benefit in eight of the nine treated patients despite the occurrence of vector-related leukemia in some of them. The sustained presence of transduced T cells is observed for up to 12 years following gene therapy. Seven patients (including the three leukemia survivors) exhibited sustained immune reconstitution, with the presence of various T cell subsets and functions. Remarkably, most patients (including the 3 who received chemotherapy) have naïve T cells, strongly suggesting the presence of ongoing, long-term thymopoiesis that could originate from transduced progenitor cells with persistent self-renewal capacity. The T-cell receptor (TCR) repertoire is still diverse in all patients. Despite the lack of persistent transduced B cells, only 3 patients are requiring immunoglobulin replacement therapy. Correction of the immunodeficiency restored the patients' general state of health and all are able to lead a normal life.

Significant advances in viral vector technology have enabled the design of safer vectors with the development of enhancer-deleted LTR-SIN vectors containing an internal promoter. It will be critical to see if the SIN vectors are as safe as expected notably in the current SCID-X1 n°2 gene therapy trial.

Inv 18

A phase I/II clinical trial entailing peripheral vein administration of a novel self complementary adeno-associated viral vector encoding human factor for haemophilia b gene therapy

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A distinct approach for haemophilia B (HB) gene transfer is being tested in the clinic. This Phase I/II clinical trial entails peripheral vein administration of a single dose of our novel self complementary AAV (scAAV2/8-LP1-hFIXco) vector encoding a codon optimised human FIX transgene into adult subjects with severe HB. Using a typical dose escalation design, scAAV2/8-LP1-hFIXco vector has been administered without immunosuppression at three dose levels consisting of the low (2×10^{10} vg/kg), intermediate (6×10^{10} vg/kg) and high (2×10^{11} vg/kg) dose, with two subjects treated at each dose level. The safety and efficacy of this approach will be discussed.

Inv 19

Limbal stem-cell therapy and long-term corneal regeneration

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Corneal renewal and repair are mediated by stem cells of the limbus, the narrow zone between the cornea and the bulbar conjunctiva. Ocular burns may destroy the limbus, causing limbal stem-cell deficiency. We investigated the long-term clinical results of cell therapy in patients with burn-related corneal destruction associated with limbal stem-cell deficiency.

We used autologous limbal stem cells cultivated on fibrin to treat 112 patients with corneal damage, most of whom had burn-dependent limbal stem-cell deficiency. Clinical results were assessed by means of Kaplan-Meier, Kruskal-Wallis, and univariate and multivariate logistic-regression analyses. We also assessed the clinical outcome according to the percentage of holoclone-forming stem cells, detected as cells that stain intensely (p63-bright cells) in the cultures.

Permanent restoration of a transparent, renewing corneal epithelium was attained in 76.6% of eyes. The failures occurred within the first year. Restored eyes remained stable over time, with up to 10 years of follow-up (mean, 2.91 ± 1.99 ; median, 1.93). In post hoc analyses, success - that is, the generation of normal epithelium on donor stroma - was associated with the percentage of p63-bright holoclone-forming stem cells in culture. Cultures in which p63-bright cells constituted more than 3% of the total number of clonogenic cells were associated with successful transplantation in 78% of patients. In contrast, cultures in which such cells made up 3% or less of the total number of cells were associated with successful transplantation in only 11% of patients. Graft failure was also as-

sociated with the type of initial ocular damage and postoperative complications.

Inv 20

Delivering on RNAi Therapeutics, Molecular Imaging and Theranostics; what do we think that we know?

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The quest for safe, efficient synthetic vectors for delivery of therapeutic nucleic acids has been proceeding for at least the past 20 years. In our own work towards this objective, we described the synthetic, self-assembly **ABCD** nanoparticle paradigm that defines critical requirements for successful vector-mediated nucleic acid delivery *in vivo*. **ABCD** nanoparticles are highly regular and comprise a nucleic acid such as small interfering RNA (siRNA) or plasmid DNA (pDNA) (**A**-component) condensed into **AB** core particles by means of cationic liposomes (or even cationic polymers) (**B**-component). Core particles are coated by post-modification (or post coupling) procedures with variable amounts of a stealth/biocompatibility polymer (such as polyethylene glycol, PEG) (**C**-component) to confer stability in biological fluids with passive targeting. Biological ligands (**D**-component) may be similarly introduced sequentially thereafter if required for more active targeting. **ABCD** nanoparticles are self-assembled from synthetic tool-kits of chemical components and so in principle should provide for tailor-made delivery solutions. Here we report how this paradigm has been used successfully to develop several new functional synthetic self-assembly **ABC** and **ABCD** nanoparticles as follows:

- **ABC** (and even **AB**) nanoparticles for pDNA delivery to murine lung *in vivo*
- **ABC** nanoparticles for the delivery of siRNA to the murine liver *in vivo*

Following this, we report on our latest data concerning the adaptation of the **ABCD** nanoparticle paradigm for the development of synthetic self-assembly Gd-**ABC** and Gd-**ABCD** imaging nanoparticles capable of multimodal (magnetic resonance imaging [MRI]-fluorescence) imaging of tumours *in vivo* post i.v.-injection. This work has now led to the creation of long-term circulation theranostic siRNA-**ABC** nanoparticles. These nanoparticles are shown to be capable of mediating functional delivery of siRNA to tumours *in vivo* (leading to phenotypic reductions in tumour growth rates), and shown to enable simultaneous real time/diagnostic imaging of siRNA delivery plus tissue biodistribution. In conclusion, we discuss how close we might be to therapy given successes with proof of concept experiments. What do we need to do next to make therapy happen?

SFTCG 2011 Oral Presentations

Or 1

Genomic targeting with group II introns: characterization of PI.LSU/2 intron protein and evidence for intron splicing activity

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Gene therapy trials using retroviral vectors result in the pseudo-random integration of the transgene cassette in the genome of target cells leading to two kinds of issues: 1) Insertional mutagenesis risk and 2) poor regulation of gene expression due to the variety of genomic context neighbouring each insertion site. Both of these issues could be overcome by targeting the insertion of the transgene into a previously chosen locus. Specific genomic site targeting could be done by using a special class of mobile element ("group II" intron) which is naturally able to perform a site specific transposition.

In contrast with the "homologous recombination" approach (Zn-finger nucleases or méganucléases) specific target recognition by group II introns depends on "RNA-DNA" rather than "protein-DNA" interactions. Thus, the engineering of a vector targeting a specific sequence in the genome should be "a priori" easier to accomplish.

We are working with the group II intron PI.LSU/2 from the brown alga *Pylaiella littoralis*. Like most of group II introns, PI.LSU/2 should be able to transpose in a "site specific manner" through a mechanism which is called "homing". Homing requires both the spliced RNA intron itself in its lariat form and an intron encoded protein (IEP). We choose the PI.LSU/2 intron because of its ability to fold into an active form *in vitro* under salinity conditions similar to those encountered in eukaryotic cells.

We will present our work concerning both the PI.LSU/2 splicing in eukaryotic cells (an essential step in the homing process) and the *in vitro* IEP characterization.

Or 2

Gene therapy of cystic fibrosis: plasmid DNA contribution on *in vivo* lipid-based transfection efficacy

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Synthetic carriers, such as cationic lipids, have been developed for gene therapy of cystic fibrosis as an alternative to viral-based systems which are still associated with immunogenic and oncogenic side effects. Our objective was to evaluate the contribution of two luciferase encoding plasmids when using two of our original cationic lipids for *in vivo* gene transfection. These plasmids were pTG11033, a CpG containing plasmid, and pGM144, a completely devoid CG-dinucleotides DNA. This latter was shown to elicit significantly reduced inflammation. Following a systemic delivery of our lipoplexes in Swiss mice, pTG11033-based lipoplexes mediated detectable bioluminescent signals up to 4 days whereas those incorporating pGM144 allowed sustained transgene expression at least for 20 days. Moreover, whereas bioluminescence was specifically observed around lungs when using pTG11033, additional signals co-localized with many other organs (heart, kidneys...) when using pGM144. We also demonstrated that re-administration of lipoplexes allowed recovering a high, as well as sustained, reporter gene expression but only when using pGM144, not pTG11033. Whatever the plasmid used, a transitional hepatotoxicity was detected during 4 days post-delivery. Recently, the intratracheal delivery route was also evaluated using the pGM144. Again, bioluminescence signals were observed in the lungs for at least 20 days. Immunohistochemical stainings showed that bronchial epithelial cells were specifically transfected. Altogether, these results highlight the important contribution of the plasmid DNA component showing that a good vector but also a good plasmid are together required in order to obtain an efficient as well as sustained *in vivo* gene transfer.

Or 3

Evaluation of the tumorigenic potential of AAV transduction in the newborn rat liver

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Delivery of AAV vectors to the newborn liver results in a rapid loss of episomal vector due to hepatocyte division. Integration of the rAAV genome can lead to a sustained expression of the transgene in selected hepatocytes. The safety of *in vivo* gene therapy with rAAV has been questioned by a study reporting a high incidence of hepatocellular carcinoma (HCC) in mice that received an AAV injection at birth, associated with provirus integration events.

In order to reveal the tumorigenic potential of AAV transduction in the liver, groups of newborn rats received intravenous injection of either self-complementary AAV vectors encoding GFP (scAAVGFP), or PBS, or DiethylNitrosamine (DEN), a well-known tumor-initiator as a control. After six weeks, rats were fed

on a diet containing 2-acetylaminofluorene (2-AAF), a potent liver tumor-promoting agent. After 2 months, animals were sacrificed and their liver analyzed. Neoplastic nodules were identified by GSTp staining, and GFP expression detected by immunohistochemistry.

The number of GSTp positive clones were comparable in the PBS and the scAAVGFP groups and were significantly reduced compared to the DEN group. There was a loss of GFP expression in hepatocytes over time except for some clusters. A lower frequency of GFP clones in GSTp positive areas compared to normal parenchyma was probably due to higher proliferation rate, leading to increased loss of the vector genomes.

In conclusion AAV injection into newborn rats does not appear to induce an increased risk of liver tumorigenesis. To complete this study, vector genome integration events are currently being investigated.

Or 4

PEG-detachable cationic polymer for FGFR-targeted delivery of p53

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One of the most challenging issues in cancer gene therapy is the shortage of efficient and safe vectors that can specifically deliver therapeutic DNA to the target site with minimum toxicity. In the past twenty years, cationic polymers have attracted much attention as potential alternatives to viruses because of their safety and tailor-made features for specific therapeutic application. Although PEGylation has been successfully applied to improve serum stability and lower the toxicity of cationic polymer/DNA complexes (polyplexes), this approach was found to decrease transfection activity and hampered the endosomal escape of polyplexes. To overcome the PEG dilemma, a new PEG-detachable non-viral vector, where the disulfide bond was inserted between the PEG chain and fibroblast growth factor receptor (FGFR)-targeted gene vector, was developed in the present study.

This vector, termed as MPC-ASP, was able to effectively stabilize polyplexes against salt or BSA-induced aggregation. *In vitro* gene transfection and cytotoxicity experiments in different carcinoma cell lines expressing FGFR showed that MPC-ASP could mediate significantly higher transfection efficiency than the non-PEG-detachable gene vector, and could achieve comparable or even higher transfection efficiency but induced much lower cytotoxicity than PEI of 25 kDa. p53 delivered by MPC-ASP showed that MPC-ASP is not only able to efficiently target different types of carcinoma cells expressing FGFR, but also could effectively inhibit the proliferation of these cancer cells. These results suggest that the MPC-ASP could be a safe and efficient non-viral vector for FGFR-mediated targeted delivery of p53 for cancer gene therapy.

Or 5

New AAV-based strategies for gene therapy of a feline SMA model

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Domestic cats exhibiting a recessive form of lower motor neuron (MN) degeneration associated with a deletion of the *lix1* gene, represent a large animal model of type III spinal muscular atrophy (SMA). In this study, we tested intravenous and intracisternal delivery of GFP-expressing self-complementary AAV vectors (scAAV5,8,9.gfp) to mediate gene transfer into the MNs of both neonatal and 7-weeks old adult SMA cats.

A high number of GFP-expressing MNs were found in the whole spinal cord after both intravenous and intracisternal injection of scAAV9.gfp. In contrast, MNs were slightly transduced using intravenous scAAV5.gfp delivery and only GFP+ nerve fibres were observed within the dorsal columns using intravenous scAAV8.gfp injection. Following these preliminary results, we investigated *lix1* overexpression into the spinal cord of SMA cats using scAAV9 recombinant vector for the feline *lix1* gene under control of the CMV promoter. After neonatal intravenous injection of scAAV9.*lix1* vector in three newborn SMA-affected kittens, the viral genome and its expression were revealed in the whole spinal cord using qPCR analyses. Intracisternal injections will soon be performed in three other neonatal SMA-kittens. The therapeutic impact and the expression of LIX1 in the spinal cord will be assessed and compared with those obtained after intravenous delivery of the vector.

Results of this study will be informative for the application of similar strategies in human SMA.

Or 6

Physiologic oxygen levels improve *in vitro* generation of regulatory T Lymphocytes

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Regulatory T (Treg) cells are a central element of cell-mediated immune tolerance. In this regard, CD4 + CD25 + Foxp3 + Treg cells could be used for immunotherapy, as well as for immune tolerance to therapeutic proteins expressed by means of gene transfer. Such cells can be obtained *in vitro* by stimulation of naïve T cells with anti-CD3/CD28 beads, in the presence of TGF- β and IL-2. It has been reported that the oxygen levels used for the culture of primary lymphocytes can impact on their proliferation rate and function, and modulate the activity of some proteins involved in the maintenance of Treg cell compartment. We raised the question of whether the oxygen level supply (atmospheric (20%) or physiologic (5%) oxygen levels), could influence the efficiency of Treg induction. Using splenocytes from Foxp3 KI-GFP anti-male TCR transgenic mice, we compared the percentage of CD25 + GFP+ (Foxp3+) obtained after stimulation of CD4 + GFP-, in 20% or 5% O₂ conditions. We show that a better induction of Tregs is obtained under 5% in comparison to 20% O₂: (i) with 92.9 + / - 0.5% and 79.6 + / - 3.6% of CD25 + GFP+ (Foxp3+) obtained at day 11, respectively; (ii) with a higher mean fluorescence intensity (MFI) of CD25 on

CD4 + GFP + (Foxp3+) cells; (iii) with a higher proliferation index of the CD4 + GFP + (Foxp3+) cell population. These results have important implications in developing *in vitro* protocols for optimization of the induction of Treg lymphocytes from naïve T cells.

Or 7

Using engineered stromal cells for the targeted therapy of liver carcinoma

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Introduction: Tumor growth is supported by a complex microenvironment that includes a wide variety of non-epithelial cell types, which form the tumor stroma. These stromal cells are attracted to many tumor types to favour their growth. Thus, adult stromal cells make excellent cell vectors to target cancerous lesions. In this work, we asked whether adult mesenchymal stromal cells (MSCs) can be engineered to target and destroy liver cancer-derived cells.

Methods: MSCs are genetically modified using lentiviral vectors to express human IFN β or IL24 as therapeutic genes. Control MSCs express GFP. Transgene expression is measured by FACS analysis, Western blotting analysis and RTPCR. Cell proliferation is measured by cell counting. Cell migration is measured using Boyden chambers.

Results: MSCs were transduced with great efficacy, in the absence of selection. MSC viability is not impaired by transduction lentiviral vectors. Cell medium collected from MSC expressing IFN β or IL24 strongly inhibits the proliferation of human cells derived from liver cancer (HepG2 and HuH7). In addition, transwell assays demonstrated that MSC encoding for IFN β or IL24 actively inhibit cancer cell proliferation. Last, migration assays demonstrate that conditioned medium from liver cancer cells has a chemotactic effect on therapeutic MSCs.

Conclusion: We demonstrate that 1) MSCs are easily engineered using lentiviral vectors and 2) MSCs expressing soluble therapeutic genes are attracted to and strongly impair liver derived-cancer cells. This work strengthens the rationale for using MSCs as anticancer vehicles and opens an avenue to help fight disseminated liver cancer.

Or 8

Characterization at the clonal level of Mesenchymal stem cells from human fetal liver

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Mesenchymal stem cells (MSCs) from bone marrow (BM) are multipotent cells with adipocytic (A), osteoblastic (O), chondrocytic (C), and vascular smooth muscle (V) differentiation potentials. They represent the stromal component of the hematopoietic stem cell "niche". We investigated whether such MSCs were present in human 12-13 week fetal liver (FL). Cells were selected by adherence on plastic then sorted by FACS and

individually seeded. Clonogenic cell frequency was 1 in 3-5 and these cells were identified as MSCs having A, O, C, V differentiation potentials. We found gene expression of transcription factors PPAR γ , RUNX2, SOX9, and MYOCD for A, O, C, V differentiation by Q-PCR and protein expression of RUNX2, SOX9 by immunofluorescence (IF). Moreover, we evaluated the presence of other differentiation potentials in our clonogenic cells. We found no trend to endothelial and hematopoietic differentiation but a low level of MYOCD (V) and SOX2 (C) gene expression, besides a significant higher expression for skeletal muscle (SKM) and pluripotency markers in FL compared to BM MSCs. We found a low expression of cytokeratin 19 and 8/18 by IF, and low expression of HNF3B, HNF1A and high expression of HNF1B by Q-PCR, compared to hepatic cell lines. Remarkably, HNF3B and HNF1B expression in FL were obviously higher than in BM, and very high gene expression of Snail, Slug, and Twist epithelial mesenchymal transition (EMT) inducers was observed.

Our study, at clonal level, suggests that MSCs from FL may have some potential for SKM and hepatoblastic differentiation, this latter one being impaired by high expression of EMT inducers.

Or 9

Progress towards gene therapy of HIV infection in humanized mice

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The recent description of the first cure of HIV infection through graft of CCR5-deficient hematopoietic progenitors represents a strong argument in favour of HIV gene therapy strategies targeting CCR5. We have undertaken such a strategy by combining a fusion inhibitor and a CCR5 agonist in a lentiviral vector to prevent viral entry into CD4+ T cells. *In vitro*, the expression of both transgenes could be observed: the fusion inhibitor prevented HIV infection whereas the agonist diminished CCR5 expression. The combined effect of the two genes on HIV replication is currently examined. To test the efficacy of this strategy with the real virus in human cells *in vivo*, we developed a small animal model susceptible to HIV infection. We show that NOD.SCID.gc^{-/-} mice reconstituted with human progenitors readily develop CD4+ T cells that are susceptible *in vivo* to minimal doses of X4 or R5-tropic HIV. The infection is correlated with CD4 depletion in the blood, but is not always observed despite high viral loads. Gene transfer into CD34+ cells resulted in expression of the therapeutic transgenes *in vitro*. Mice reconstituted with modified progenitors will be challenged with HIV and various parameters will be measured. Overall, this project aimed at developing the first French clinical trial for gene therapy of HIV infection.

Or 10

Silencing of NMDA receptor NR1 subunit using HSV-1-derived amplicon vectors impairs cognitive functions in rats and reduces amyloid-beta oligomers binding and toxicity in cultured neurons

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Ionotropic N-Methyl-D-aspartate glutamate receptors (NMDAR) are the predominant molecular devices for controlling synaptic plasticity and memory function. NMDAR are tetramers formed by two NR1 subunits and different NR2 and/or NR3 subunits. To study the role of hippocampal NMDAR in cognitive functions such as learning and memory formation, we developed HSV-1-based amplicon vectors expressing antisense RNA sequences against NR1 to induce silencing of this subunit. Upon inoculation into the rat hippocampus the vectors induced high-levels of antisense NR1 RNA and considerably decreased expression of NR1. When inoculated into the CA1 layer of rat hippocampus, the vectors caused a significant impairment in the performance of behavioural tasks, thus indicating an involvement of hippocampal NMDAR in the control of the cognitive functions under study. The hippocampal formation is one of the earliest regions damaged in Alzheimer's disease (AD). Amyloid-beta ($A\beta$)-oligomers accumulate in AD brains, where they target excitatory post-synaptic terminals and trigger synapse deterioration, a mechanism likely underlying memory loss in early-stage AD. When used to infect primary cultures of rat hippocampal neurons, the amplicons suppressed up to 90% NR1 expression in dendritic spines and significantly reduced the binding of exogenously added soluble $A\beta$ -oligomers to the spines. Furthermore, the amplicon-induced NR1 knockdown markedly reduced the neuronal oxidative stress instigated by binding of $A\beta$ -oligomers. Therefore, our results corroborated that NMDAR of CA1 are directly involved in learning and memory processing and strongly suggest that NMDAR are required for synaptic targeting of $A\beta$ to neurons and are at least partially responsible for their deleterious effects.

Or 11

Evaluation of cholesterol 24S-hydroxylase overexpression by AAV-gene transfer as a potential therapeutic target for Alzheimer's disease-like Tau neuropathology

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The development of Alzheimer's Disease (AD) is closely connected with cholesterol metabolism. In the brain, the synthesized cholesterol cannot be degraded *in situ* nor cross the blood-brain-barrier. The major exportable form of brain cholesterol is 24S-hydroxycholesterol generated by the neuronal cholesterol 24-hydroxylase encoded by the *CYP46A1* gene. We previously demonstrated that increasing brain *CYP46A1* gene expression through AAV-mediated gene transfer markedly reduces amyloid pathology in APP23 mice.

In a therapeutic perspective, we aimed to determine if *CYP46A1* gene overexpression not only could improve amyloid pathology but also could affect Tau pathology in the brain of Tau22 mice. This latter model exhibits progressive neuron-

specific AD-like Tau pathology devoid of any motor deficits. AAV vector expressing the human *CYP46A1* gene was injected stereotactically in the cortex and hippocampus of 3-month old Tau22 mice. Behavioural studies (Morris water maze) were performed at 9 and 12 months and mice were then sacrificed to study neuropathological and biochemical parameters (gliosis, amount of aggregated Tau protein etc.).

We demonstrate that increasing brain *CYP46A1* gene expression through AAV-mediated gene transfer reduces hyperphosphorylated Tau content and improves spatial memory defects in Tau22 mice as soon as 9 months after *CYP46A1* gene transfer. Biochemical and neuropathological studies are in progress to further characterize the mechanism of *CYP46A1* intervention on Tau pathology.

Our results suggest that overexpressing *CYP46A1* in specific mouse brain regions not only improves amyloid but also Tau pathology and that viral vector delivery of *CYP46A1* could find therapeutic applications in Alzheimer disease.

Or 12

Recombinant AAV vectors as genetic vaccines to induce a neutralising humoral response against NIPAH virus

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During the last decade, recombinant vectors derived from the Adeno-associated virus (rAAV), a single-stranded DNA parvovirus, have mainly been used for gene therapy. Indeed, these vectors are able to efficiently transduce several tissues *in vivo*. In addition, several AAV serotypes are available displaying different transduction capacities depending on the target tissue. The aim of this project is to determine if rAAV vectors can be used as genetic vaccines inducing a protective immune response against an emerging virus.

Nipah virus (NiV) is a paramyxovirus that emerged thirteen years ago in Malaysia, associated with a 75% mortality rate in humans. It is classified as a Biosafety Level 4 (BSL4) pathogen and there are currently no treatments or vaccines available.

To evaluate the potential of rAAV vectors for vaccination, we used the G glycoprotein of NiV as a viral antigen. Three rAAV-NiV.G vectors of serotype 1, 8 and rh32/33 were injected in mice to determine their ability to induce a neutralising antibody response against NiV.G. RAAV8 vectors induced the most efficient antibody response in two different injection routes. Surprisingly, a boost injection with a different AAV serotype did not significantly enhance the antibody titre. Further studies were conducted in the hamster model that is highly susceptible to Nipah virus infection. Vaccinated animals developed a strong humoral immune response against NiV.G after a single rAAV injection. The analysis of the protection efficiency against a challenge with Nipah virus is currently under way and results will be presented.

Or 13

Vaccine strategy based on DNA vaccines forming E7 recombinant retrovirus-like particles for the treatment of HPV-induced head and neck cancers

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Tobacco and alcohol consumption are the main risk factors of head and neck squamous cell carcinomas (HNSCC). However, papillomavirus (HPV) infection has been recently associated with the development of these cancers particularly in the lingual and palatine tonsils. In this context, HPV-16 is the most frequent serotype known to express the E6 and E7 oncoproteins. While vaccines containing L1 and L2 capsid HPV proteins are efficient for the prevention of HPV infection, they are not adequate for the treatment of established tumors. Then, development of innovative vaccine therapies targeting E6/E7 is important for controlling HPV-induced cancers. Thus, we engineered a plasmovLP DNA vaccine encoding a non-oncogenic mutated E7 antigen displayed on retrovirus based virus-like particles (VLPs) formed by murine leukemia virus Gag proteins and VSVG envelope glycoproteins. We studied the ability of plasmovLPs/E7 to generate specific immune responses as well as *in vivo* anti-tumor effects using the TC-1 epithelial cells that over-express the HPV-16 E6/E7 oncoproteins. First, we demonstrated by FACS and ELISpot that human dendritic cells incubated with VLPs/E7 specifically elicit *in vitro* anti-E7 IFN γ T cell responses. Next, we showed by ELISpot analysis that intradermic vaccinations of mice with plasmovLPs/E7 introduced by *in situ* electroporation induce an anti-E7 response. Finally, vaccinated mice were challenged either in a preventive or a curative way with E7-bearing TC-1 cells. Data indicated that preventive vaccination fully protect mice, since curative vaccination had antitumor effects and prolonged the survival of vaccinated mice. Thus, plasmovLPs may be an efficient strategy for HPV-induced HNSCCs.

Or 14

Impact of the immune system on rAAV2/4.hrpe65 mediated gene delivery in retina: Efficacy of sequential subretinal readministration of rAAV2/4.hrpe65 in RPE65^{-/-} dogs

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Leber's Congenital Amaurosis (LCA) is a group of hereditary retinal degenerations leading to total blindness. We previously reported that subretinal injection of recombinant adeno-associated virus serotype 4 (rAAV2/4) allows a dramatic restoration of vision in a LCA model of RPE65^{-/-} dogs. We evaluated the impact of the immune system on subretinal rAAV2/4.hrpe65-mediated gene delivery in RPE65^{-/-} dogs.

Three RPE65^{-/-} dogs were efficiently treated by subretinal injection of rAAV2/4.hrpe65 in one eye. ELISA analyses showed that none of the animals developed detectable antibodies against the transgene product and/or the AAV4 capsid in sera in the 4 months post-injection although AAV4-neutralizing factors were detected in 1/3 dogs. IFN γ —ELISpot assays performed on PBMCs did not show any anti-AAV4 cellular response.

At four months post-first injection, the contralateral eye of these dogs was similarly treated and showed a successful rescue of retinal function. Both anti-AAV4 and/or anti-RPE65 antibodies were not detected by ELISA in sera meanwhile we noticed

an increase of the AAV4-neutralizing factors in 1/3 dogs. No anti-AAV4 cellular immune response was found either.

Because anti-AAV4 pre-existing immunity could exist in human patients, high titres of circulating anti-AAV4 antibodies were induced in 2 dogs by AAV4 immunization before subretinal injection of rAAV2/4.hrpe65. Evaluation of the retinal function after the injection showed that a strong pre-existing immunity to AAV4 did not block rAAV2/4.hrpe65-mediated gene delivery and retinal function recovery in RPE65^{-/-} dogs.

These findings, (i) sequential subretinal readministration of rAAV4 is safe and potent (ii) pre-existing immunity to AAV4 does not block subretinal AAV2/4.hrpe65 mediated gene transfer, have important implications for the treatment of LCA patients.

Or 15

The cross-priming of tumor reactive cytotoxic T cells by synthetic long peptides depends on a sufficient HLA binding of the CD8 epitope

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Induction of a potent CTL response requires long-lived and strong enough antigen cross-presentation by dendritic cells (DC). Development of cancer vaccines fulfilling these requirements is therefore a major aim. It has been observed that long peptide (LP), from human melanoma antigens, induce a long lasting cross-presentation by human monocyte-derived dendritic cells (moDC) *in vitro*. We studied here, *in vitro* and *in vivo* (with HLA-A2 healthy donor PBL and HHD mice), the cross priming potential of a LP from the melanoma antigen Melan-A/MART-1, bearing a CD8 epitope optimized or not for HLA-A2 binding (Melan-A 16-40 and 16-40_{A27L}) also containing CD4 epitopes. We show that both natural and modified LP induce a long-lived cross-presentation by moDC. However, only the modified LP efficiently prime long-lived tumor-reactive CTL. We further show that CD8 T cell priming and expansion by this LP is increased by CD4 lymphocytes. These data underscore that sufficient HLA binding affinity and/or TCR ligand potency of the cross-presented epitopes are critical requirements for tumor antigen LP immunogenicity. Together this supports the design and use of LP bearing optimized tumor epitopes as tumor vaccines. Further strengthening this conclusion, we show that cross-presentation of the modified Melan-A LP is a property restricted to antigen presenting cells.

Or 16

Therapeutic interest of RNA interference molecules in two rare bone diseases

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RNA interference is a physiological pathway that allows post-transcriptional inhibition of gene expression through the recognition of a specific sequence of mRNA by complementary sequences of small interfering RNAs (siRNAs). Since its discovery in mammalian cells in 2001, RNAi is being explored as a potential treatment for various diseases.

Intra-tumoral injections of siRNAs directed against the pro-survival cytokine Rankl (Receptor Activator of Nuclear Factor- κ B Ligand or Tumor Necrosis Factor Superfamily member 11, Tnfs11), implicated in the vicious cycle established between tumor development and osteolysis at bone site, reduce the Rankl production in serum and protect bone from para-tumor osteolysis. These Rankl-directed siRNAs significantly prevent tumor relapse when combined with ifosfamide as compared to chemotherapy alone in two osteosarcoma model.

Short hairpin RNAs (shRNAs) also permit to specifically target a mutated transcript that differs from the wild-type mRNA by only two nucleotides in a model of osteogenesis imperfect (OI). Our data support the use of shRNAs cautiously designed and lentivirus delivered as a strategy to specifically suppress the mutant allele making appealing the modification of mesenchymal stem cells of OI patients for autograft transplantation.

The increasing number of preclinical and clinical studies and our results highlight the power of this new therapeutic tool, suggesting a large-scale clinical development of siRNAs and shRNAs molecule in therapy.

Or 17

***In vivo* targeting of microRNA 21 using lentiviral vectors for the gene therapy of pancreatic cancer**

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Pancreatic ductal adenocarcinoma has a poor prognosis and is one of the most common malignancies worldwide. Therefore, there is an urgent need to develop new therapeutic strategies for pancreatic cancer. We recently demonstrated that oncogenic microRNA miR-21 is induced early in pancreatic cancer and that its inhibition decreases cell proliferation and induces cell death by apoptosis *in vitro*. The aim of the present study was to ask whether targeting miR-21 using gene delivery may impair pancreatic cancer development *in vivo*. We designed lentiviral vectors (LV) delivering siRNA against miR-21 (LV a/miR21). Pancreatic cells MIA PACA-2 were first transduced with a secreted luciferase, the Gaussia Luciferase (GLuc), encoding LV. These cells were able to develop tumors when injected subcutaneously or directly into the pancreas of immunosuppressed mice. GLuc secretion was detected by simple plasma sampling. Few weeks after implantation, tumors were injected with LV a/miR21 or LV GFP, as a control. We showed that plasmatic GLuc levels were directly correlated to tumor size, allowing us to non-invasively monitor the viability of tumors during their development. Intratumoral injection of LV a/miR21 drastically reduced the viability and the volume of pancreatic tumors in comparison with tumors injected with LV GFP. In addition, huge areas of necrosis and apoptosis were observed at the injection site. These data suggest that targeting miR-21 using LVs strongly impairs pancreatic cancer development *in vivo*. This study will

help to define new therapeutics for pancreatic cancer to further improve the gene therapy clinical trials currently performed in our team.

Or 18

Brain gene therapy for Metachromatic Leukodystrophy using AAVrh10 vector: optimized clinical protocol

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Metachromatic Leukodystrophy (MLD) is a lethal neurodegenerative disease caused by deficiency of ArylsulfataseA (ARSA). The most severe form starts around 1-2 years, leading to death within a few years, without available treatment.

We have demonstrated: (1) in MLD mice and non-human primates (NHP) the efficiency and safety of a brain gene therapy strategy, using intracerebral delivery of adeno-associated-vector serotype 5 (AAV5) encoding human ARSA and (2) the superiority of AAVrh.10 serotype upon AAV5 to more rapidly improve MLD mice and normalize sulfatide isoforms that accumulate specifically in oligodendrocytes.

In a clinical perspective, we have optimized in NHP the neurosurgical procedure to allow simultaneous infusion of vector at 12 different brain sites in less than 2 hours. We also developed a brain MRI protocol to select the injection sites and evaluate the tolerance of the procedure. Following the same protocol planned to be used in patients, we demonstrated in NHP that intracerebral injections of AAVrh.10/ARSA vector in the white matter is well tolerated and results in the diffusion of the vector in up to 90% of the injected hemisphere and up to 31% increase in ARSA activity, reaching foreseeable therapeutic levels. Toxicological studies are in progress towards phase I/II tolerance and efficiency clinical trial in late 2011. This trial will enrol five patients with rapidly progressing MLD. Safety and efficiency parameters will be evaluated up to 2 years, a period that will be sufficient enough to assess the potential therapeutic efficiency of this strategy in rapidly progressing forms of MLD.

Or 19

Effective limb transduction and phenotypic correction after injection of rAAV8-U7snRNA in GRMD dogs

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In Duchenne Muscular Dystrophy (DMD) the selective removal by exon skipping of exons flanking an out-of frame mutation in the dystrophin messenger can result in in-frame mRNA transcripts that are translated into shorter but functionally active dystrophin. The goal of our project was to determine in GRMD, the effective dose of our therapeutic product defined as a recombinant Adeno-Associated Virus serotype 8 (rAAV8) expressing a modified U7 snRNA specific for the skipping of exons 5 to 10 of the GRMD dystrophin transcript. The mode of delivery was the locoregional high-pressure intravenous (IV) injection of a forelimb. Several groups of GRMD dogs were exposed to differ-

ent rAAV8-U7snRNA doses. Each dog was followed ≈ 3 months after injection. The primary outcomes were the restoration of dystrophin expression and the improvement of the tissue pathology in the injected limb compared to the contralateral limb. The secondary outcomes were the muscle strength correction, the biodistribution and shedding patterns as well as the immune response against rAAV8 capsid and dystrophin. We built a unique network of laboratories with complementary skills to deliver a GLP-compliant set of preclinical data to further define the regulatory toxicology studies. The organization of our network and the results obtained in our GRMD dogs study will be presented.

SFTCG 2011 Poster Presentations

P 1

Creation of novel rat models for cancer research using zinc finger nuclease (ZFN) technology

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For many human conditions, rats are superior to mice as model organisms, due to their physiology, responses to drugs, and performance during behavioural tests. Their larger size facilitates molecular cell biology and physiology experimentation. Therefore, rats are the preferred model organism for studies of human diseases including cancers such as breast, prostate, and bone marrow metastases. However, the lack of convenient experimental tools to manipulate the rat genome has largely limited their use as genetic models for disease, until the recent creation of the first targeted knockout rats by using zinc finger nuclease technology. ZFN-assisted genetic modifications can be introduced into the rat genome at specific sites, and in a significantly shortened timeframe (compared to conventional ES cell technology in mice). At SAGE Labs, we are uniquely positioned to create rat knockout models for all major disease categories. With regard to cancer research, we have developed Tp53 knockout rats, which will complement and expand the p53 studies performed in mice to date. In the same capacity, we are also creating a suite of immunocompromised rats, knocked out for the RAG1, RAG2, and DNAPK gene. The models may be used, for example, in the generation of xenografts for metastasis studies and bone marrow transplantation for cancer therapy. In addition, we have developed a set of toxicology models, including knockouts of MDR1a, PXR, BCRP, MRP1 and MRP2, which will enable studies on drug delivery and metabolism in a more physiologically relevant model. Model generation, their applications and future models will be discussed.

P 2

Gene targeted correction of inherited liver defects using zinc-finger nucleases: application to Crigler Najjar disease

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Zinc-finger nucleases (ZFNs) can induce a locus-specific DNA double strand break (DSB). DNA repair can be performed through homologous recombination with donor DNA homologous to the DSB site. *In vitro*, it allows efficient knock-in and gene repair. We aim to evaluate the feasibility and efficiency of ZFNs-

mediated knock-in and gene repair for *in vivo* gene therapy of inherited liver diseases, especially for type I Crigler-Najjar disease, a deficiency in UGT1A1 enzyme.

For the knock-in approach, we constructed a liver-specific lentiviral vector encoding IgM-ZFNs. As IgM is not expressed in hepatocytes, it represents an innocuous insertion site. Integrative lentiviral vectors (LV) and integration deficient lentiviral vectors (IDLV) were produced. They were tested *in vitro* in FAO rat hepatocytes by mutations detection assay based on PCR products heteroduplexes formation and T7 endonuclease I.

LV and IDLV encoding IgM-ZFNs were produced at a titer of 1×10^9 TU/mL. FAO were highly transduced (>90%) with LV. Transduced FAO contained more DSB with LV than IDLV. Knock-in at the IgM locus is being evaluated first in FAO cells. We also verified that UGT1A1-exon4-ZFNs can be designed and we will construct the corresponding donor DNA for gene repair.

We will evaluate this approach in primary hepatocytes and *in vivo*. We will also construct a vector encoding IgM-ZFNs and carrying a donor DNA encoding GFP or UGT1A1 flanked by sequences homologous to the target. For gene repair, we will construct a vector carrying UGT1A1-ZFNs and a promoter-less wild-type exon 4.

P 3

Gene therapy for the severe virilizing forms of congenital adrenal hyperplasia using direct intra-adrenal injection of recombinant AAV vector encoding human CYP21

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21-Hydroxylase deficiency (21OH-D, 1/40.000) due to mutations in *CYP21* gene blocks cortisol and aldosterone synthesis, leading to adrenal insufficiency, excessive ACTH feedback, hyperplastic adrenals and androgen overproduction. In the most severe forms (5% of cases), newborn girls have a complete sexual ambiguity. Despite medical treatment, severely affected girls endure multiple surgical corrections and remain exposed to lifelong virilization, almost complete restriction of sexual life and lack of fertility. We decided to initiate, for severe forms of 21OH-D, a gene therapy approach based on intra-adrenal injection of Adeno Associated Virus (AAV) encoding human *CYP21*. The objective is to restore a life-long enzymatic activity allowing normal secretion of gluco- and mineralo-corticoids and reduction of androgen excess.

Among several serotypes of AAV, we showed that AAV5 and AAV8 vectors were the best serotypes to target adrenal cells. Six

weeks after a single intra-adrenal administration (1.0 to 1.7×10^{10} vg) in rats, the mean number of vector genome copies/cell in the injected adrenal ranged from 5.5 ± 1.5 to 89.5 ± 18.2 and 1.8 ± 0.5 to 10.6 ± 2.3 with AAV5/GFP and AAV8/GFP, respectively. Both vectors shared a strong tropism for adrenal fasciculate and glomerular zones, where cortisol is produced. The percentage of adrenal cells expressing GFP reached up to 8.7% after injection of AAV5/EGFP vector (13% around the injection site) and 6.5% after injection of AAV8/GFP vector (14% around the injection site).

In the view of these results, both AAV5 and AAV8 will be used to evaluate the efficiency of our strategy in a mouse *CYP21^{-/-}* model of the disease.

P 4

Genetic modifications at safe harbor loci in hematopoietic stem cells and T cells for gene therapy

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Targeted gene therapy approaches offer the possibility of precisely editing genomic sequences. They present a lower risk of adverse consequences compared to non-specific integration but rely on the availability of accurate genomic tools. Meganucleases are natural highly specific endonucleases capable of inducing high frequency of homologous recombination at their target site. Redesigning their protein-DNA interface allowed us to engineer dozens of custom meganucleases with chosen specificity and to target defined loci in the human genome. We defined specific genomic loci where insertion of a gene expression cassette would have a better therapeutic benefit/risk ratio (called safe harbor loci) according to different criteria such as the cell type where the modification is made. We first characterize potential safe harbor meganucleases by measuring their ability to induce mutagenesis or homologous recombination-mediated gene targeting in model cell lines. Then, we set up initial assays to examine the safety and the capacity for the locus to support transgene expression.

Because they can be manipulated *ex vivo*, cells of the blood lineage are an attractive target for gene therapy. Among them, we focus our efforts on hematopoietic stem cells (HSCs) that could be of interest in a variety of indications such as immune deficiencies, hemophilia or thalassemia and T cells for adoptive immunotherapy in the treatment of malignancies and infectious diseases.

We will present our latest results on safe harbor meganucleases and on genetic modifications of HSCs and T cells.

P 5

Novel drug delivery system of siRNA based on chitosan, pegylated chitosan and polyethyleneimine

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Gene silencing mediated by double-stranded small interfering RNA (siRNA) appears to be a good strategy for the treatment of

many diseases. However, the use of siRNA is limited by poor blood stability and low cellular uptake. Hence vectors are needed to avoid opsonisation and to deliver the siRNA into the cytoplasm.

The aim of this study is to design and evaluate chitosan-based nanoparticles able to reach endothelial tumor cells and to deliver anti-tumoral siRNA.

The weak buffering capacity of chitosan does not permit complete endosomal disruption, therefore only a low gene silencing was observed on B16F10 melanoma cells stably expressing luciferase. Based on these findings, we included in the formulations the primary amine rich polymer PEI which led to a high gene silencing efficiency without toxicity. The nanoparticles presented a narrow size distribution with a mean diameter of 200nm. The siRNA encapsulation efficiency was close to 100%. Confocal microscopy showed that the nanoparticles were well-endocytosed by the cells after 30 minutes. We also assessed the influence of the chitosan molecular weight on the transfection efficiency.

In this work, we demonstrated that the combination of chitosan, PEGylated chitosan and polyethyleneimine (PEI) was an efficient vector for siRNA delivery *in vitro*. Further studies are ongoing to predict the behaviour of these carriers in biological fluids.

P 6

Synthesis of new neutral pH-sensitive lipids for nucleic acids delivery

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Colipids or helper-lipids, a class of lipids uncharged at physiological pH, were found to be essential components in the final formulation of liposomes used for gene transfection.

Lots of cationic lipids developed have often been associated with a zwitterionic colipid such as dioleoylphosphatidylethanolamine (DOPE), to allow efficient gene transfer. Indeed, the presence of DOPE in the formulations provides better transfection efficiency by its fusogenic properties that facilitate endosomal escape, by destabilizing the membranes of endosomes. However it is also known that DOPE may in some cases reduce the efficiency of vectorisation, because it can promote the agglomeration of the lipoplexes with blood lipoproteins, and also promote toxicity.

The synthesis of new neutral lipids, in place of DOPE, is therefore an extremely important issue. It seemed important to consider the synthesis of new neutral pH-sensitive lipids bearing one or more imidazole groups. These novel compounds may favor a different mechanism of endosomal release, related to the failure of the endosomal membrane by a proton sponge mechanism as induced with the cationic polymer polyethyleneimine, and not connected to a mechanism of membrane fusion as in the case of DOPE.

We therefore present the synthesis of new neutral pH-sensitive lipids, their physicochemical properties and their transfection efficiencies of nucleic acids (DNA and siRNA).

P 7

Tissue-specific regulation of transgene expression by recombinant lentivirus

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Tight control of gene expression is a prerequisite for clinical applications of gene therapy. To this end we have designed a unique system that combines the tTR-Krab doxycyclin sensitive regulation system with endogenous miRNA silencing to achieve specific and high expression in hepatocytes.

We generated lentiviral vectors containing the tet-O sequence and two different expression cassettes in reverse orientation. The first cassette contains an ubiquitous promoter driving the TetR-KRAB sequence and 4 target sequences of the liver specific miR 122. The second cassette contains and the GFP gene under control of an ubiquitous or liver specific promoter. The regulation of GFP expression was evaluated *in vitro* in cells from hepatic or non hepatic lineage.

In non hepatic cells (miR122negative), withdrawal from doxycycline resulted in a decrease of mean fluorescence from 50 to 95% depending on the promoters used. In contrast, in hepatic cells in which miR122 binds to its target sequence transgene expression remained stable at high level in the presence or absence of doxycycline. As a control we used target sequences for miR 142 which is specifically expressed in cells from hematopoietic lineage and we got similar results.

We will now evaluate *in vivo* the tissue specificity of our approach. Also we seek to determine whether tight tissue specific expression may thwart immune response to the transgene product. Furthermore if an immune response to the tTR-Krab develops, it will specifically eliminate the non hepatic cells but should spare hepatocytes that do not express the tTR-Krab protein.

P 8

Tools for gene targeting using DNA double strand break-induced homologous recombination in human cells

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Meganucleases (MN) are site specific endonucleases, with 12 to 45 bp DNA recognition sites. They generate double-strand breaks that can be repaired by homologous recombination if a DNA template is present. MN may be engineered for custom recognition of any genetic locus and used for gene targeting. Our interest is to develop efficient means of targeting transgenes at specific loci for the purpose of gene therapy.

We are studying single chain MN derived from I-CreI that recognizes sequences within the human Rag-1 gene or at two candidate safe harbor loci in the human genome. The MN are introduced into the target cells using integrative and integration-deficient lentiviral vectors encoding them, or as proteins associated with the lentiviral vector particle. The later approach avoids the prolonged expression of the MN which is reportedly toxic. It is achieved by fusing the MN to Vpr, a lentiviral protein which is associated with the capsid and is able to drive the incorporation of foreign proteins into the virion.

We have constructed a recombination detection system based on luciferase, allowing for a quantitative assay that could also be used for detecting the MN activity after delivery into human cells, or live animals. A duplication-inactivated thermostable Firefly luciferase gene was constructed and used in different assay format in human 293 cells. This luciferase-based system is

fast, sensitive and quantitative, and will be useful for the detection of MN activity into experimental animals.

We present here the latest results on the activity of our MN-Vpr constructs.

P 9

***In vivo* transformation of lung cells with apoprotein E derived peptide conjugated to polylysine (apoEdp-PLL): a non-viral vector for gene therapy**

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Gene therapy involves the delivery of genes, small DNA or RNA molecules to human cells, tissues or organs to correct a genetic defect, or to provide new therapeutic functions for the ultimate purpose of preventing or treating diseases. Two diseases, cystic fibrosis (CF) and α_1 -antitrypsin (α_1 -AT) deficiency are relatively common single-gene disorders for which the genetic basis is known and current treatment strategies are not curative. On the other hand lung cancers of multifactorial nature are also common. Attempts have been made to treat both categories by gene therapy. For any sort of gene therapy we need to develop therapies that are non-toxic, non-immunogenic, cost effective with good transformation/expression efficiency for the target organ.

Here we report the development of a non-viral vector using a LDL receptor mediated pathway for transporting genetic materials to the lung tissue. A tandem dimmer sequence of apoprotein-E conjugated to polylysine was used as the DNA delivery vector for *in vivo* transformation of the lung. pCDNA3.1 plasmid complexed with a apoEdp-PLL harboring beta-galactosidase reporter gene was injected to the tail vein of 5 male Balb/c mice.

Frozen sections were prepared two days after injection from lung tissue and stained with X-gal for transgene activity assay. A Beta-galactosidase reporter gene activity detection kit was used for quantitative assessment of gene expression. The beta-galactosidase activity level of 180 ng/mg of protein was detected where as in all control tissues tested the values were 0.05 ng/mg.

Using a specific promoter for exclusive gene expression in lung tissue, therapeutic genes, either for cancer or monogenic diseases, would be delivered to the lung tissue.

P 10

"Double-Edged" arsonium-containing lipophosphoramides: gene transfection compatibility with antibacterial properties

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Gene therapy of diseases such as cystic fibrosis consists of the delivery of a gene medicine in a complex environment where colonization by multiple bacterial strains can compromise its success. In the course of the development of novel gene delivery systems, our group has reported over the past decades new classes of cationic lipids as efficient gene transfer agents for both *in vitro* and *in vivo* applications. In the present study, we wondered

whether these compounds could also behave as cationic biocides when mixed with a bacterial strain. By conducting a systematic screening of the properties of diverse chemical structures, we found that arsonium-containing cationic lipids can display efficient to very efficient antimicrobial activities against various bacteria, including multi-resistant virulent clinical strains. Interestingly, their antibacterial properties were compatible with their gene transfer activity as (i) corresponding efficient concentrations were of the same order of magnitude, (ii) DNA condensation and protection against degradation were not impaired, and (iii), using *in vitro* cell culture models, it was possible to inhibit bacterial growth while still transfecting eukaryotic cells. Thus, arsonium-containing cationic lipids appear as promising versatile multifaceted agents which could find various applications for the treatment of inherited as well as acquired diseases.

P 11

Hepatic differentiation of simian pluripotent stem cells: a tool for cell therapy applications

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Hepatocyte transplantation is becoming an alternative to orthotopic liver transplantation for the treatment of metabolic diseases. However, there is a lack of donor organs and isolated mature hepatocytes are difficult to manipulate *in vitro*. Pluripotent stem cells, either embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), may become an alternative source of hepatocytes. However, it is necessary to develop large animal models, which include non-human primates, of ESC- and iPSC-derived hepatic cells before envisaging clinical trials. These models will also allow for the *in vivo* evaluation of the degree of liver function replacement obtained and safety issues. The aim of this work is to generate hepatic progenitor cells and hepatocytes from monkey ESCs and iPSCs and to characterize them *in vitro* for their phenotype markers and functions. We have set up chemically defined conditions devoid of serum, to derive rhesus monkey ESCs into endodermal cells that express specific markers such as Sox17, FoxA2. iPSCs from cynomolgus monkey fibroblasts were generated using four Moloney-derived retroviral vectors that express human Oct4, Sox2, Klf4 and c-Myc. The isolated iPSC clones express pluripotency markers that include Oct4, SSEA4, Nanog. These cells were then differentiated into endodermal cells expressing the aforementioned markers. We are presently defining optimal conditions to generate hepatic progenitors from both ESC-derived and iPSC-derived cells and compare their phenotypes. We will then generate pluripotent cells from fibroblasts isolated from skin biopsies of three cynomolgus monkeys, differentiate them into hepatic cells and transplant them into the autologous donor liver after cell expansion.

P 12

Vevo-Micromarker[®] microbubbles: A new and promising generation of microbubbles for enhanced *in vitro* gene delivery by sonoporation

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Among the non-viral methods for *in vitro* gene delivery, the use of ultrasound waves with gas microbubbles as a safe tool to deliver pDNA to tissues and organs has been rapidly developing throughout the past decade. This method transiently increased the native permeability of cell membranes when submitted to ultrasound in the presence of microbubbles. This process is commonly known as sonoporation.

In this report, we investigated the use of new generation of microbubbles, Vevo-Micromarker[®], for *in-vitro* gene transfer and we compared to BR14[®] and SonoVue[®]. The acoustical properties of the microbubbles were then measured to help understand the biophysical mechanism involved in the pDNA delivery.

Human glioblastoma (U-87MG) cells are transfected with GFP-encoding plasmid. 24h and 48h later, the transfection level and the cell viability are measured by flow-cytometry after propidium iodide staining. Our results showed that the transfection level achieved with Vevo-Micromarker[®] was much higher than BR14[®] and SonoVue[®] with a comparable cell viability. For the first time, a transfection rate of nearly 70% was reported using the combination of ultrasound and microbubbles. Furthermore, the persistence of the microbubbles to insonation was directly correlated to the induced transfection efficiency. Indeed, Vevo-Micromarker[®] exhibited the lowest attenuation coefficient than BR14[®] and SonoVue[®]. These findings indicated that the attenuation and the destruction of microbubbles might be involved in the sonoporation efficiency.

In conclusion, our results demonstrated that the combination of ultrasound and Vevo-Micromarker[®] could be a promising method for efficient gene delivery.

P 13

Transendothelial passage of polyplexes across a pulmonary vascular endothelium

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To treat cystic fibrosis by gene therapy, systemic administration of CFTR gene complexed with synthetic vectors is an interesting alternative compared to the aerosol administration. Indeed, the presence of thick mucus reduces the access to airway epithelial cells. However, the endothelial barrier of pulmonary capillaries represents a limiting step for transfection of underlying airway epithelial cells.

We have assessed the transendothelial passage of polyplexes (DNA/polymer complexes), in a human lung endothelium model constituted of HMVEC-L cells cultured on a polyethylene

terephthalate insert with 3 μm pore size. The formation of the endothelial monolayer was controlled by the transendothelial electrical resistance. The human tracheal epithelial ($\Sigma\text{CFTE29}\alpha$) cells from a CF patient homozygous for the ΔF508 mutation of CFTR gene were seeded at the bottom of the well. Their transfection efficiency was used to measure the transendothelial passage of a series of polyplexes formed with linear polyethyleneimine substituted by histidine groups. The results indicate that His₁₆-IPEI polyplexes were the most efficient both in terms of transendothelial passage of HMVEC-L cells, transfection efficiency of $\Sigma\text{CFTE29}\alpha$ -cells, and cell viability. Quantitatively, we found that about 45% of polyplexes passed through the endothelial monolayer.

Further work is in progress to delineate mechanisms of polyplexes transendothelial passage. Subsequently, this system will be used to assess the capacity of transendothelial passage of different types of DNA complexes including lipopolyplexes and lipopolyplexes.

P 14

Isolation of cardiac progenitors from NKX2.5-eGFP transgenic human embryonic stem cells

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Embryonic stem cells (ESCs) are a promising source for cardiac cell therapy but their potential use in human has been hampered by major safety concerns. Here, we describe the generation of transgenic human ES cells (hESCs) that allow the isolation of a population of committed human cardiac progenitors depleted of undifferentiated cells. This approach is based on the use of the *Nkx2.5* cardiac-restricted promoter to drive the expression of a reporter gene (eGFP). Sorted *Nkx2.5*-GFP⁺ hESCs showed a gene profile of cardiac progenitors with a concomitant expression of *GATA4*, *ISL1*, *MEF2C* and *TBX5* and the absence of *Nanog* and *Oct-4*, two genes reflecting the undifferentiated state of hESC. Loss of tumorigenic potential was confirmed in a model of Nod SCID mice, in which no teratoma was detected after 2 months. In *in vitro* differentiation assays, these cells gave rise mainly to smooth muscle cells and cardiomyocytes, and at a lesser extent to endothelial cells. After administration in a cardiac environment, *Nkx2.5*-GFP⁺ hESCs could still be detected after 2 months and their cardiac, endothelial and vascular smooth muscle development potential was confirmed at a molecular and phenotypic level. These results demonstrated the potential for this approach to provide an innovative tool for cardiovascular research and regenerative medicine.

P 15

Value of large-scale expansion of tumor infiltrating lymphocytes in a compartmentalised gas-permeable bag: interests for adoptive immunotherapy

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Background: Adoptive cell therapy (ACT) has emerged as an effective treatment for patients with metastatic melanoma.

However, there are several logistical and safety concerns associated with large-scale *ex vivo* expansion of tumour-specific T lymphocytes for widespread availability of ACT for cancer patients. To address these problems we developed a specific compartmentalised bag allowing efficient expansion of tumour-specific T lymphocytes in an easy handling, closed system.

Methods: Starting from lymph nodes from eight melanoma patients, we performed a side-by-side comparison of Tumour-Infiltrating Lymphocytes (TIL) produced after expansion in the compartmentalised bag versus TIL produced using the standard process in plates. Proliferation yield, viability, phenotype and IFN γ secretion were comparatively studied.

Results: We found no differences in proliferation yield and cell viability between both TIL production systems. Moreover, each of the cell products complied with our defined release criteria before being administered to the patient. The phenotype analysis indicated that the compartmentalised bag favours the expansion of CD8⁺ cells. Finally, we found that TIL stimulated in bags were enriched in reactive CD8⁺T cells when co-cultured with the autologous melanoma cell line.

Conclusions: The stimulation of TIL with feeder cells in the specifically designed compartmentalised bag can advantageously replace the conventional protocol using plates. In particular, the higher expansion rate of reactive CD8⁺ T cells could have a significant impact for ACT.

P 16

Gene therapy for retinal dystrophies using human cellular models: differentiation of disease-specific iPS cells into retinal pigment epithelium

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The retina is highly amenable to, and many retinal dystrophies ideal candidates for, gene therapy. The first such clinical trials were carried out in 2008 using an AAV vector with positive results. Consequently, the requirements for future preclinical studies of other diseases will be less stringent, notably concerning the need for large animal disease models. However, a growing number of diseases lack even an appropriate small model, compromising their chances of one day reaching a clinical trial. In such cases, a viable alternative would be to perform preclinical studies on human cellular models of the pathogenic retina.

As it is impossible to obtain retinal cells from a patient, the aim of our work is to generate iPS cells from skin fibroblasts of patients with a retinal dystrophy and differentiate these cells into retinal pigment epithelium (RPE). As a pilot project we are concentrating on the X-linked disease choroideremia that represents 2% of retinal dystrophies and is due to mutations in the *CHM* gene encoding the Rab escort protein 1 (REP1) protein; the mouse and zebrafish REP1-deficient models are lethal. We have obtained the first iPS clone of a choroideremia patient with the aim of transducing the future REP1-deficient RPE with a *CHM*-expressing AAV vector and assaying for the restoration of a normal cellular phenotype. The use of such innovative human disease systems are more economical and less time-consuming alternatives to animal models, as well as more biologically

relevant for further understanding disease pathogenesis and preclinical therapeutic studies.

P 17

Biodistribution and shedding of rAAV8-U7 snRNA vectors after locoregional injection in the forelimb of GRMD dogs

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In Duchenne Muscular Dystrophy (DMD) the selective removal by exon skipping of exons flanking an out-of frame mutation in the dystrophin messenger can result in in-frame mRNA transcripts that are translated into shorter but functionally active dystrophin.

The goal of our project is to determine in Golden Retriever Muscular Dystrophy (GRMD) dogs the effective dose of our therapeutic product defined as a recombinant Adeno-Associated Virus serotype 8 (rAAV8) expressing a optimized U7 snRNA specific for the correction by exon skipping of the GRMD dystrophin transcript. The mode of delivery is the locoregional high-pressure intravenous (IV) injection of a forelimb.

We established a unique network of laboratories with complementary skills to deliver a comprehensive set of preclinical data. Several groups of GRMD dogs were exposed to different rAAV8-U7snRNA doses (2,5E12 to 2,5E13 vg/kg). To assess the biodistribution and shedding patterns of the vector, biological fluids were repeatedly collected as soon as 6 hours post-injection and subsequently for weeks. Ultimately, all animals were sacrificed \approx 3 months after injection of the vector. All muscles of the injected limb and of the contralateral limb were precisely sampled, as well as several skeletal muscles at distance and major organs.

The complete biodistribution and shedding data, obtained after quantitative PCR analysis of these different samples will be presented.

P 18

Gene therapy approach for Huntington's disease

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Huntington's disease (HD) is a fatal dominant hereditary neurodegenerative disease, that typically emerges in adulthood and results in motor, cognitive and behavioural abnormalities. HD has a single genetic cause (abnormal expansion of CAG trinucleotide repeats in the gene encoding huntingtin) and a well-defined striatal neuropathology.

There is no available treatment for HD. The therapeutic approaches are targeted on mitochondrial dysfunctions, or excitotoxicity. Recently, it has been shown that the brain cholesterol

metabolite, 24S-hydroxycholesterol, is decreased in the plasma of HD patients. We recently showed that expression of CYP46A1 mRNA, the enzyme responsible for the conversion of cholesterol into 24S-hydroxycholesterol is decreased into the striatum of the R6/2 mouse model of HD at early stages (6 weeks). Furthermore, overexpression of CYP46A1 is neuroprotective in a cellular model of HD. We thus aimed to restore CYP46A1 expression in the striatum of R6/2 HD mouse models, using a *new serotype (rh10) of AAV vector (AAV10)*. We characterized transduction and tropism of AAV10rh-GFP, and showed widespread expression of the transgene within the striatum (around 63% of the whole rostro-caudal extension of the striatum), along with the synaptically connected brain regions. As expected, the transgene showed a particular tropism for neuronal cells. Ongoing experiments aim to demonstrate the effects of CYP46A1 overexpression on the hallmarks (behavioural, biochemical and neuropathological) of HD pathology in R6/2 mice. Overall these studies will provide a body of preclinical information to address the feasibility and the potential efficacy of gene therapy in HD patients based on targeted overexpression of CYP46A1 enzyme.

P 19

In vivo inhibition of cholesterol-24-hydroxylase induces a sequential and progressive neuronal loss in hippocampus of C57BL/6 mice

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The abnormalities of cholesterol metabolism are closely related to neurodegenerative disorders. The neurons cannot degrade cholesterol by themselves and the blood-brain barrier restricts its export out of the brain. The major mechanism of cerebral cholesterol clearance involves the conversion of cholesterol into 24S-hydroxycholesterol by neuronal cholesterol 24-hydroxylase, an enzyme encoded by the CYP46A1 gene. 24S-hydroxycholesterol can freely cross the blood-brain barrier and is degraded in the liver.

Intracerebral injection of AAV5 vector encoding CYP46A1 in a mouse model of Alzheimer disease (APP23) decrease amyloid pathology and improve cognitive function.

To further characterize the role of CYP *in vivo*, we studied the effect of cholesterol 24-hydroxylase gene down-regulation through RNA interference strategy.

Among eleven shRNA, only AAV5-sh271 was chosen for its highest efficiency to decrease CYP46A1 expression and brain 24S-hydroxycholesterol content (-40%) *in vivo* after injection in the hippocampus of C57BL/6 mice.

One month after injection in the cortex and the hippocampus of three month old C57BL/6 mice, neuronal loss in the CA2 and CA1 layer of the hippocampus were observed which progress to the CA3 layer to finally lead to a complete destruction of hippocampus three months after injection. The emergence of hippocampal lesions was accelerated in APP23 mice and was associated with 3-fold increase of A β 40/42 peptides. This observed toxicity on neurons is specific for CYP downregulation and not to a non specific one of the RNAi strategy.

Electrophysiological and neuropathological investigations are ongoing to understand the mechanisms of neurotoxicity. These results confirm the crucial role of CYP46A1 in the hippocampus.

P 20

Therapeutic approach by “genetic compensation” for dysferlinopathies

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Mutations in DYSF encoding dysferlin cause primary dysferlinopathies, autosomal recessive diseases that mainly present clinically as Limb Girdle Muscular Dystrophy type 2B and Miyoshi myopathy. Nowadays, there is no curative treatment but some therapeutic approaches are being investigated.

Dysferlin is composed of homologous C2 domains and a C-terminal transmembrane domain. Even if, these C2 domains differ from each other by the interaction established with dysferlin partners, there is some evidence that they could have redundant or independent functions. Based on the clinical observation that some deletions have been associated with moderate phenotype, we are investigating two approaches of “genetic compensation” for dysferlinopathies: exon skipping and “miniproteins” transfer.

Firstly, an in-frame deletion of exon 32, resulting in “quasi-dysferlin” expression, has been found in a patient presenting with a mild and late-onset phenotype. Thus, we have postulated that exon 32 of dysferlin could be a target for an exon-skipping therapeutic strategy. We have then developed Antisense Oligo-Nucleotides designed to block essential sites for the maintenance of exon 32.

Similarly, a “minidysferlin”, composed only by the two last C2 domains and the transmembrane domain has been discovered in a patient with no severe symptoms. Here again we used this natural proof of concept for a gene therapy. The minigene approach overcomes the limited packaging size of AAV vectors, the best vector so far for muscular disorders. Several combinations of dysferlin domains will be tested to obtain the most functional “minidysferlin”.

Finally, we think that both approaches, based on clinical data, could be the starting point for a therapy in the dysferlinopathy fields.

P 21

Therapeutic approach of TMD and LGMD2J by titin trans-splicing

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Titin is a giant protein expressed in both skeletal muscles and heart. Several pathogenic mutations were identified in its last coding exon. The most frequent mutation commonly referred to as FINmaj, results in the replacement of 4 amino acids and affects a number of patients in Finland. The mutation causes a Tibial Muscular Dystrophy (TMD) when present on one allele and a Limb Girdle Muscular Dystrophy phenotype 2J (LGMD2J) when present on both alleles.

To date, there is no treatment for these diseases. Considering the large size of the titin cDNA (about 100kb), classical gene transfer strategies are not feasible as therapeutic approach. To bypass this

hurdle, spliceosome-mediated RNA trans-splicing (SMaRT) is being tested to replace titin last exon at mRNA level. HER911 cells expressing a titin minigene with the FINmaj mutation were transfected with a plasmid coding for a 3' pre-trans-splicing molecule encoding a binding domain (BD) specific for the last intron, an intronic sequence followed by the wild-type last exon. RT-PCR and western blot analyses showed that specific replacement of the mutated 3' portion of the titin minigene transcript occurred through trans-splicing. PTM constructs carrying different BD were tested and the result showed that localization of these BD modifies the trans-splicing efficacy. Following this proof-of principle at cellular level, experiments to deliver trans-splicing RNAs in a murine model carrying the FINmaj mutation are currently being performed in our laboratory.

P 22

Trans-splicing approach for dysferlinopathies

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Deficiency in **Dysferlin** causes two major phenotypes: **Limb Girdle Muscular Dystrophy type 2B** (LGMD2B) and **Miyoshi Myopathy** (MM). There is no treatment to date for these diseases. Besides the traditional viral-mediated gene transfer strategy, which is complicated by the large size of the dysferlin cDNA, there are alternative strategies that can be tested such as the **RNA reprogramming by spliceosome mediated RNA trans-splicing**. *Trans-splicing* is achieved using a nuclear pre-mRNA splicing event in *trans* between the mutated endogenous pre-mRNA and the exogenous therapeutic **pre-mRNA trans-splicing molecule (PTM)** which provides the correct RNA sequence. In the case of dysferlinopathies, this strategy would have the advantage of by-passing the possibility of any **toxicity** related to dysferlin over-expression since the endogenous transcriptional control is conserved. In addition, it would be applicable to **any type of mutations** with the exception of very large deletions.

To obtain a **proof of feasibility**, we constructed a PTM targeting the last exons of dysferlin and a human dysferlin **minigene** to be used as *trans-splicing* target. Following co-transfection of these constructs in HER911 cells, we validated transcription of the minigene and PTM by RT-PCR, and demonstrated that **trans-splicing had occurred**. Western Blot analysis using antibody to detect specifically the *trans-spliced* protein showed a band indicating that the **trans-spliced RNA had been translated**. Following these preliminary results, we are now testing this PTM on human myogenic cells from dysferlin patients and are also evaluating a similar strategy to reverse the pathological signs observed in dysferlin deficient mouse model.

P 23

Use of miRNA-regulated AAV vector to reduce humoral immune responses against alpha-sarcoglycan

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Alpha-Sarcoglycanopathy (Limb-Girdle Muscular Dystrophy type 2D, LGMD2D) is a recessive muscular disorder caused by

deficiency in α -sarcoglycan, a transmembrane protein part of the dystrophin-associated complex. We previously reported efficient AAV-mediated transfer of α -sarcoglycan in α -sarcoglycan deficient mice (*Sgca*-null mice) resulting in correction at the biochemical, histological and functional levels. However, specific humoral immune response directed against the transgene was induced after intramuscular injection, leading to disappearance of transgene expression in muscle fibres. The use of a muscle-specific promoter like desmin improved transgene expression but continued to elicit a weak humoral response. Since expression of a neotransgene in antigen-presenting cells (APC) can effectively prime immune responses, we decided to switch off the expression of the transgene in APC by using microRNA (miRNAs) regulating properties. Indeed, miRNAs are small noncoding RNAs that have very specific expression profiles and regulate gene expression by repressing translation of target cellular transcripts. We incorporated a target sequence of miRNA142-3p (miR142-T) in the AAV cassette containing the α -sarcoglycan transgene under the transcriptional control of the ubiquitous promoter CMV. After viral preparations in serotype 1, we injected the constructs with and without miR142-T intramuscularly in the *Tibialis Anterior* of *Sgca*-null mice. Analyses of muscle biopsies at day 84 and of blood sera showed that the use of miRNA-regulated vector led to increased survival of muscle fibres associated with long-term expression of α -sarcoglycan and reduced humoral immune responses against the transgene. This promising strategy could facilitate sustained therapeutic effects of rAAV gene transfer in muscle.

P 24

Efficient motor neuron and sensory neuron transduction by intrathecal AAVrh10 gene transfer in rats

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Recombinant AAV vectors are promising tools for safe and efficient clinical gene therapy. They lead to long-term gene expression in brain and spinal cord with no detectable toxicity and are thus attractive vectors for gene transfer to the central nervous system (CNS). Currently, direct CNS injections of rAAV vectors are performed to treat neurodegenerative diseases. However direct delivery into the brain is invasive and requires multiple injections to treat diffuse CNS diseases because of its relatively local transduction. In order to investigate broader CNS gene transfer methods we initiated a gene therapy strategy based on intrathecal injection of Adeno-Associated Virus of serotype 10 (AAVrh10) encoding EGFP.

We show that AAVrh10 has a strong tropism for CNS both in neonates and adults. Four weeks after a single intracisternal administration (1.0 to 4.10¹¹ vg) in rats, single or double-stranded vectors lead to a strong transduction of the brain, cerebellum and spinal cord. Neurons are mainly targeted: EGFP is strongly expressed in pyramidal and subcortical neurons, Purkinje cells and motor neurons from cervical to lumbosacral spinal cord. Sensory neurons in dorsal root ganglia are also transduced. A strong expression of the transgene is observed in peripheral nerves and dorsal ascending spinal tracts as a consequence of the high proportion of transduced motor

and sensory neurons respectively. This widespread transduction of upper and lower motor neuron and of sensory neurons offers promising perspectives in the field of motor neuron diseases, sensory neuron diseases, and chronic pain therapies.

P 25

Antigen-presenting cells interact with rAAV1 vectors and differentially present capsid and transgene MHC class II determinants

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rAAV vectors have been successfully used for long-term expression of therapeutic genes. It is postulated that the maintenance of gene-modified cells upon rAAV1 transfer is due to its relative inefficiency to transduce antigen-presenting cells (APC). However the interactions between APC and rAAV1, as well as the functional consequences in term of anti-capsid and anti-transgene immune responses, remain unexplored.

We generated Alexa-stained rAAV1 vectors to analyze their ability to target different APC populations, ie CD11c+, CD11b+, CD19+ cells, both *in vivo* and *in vitro* in mice. We found that rAAV1 vectors interact with all the populations tested, but mainly macrophages both *in vivo* and *in vitro*. Moreover, APC capture rAAV1 in a dose-dependent manner that could be completed by non-labelled rAAV1, suggesting the presence of specific receptors on APC. To further analyze the immunological outcome, we examined if APC can present antigens from the capsid and the transgene to activate specific CD4+ T cells *in vivo*. We observed that CD11c+ cells present both capsid- and transgene-derived antigens, whereas CD19+ and CD11b+ cells only present capsid and transgene determinants respectively. This antigenic presentation was functionally relevant since rAAV1-loaded CD11c+ trigger anti-capsid and anti-transgene CD4+ T cell responses in mice, while rAAV1-loaded CD11b+ only induce an anti-transgene immune response.

In conclusion, macrophages efficiently capture rAAV1 particles and are transduced since they present transgene determinants, but they inefficiently present capsid epitopes. rAAV1 uptake by dendritic cells is not as efficient, but results in a strong immunogenicity to capsid and transgene determinants.

P 26

DNA immunization in an allergic asthma mouse model

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Asthma is a chronic respiratory disease due to repeated allergen sensitization, in particularly *Dermatophagoides farinae* allergen. Specific immunotherapy, and particularly DNA immunization is a promising approach to treat and prevent allergic diseases. The aim of this study is to understand immunological mechanisms of DNA immunization against Derf1 in a mouse model of allergic asthma induced by *Dermatophagoides farinae*.

Method: Derf1 encoding plasmid formulated with synthetic vector was administered using i.m. injection following a prime-boost protocol in healthy or asthmatic mice. Immune response

was measured by enzyme-linked immunosorbent assay and ELISPOT analysis.

Results: Intramuscular administration of Derf1 encoding plasmid formulated with synthetic vector lead to a strong and specific humoral response against Derf1 antigen. We have tested this formulation in a mouse model of allergic asthma, in order to evaluate the ability of our immunization protocol to bias the T-helper (Th) cell response to a Th1 phenotype.

Discussion: DNA immunization against Derf1 allergen following a prophylactic approach would prevent allergic response against house dust mite, which will be a promising approach to prevent allergic respiratory diseases.

P 27

Intramuscular AAV8 delivery in mice results in intricate vector interactions with the host immune system and dendritic cell transduction

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Recombinant AAV (rAAV) vectors appear to be vehicles of choice for gene transfer to the skeletal muscle. However, recent gene transfer studies into animal models and even humans indicate that the risk of transgene and/or capsid-specific immune responses is high following intramuscular (IM) rAAV delivery. It has been hypothesized that these immune responses are correlated to intricate interactions of the vector with the host immune system, due to high vector concentration at the site of injection. We explored the early relationship between the host immune system and an AAV8 vector expressing GFP, following its IM delivery in a mouse model. Kinetics of vector biodistribution and transcript detection in draining lymph nodes and spleen were analyzed and immune cell transduction was explored. We detected viral genomes as early as day 1 post-injection in lymphoid organs. Transgene transcripts were detectable from day 4 post-injection in draining lymph nodes. Interestingly, we were able to detect both viral genomes and GFP transcripts in dendritic cells (DC). DC transduction was further confirmed by immunohistochemistry in draining lymph nodes as well as spleen. Moreover, we were able to observe DC transduction, with both self complementary (sc) and single stranded (ss) AAV8 vectors. These results indicate that transgene immune rejection could be initiated by DC-mediated direct antigen presentation, in addition to the crosspresentation mechanism that was favoured in early AAV studies.

P 28

Intrathymic injection of lentiviral vector curtails the peripheral immune response in BALB/c mice

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Lentiviral vectors are promising tools for gene therapy. Here, we have investigated whether pre-conditioning of normal BALB/c mice with intrathymic injection of lentiviral vectors expressing the hemagglutinin (HA) of the Influenza virus could prevent the immune response against this antigen in the periphery. We first evaluated the distribution and persistence of a lentiviral vector expressing luciferase after intrathymic injection. Luciferase signal could be detected in the thymus for over 40 days and rapidly vanished thereafter. In some mice, the signal was also observed in the periphery, showing that even surgically-performed intrathymic injections could leak outside of the thymus. We then observed that the vector expressing HA was able to induce a robust CD4 and CD8-mediated immune response in the periphery that could prevent the development of a tumor expressing HA, illustrating the power of lentiviral vectors for vaccination purposes. After intrathymic injection, some treated mice presented an efficient HA-specific lytic activity in the spleen without peripheral immunization, suggesting that leakiness of the injection could result in immunization. We also observed that mice surgically manipulated were resistant to immunization few days after the surgery. This immuno-incompetence was time-dependent as a normal immune response against HA was recovered 3 weeks after the procedure. At that time, we show a dose-dependent effect of intrathymic injection of the vector on the immune response elicited in the periphery. Altogether, our results establish the conditions and feasibility of intrathymic injection of lentiviral vectors for manipulating T cell tolerance in the thymus for future clinical applications.

P 29

An experimental model set-up to study MHC class II targeted gene transfer in antigen-presenting-cells

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Our objective is to target gene transfer into various types of MHC-II⁺-antigen-presenting cells (APC) to study the impact on the cellular immune responses against the transgene. We herein report the experimental model set-up for this study. A novel immunogenic transgene was generated by fusing GFP to murine male antigenic epitopes from the HY gene. Cells expressing the GFP-HY transgene were detected by FACS with reduced (about a log) fluorescence levels compared to native GFP. To target APCs for gene transfer *in vivo*, we used lentiviral vectors pseudotyped with engineered measles envelope glycoproteins targeted to MHC class II with a ScFV (Ageichik *et al.*, 2011). The vector is produced by co-transfecting 5 plasmids in 293T cells. Under optimal conditions, viral stock containing 90 ± 60 ng/ml p24 (n=7) is produced. The vector concentrated by ultracentrifugation (2500g, 12h, 4°C) yields 15-25% of p24 and concentration increased p24 titers by about 50 fold. Concentrated preparations contained about 1500 ng/ml reverse transcriptase (RT). Intravenous injection of a concentrated vector lot (30 to 40 ng of RT i.e. 120 or 200ng of P24 per female mouse) induced a clear and

specific immune response. Fourteen days after injection, spleen cells re-stimulated *ex vivo* with the Dby or Uty peptides (respectively CD4 and CD8 epitopes) generated 200-400 IFN γ SFU/millions lymphocytes whereas PBS-injected animals did not respond. Future experiments will consist of directing transgene expression in different MHC Class II+ APCs through microRNA usage and study the effects on immune responses.

P 30

Agonistic anti-CD137 antibody treatment leads to anti-tumor response in mice with orthotopic liver tumor

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Immunotherapy represents a potential therapeutic option for patients with hepatocellular carcinoma (HCC). Patient's survival is directly correlated to the type and number of tumour infiltrating immune cells, indicating that immune responses have a direct effect on the clinical course of the disease. CD137, a member of the TNF receptor family, is expressed on various immune cells and plays an important role in sustaining T cells activation, amplifying cytotoxic T lymphocyte response, and co-stimulating both Th1 and Th2 cytokine production. We have assessed the potential of immunotherapy with an agonistic anti-CD137 antibody in an orthotopic murine model of HCC.

Injection of 2.5×10^6 Hepa1.6 hepatoma cells into the portal vein of C57Bl/6 mice results in orthotopic liver tumors which develop within 3 weeks. Groups of mice received intraperitoneal injections of 100 μ g of anti CD137 antibody or control rat IgG on day 4 and 8 after tumor challenge and were sacrificed on day 18 to monitor tumor development. All animals in the control group showed an important tumor burden whereas 57% (4/7) of the anti-CD137 antibody-treated animals were completely free of tumor growth. Moreover, these mice remained tumor-free when re-challenged with a new load of Hepa1.6 cells, demonstrating a long-term protection. *In vivo* mAb depletion experiments demonstrated that this therapeutic effect required CD8 and NK cells.

In conclusion anti-CD137 antibody demonstrated a potent anti-tumor effect on a preclinical *in situ* liver tumor model and is therefore a promising strategy for the treatment of HCC.

P 31

Osteoprotegerin administrated by non viral gene transfer decreased lytic bone lesions and prevents tumor development in a xenogenic model of Ewing's sarcoma by inhibiting RANKL produced by tumor cells

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Ewing's sarcoma (ES) is a solid tumor typically arising in the bones of children and adolescents. Long-term survival rates reach 50% at 5 years but only 20% for patients with clinically detectable metastases at diagnosis, not responding to therapy or with disease relapse. Osteoprotegerin (OPG), an anti-bone re-

absorption molecule may represent a promising candidate to inhibit the osteolytic component of ES and subsequently to limit tumor development. OPG was administered *in vivo* by gene transfer using the amphiphilic non-ionic block co-polymer vector LUTROL. A xenogenic model of ES was induced by intra-osseous injection of 2×10^6 human TC71 ES cells. ES bearing mice were assigned to control (CT: no treatment), LUTROL (vector alone), pcDNA3.1 (LUTROL/empty pcDNA3.1 plasmid), hOPG (LUTROL/pcDNA3.3-human OPG) and mOPG (LUTROL/pcDNA3.1-mouse OPG) groups. Mice were treated once a week with $2 \times 50 \mu$ g DNA-LUTROL beginning 7 days before tumor induction. hOPG transgene expression was validated *in vitro* at the transcript and protein levels and confirmed *in vivo* at the systemic (serum) and local (tumor) levels. *In vivo*, a significant inhibition of tumor development was shown in the hOPG group as compared to control groups (CT and LUTROL). A marked osteolysis was revealed by micro-architecture analyses in all control (CT, LUTROL, pcDNA3.1) groups and also in the mice treated with mOPG, whereas osteolysis was significantly prevented in the hOPG group. By using ELISA test specific to the species, we demonstrated that RANKL over-expressed in ES was from human origin (expressed by tumor cells) rather than mouse origin (host cells).

P 32

Partial correction of liver metabolism by scAAV-mediated gene therapy in a mouse model of glycogen storage disease type 1a did not prevent the development of hepatic tumours

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Glycogen storage disease type 1a (GSD1a) is caused by the deficiency of glucose-6 phosphatase (*G6pc*) gene, encoding an essential enzyme for glucose production. Patients with GSD1a suffer from severe hypoglycemia, hepatomegaly and liver steatosis. Despite a strict diet, patients develop liver tumours with age. In this study, we analyzed the efficiency of gene therapy on the long-term liver pathology in mice with an inducible liver-specific *G6pc* deficiency (Mutel *et al.*, 2011).

At weaning, liver *G6pc* deletion was induced by tamoxifen treatment of B6.g6pc^{lox/lox}.SACre^{ERT2/+} mice to obtain Lg6pc^{-/-} mice. Four weeks later, mice were injected with scAAV-LP1-G6PC [6x10¹¹Vg/kg] via the retroorbital vein. To accelerate the development of hepatic tumours, a group of mice were fed on a high fat/high sucrose (HF/HS) diet for 9 months and nodules were detected by MRI.

During 8 months, blood glucose of treated mice fed on the standard diet was maintained after 6h fasting whereas hypoglycemia occurred after this time in untreated Lg6pc^{-/-} mice. The weight, glycogen and triglyceride contents of liver treated with scAAV-LP1-G6PC were significantly decreased compared to Lg6pc^{-/-} mice, but not completely normalized referring to wild-type mice. After 7 months on HF/HS diet, hepatic nodules were detected in only 3 treated mice by scAAV-LP1-G6PC (n=8), although more than 60% of untreated Lg6pc^{-/-} mice (n=20) developed hepatic tumours. However, all mice developed hepatic tumours after 9 months on HF/HS diet.

Our data suggest that AAV-gene therapy may delay but not prevent the development of hepatic tumours in GSD1a.

P 33

Use of lentiviral vectors to sensitize pancreatic cancer cells to chemotherapy

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Introduction: There is no efficient treatment for pancreatic cancer. Gemcitabine chemotherapy improves the patient's clinical status but survival is not prolonged. Because of the promise of lentiviral vectors, we tested their ability to deliver deoxycytidine kinase (DCK), a key enzyme in gemcitabine metabolism, so as to sensitize pancreatic cancer-derived cells to chemotherapy.

Methods and Results: We produced HIV-1 based lentiviral vectors encoding for human DCK (LV-DCK) or copGFP (LV-GFP). Human MIA Paca-2 and Capan-2 pancreatic cancer-derived cells are readily transduced using these vectors (75 to 95% of positive cells, flow cytometry analysis). Transduction with LV-DCK results in DCK mRNA and protein over-expression. We found that stable clones expressing DCK are 50 times more sensitive to gemcitabine than control cells expressing copGFP (2D and 3D cell proliferation, cell viability). In addition, pancreatic cancer cells treated with LV-DCK are blocked in S phase (cell cycle analysis) and enter apoptosis (annexin-V labeling, caspase activity, Western blotting analysis). Last, we proceeded to DCK gene transfer in proliferating MIA Paca-2 cell cultures. As a consequence, cell viability as well as cell proliferation were strongly altered.

Conclusion: Taken together, these results strengthen the high efficiency of lentiviral vectors to deliver genes into pancreatic cancer-derived cells. As a consequence, DCK transduction strongly sensitizes pancreatic derived-cells to gemcitabine chemotherapy. This study is essential to benchmark the use of therapeutic vectors for the management of pancreatic cancer, in the continuity of the THERGAP gene therapy trial currently performed by the team.

P 34

Gene therapy for ovarian cancer: initial results with intraperitoneal infection of AAV vectors

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It is known that peritoneal dissemination of cancer cells is characteristic of advanced stages of ovarian cancer. One of the novel therapeutic strategies available is cancer gene therapy with recombinant adeno-associated vectors (rAAV). The rAAV vectors represent the most promising gene delivery vehicles to cancer cells within serosal cavities. In our experiments rAAV2/2

vectors were produced in a helper-free system using an AAV-293 packaging cells, and analyzed by real-time PCR. The obtained viral vectors and non-viral preparations as a control (pDNA:PEI and pDNA:aminoprenols) were intraperitoneally administered to Balb/c mice pre-injected with L1 cancer cells. Subsequently, the mice were sacrificed and intraperitoneal effusions were analyzed for rAAV and pDNA sequences and the efficiency of gene transfer to cancer cells disseminated into peritoneal cavity was estimated. We reported an efficient infection of L1 cancer cells disseminated into the peritoneal cavity by rAAV2. The expression of reporter genes (GFP and LacZ) attributable to the rAAV cell uptake was dependent on an applied multiplicity of infection ratio (MOI). The highest infection efficiency was observed at the highest rAAV doses. This work documents the ability of rAAV to facilitate gene transferability to cancer cells disseminated in the serosal cavity, as well as the potential usefulness of these viruses as a new approach in cancer gene therapy in the clinic.

P 35

Development of a clinically-applicable lentiviral vector for the gene therapy of RS-SCID

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RS-SCID is a primary severe combined immune deficit caused by mutations in the Artemis gene. RS-SCID patients lack T and B cells and have an increased sensitivity to ionizing radiations. Several studies have already corrected models of the disease following Artemis lentiviral gene transfer. These results warrant further development of a clinically-applicable vector for a phase I/II gene therapy trial.

The proposed clinical vector expresses the human Artemis cDNA under control of an intronless EF1a promoter. To test if a post-transcriptional regulatory element is needed we evaluated the biopotency of vectors with or without WPRE in human Artemis-deficient cells (Guetel) and Sca1+ bone marrow cells of Artemis KO mice.

Transduction in Guetel cells showed a good correlation between vector copy number, mRNA and protein expression, according to the LV concentration. With a mean vector copy number equal to 1, the vector with a WPRE expressed 5 fold-higher levels of mRNA than the vector without WPRE whereas the vector without WPRE expressed normal levels of Artemis mRNA, as evaluated on single cell clones. Importantly, the expression of high levels of the Artemis transgene caused toxicity in Guetel cells and BM-Sca+ cells. It is therefore important to maintain low levels of this transgene. The vector without WPRE is a good candidate for this option. Future experiments are planned to evaluate its biopotency in murine models and a formal preclinical biosafety evaluation will be conducted to obtain clinical trial authorization for a phase I/II gene therapy trial of RS-SCID.

SFTCG 2011 Abstract Author Index*

- Abasali, Palizban, P 9
Abed, Soumeiya, P 11
Adjali, Oumeya, P 27
Adrover, Martin, Or 10
Aghighi, Saman, Inv 18
Aguirre, Alejandra, I, Or 10
Ahouansou, Ornella, P 19
Ahouansou, Ornella, Or 18
Allay, James, Inv 18
Andreeff, Michael, Inv 14
Anegon, Ignacio, P 2
Astord, Stéphanie, Or 5
Aubourg, Patrick, Or 18, P 3, P 18, P 19
Avril, Aurélie, P 20
- Baekelandt, Veerle, Inv 6
Baez, Maria-Veronica, Or 10
Baillou, Claude, P 14
Baker, Andrew H, Inv 2
Barkats, Martine, Or 5
Barnay, Frédéric, P 35
Barteau, Benoit, P 31
Barthelemy, Florian, P 20
Bartoli, Marc, P 20
Battaglia, Sèverine, Or 16
Becker, Fabienne, P 16
Beilvert, Fanny, P 6, P 26
Bellier, Bertrand, Or 13
Berchel, Mathieu, Or 2, P 10
Bergot, Anne Sophie, P 28
Berretta, Julia, P 4
Bertrand, Emilie, P 13
Betuing, Sandrine, P 18
Beuf, Olivier, P 32
Bieche, Ivan, P 19
Bigey, Pascal, Inv 15
Blancho, Gilles, Inv 7
Blondeau, Johanna, P 35
Blondel, Armelle, Or 15
Blum, David, Or 11
Boisgerault, Florence, Inv 12, Or 6,
P 23, P 25
Boni, Sébastien, P 7
Bouakaz, Ayache, P 12
Bougnères, Pierre, P 3
Bouquet, Celine, Or 18
Bourdel, Alix, P 8
Bouvattier, Claire, P 3
Brion, Régis, Or 16
Briot-Nivard, Delphine, Or 5
- Brown-kennerly, Victoria, P 1
Bucher, Thomas, Or 5
Buchholz, Christian J., P 29
Buee, Luc, Or 11
Büning, Hildegard, Inv 1
Burgain, Aurore, Inv 15
Burlot, Marieanne, Or 11
Buscaill, Louis, Or 7, Or 17, P 33
- Caboche, Jocelyne, P 18
Caccavelli, Laure, P 35
Cany, Jeannette, P 30
Carbery, Lara, P 1
Carlier, Pierre, Or 19
Carmoy, Nathalie, Or 2
Cartier, Nathalie, Or 11, Or 18, P 18, P 19
Castell, Jose V, Inv 8
Cavazzana-Calvo, Marina, Inv 17, P 35
Cedrone, Frédéric, P 4
Cedrone, Grégory, P 35
Cereso, Nicolas, P 16
Charbord, Pierre, Or 8
Charrier, Sabine, P 35
Charton, Karine, P 21, P 22
Chatin, Benoît, P 6, P 26
Chautard, Hélène, P 35
Chauveau, Christine, Or 14
Chauvin, Joë Marc, Or 15
Cheli, Veronica, T, Or 10
Cheradame, Hervé, P 13
Chérel, Yan, Or 18, Or 19, P 17,
P 24, P 27
Cheve, Christophe, Or 14
Chowdary, Pratima, Inv 18
Chrétien, Stany, P 11
Ciré, Séverine, Inv 12, P 29
Coleman, John, Inv 18
Colle, Marieanne, Or 18
Colle, Marie-Anne, P 3, P 24
Collins, Mary K., P 29
Conchon, Sophie, Or 3, P 30
Conscience, Aude, P 16
Consortium, Duchenne, P 17
Corbineau, Sébastien, P 11
Cordelier, Pierre, Or 7, Or 17, P 33
Corre, Guillaume, Or 6
Cosset, François-Loïc, Inv 3, Or 12
Costiou, Patrick, Or 5
Crystal, Ronald, G, Or 18
Cui, Xiaoxia, P 1
- Da Rocha, Sylvie, Or 6
Daniele, Nathalie, P 21
Danos, Olivier, P 8
Darocho, Sylvie, Inv 12
Davidoff, Andrew M., Inv 18
De Luca, Michele, Inv 19
De Villartay, Jean-Pierre, P 35
de Vos, John, P 16
Decker, Helena, Or 10
Delbani, Anna, P 34
Deniaud, Johan, P 3, P 24
Deschamps, Jack-Yves, Or 14, Or 18
Desseaux, Carole, P 4
Dessolin, Sofien, P 28
Djelti, Fathia, P 19
Domene, Aurélie, Or 16
Dorgham, Karim, Or 9
Dubart-Kupperschmitt, Anne, Or 8, P 11
Dubreil, Laurence, P 3, P 24, P 27
- Epstein, Alberto, Or 10
Erard, Francois, Inv 11
Escoffre, Jean-Michel, P 12
Escriou, Virginie, Inv 15, Or 16
- Faideau, Béatrice, P 28
Ferec, Claude, P 10
Ferrand, Maxime, Inv 12, P 23, P 25
Ferreira, Sergio, Or 10
Ferry, Nicolas, Or 3, P 2, P 7,
P 30, P 32
Fischer, Alain, Inv 17
Forlino, Antonella, Or 16
Fourquet, Elodie, Or 7
Fraix, Aurore, Or 2
François, Virginie, P 17
Frin, Sophie, Or 1
Fromes, Yves, Or 19, P 17
Fyfe, John, Or 5
- Galea, Sandra, Inv 13
Galletto, Roman, P 4
Gall, Tony Le, Or 2, P 10
Gallardo, Eduard, P 22
Galy, Anne, Inv 12, Or 1, Or 6, P 23,
P 25, P 29, P 35
Garcia, Luis, Inv 10, Or 19, P 20
Gautier, Benoit, Or 11

*Invited (Inv) abstracts are found on pp. A-3–A-8; Oral (or) abstracts are found on pp. A-9–A-15; and Poster (P) abstracts on pp. A-16–A-26.

- Gauttier, Vanessa, Or 3, P 30
 Gavard, Françoise, Inv 13
 Gawrychowski, Krzysztof, P 34
 Gernoux, Gwladys, P 27
 Gill, Deborah, Or 2
 Gioia, Roberta, Or 16
 Glader, Bert, Inv 18
 Goldtsein, Jérémie, P 28
 Gomez, Jean-Pierre, P 13
 Goncalves, Cristine, P 13
 Gorochov, Guy, Or 9
 Gottrand, Gaelle, P 28
 Gouble, Agnès, P 4, P 8
 Goumeaux, Cyndie, P 3
 Gray, John T., Inv 18
 Gribova, Varvara, Or 8
 Griffioen, Anja, Inv 18
 Guegan, Philippe, P 13
 Guilbaud, Mickaël, P 27
 Guilloux, Yannick, Or 15
 Guiner, Caroline Le, Or 19, P 17, P 24, P 27
- Hacein-Bey-Abina, Salima, Inv 17
 Hacein-Bey-Amina, Salima, P 35
 Hackett, Neil, Or 18
 Haghighi, Farhad, Or 8
 Hamel, Christian, P 16
 Hamidi, Sofiane, P 14
 Hamidouche, Zahia, Or 8
 Harrington, Chris, Inv 18
 Haudebourg, Thomas, P 3
 Hemion, Pierre Marie, P 2
 Hervouet, Soizic, P 31
 Heymann, Dominique, Or 16, P 31
 High, Katherine, Inv 18
 Hir, Sophie Le, P 10
 Hogrel, Jean-Yves, Or 19
 Holic-Barlet, Nathalie, Or 1
 Hordeaux, Juliette, P 24
 Horvat, Branka, Or 12
 Hu, Amélie, P 18
 Hudry, Eloise, P 19
 Huret, Christophe, Or 13
 Hyde, Stephen C., Or 2
- Iacobelli, Fabien, P 24
- Jaffres, Paul-Alain, Or 2, P 10
 Jerome, Christine, P 5
 Jerusalinsky, Diana A, Or 10
 Ji, Diana, P 1
 Jollet, Arnaud, P 8
 Jotereau, Francine, Or 15
 Joussemet, Béatrice, Or 5, P 24
- Kalatzis, Vasiliki, P 16
 Kaye, Mark, Inv 18
 Kergourlay, Virginie, P 20
 Klatzmann, David, Or 9, P 28
- Klonjkowski, Bernard, Inv 13
 Krahn, Martin, P 20
- Labarriere, Nathalie, Or 15
 Lambert, Sidonie, Or 9
 Lamoureux, François, P 31
 Langelot, Marie, P 26
 Larcher, Thibaut, P 27
 Larrieu, Pierre, Or 15
 Le Guiner, Caroline, Or 19
 Leboulch, Philippe, P 11
 Lehn, Pierre, Or 2, P 10
 Lemeur, Guylène, Or 14
 Lemoine, François M., Or 13
 Lemoine, Francois Marie, P 14
 Leroy, Florence, P 21
 Lescaille, Geraldine, Or 13
 Levy, Nicolas, P 20
 Li, Jun, Or 4
 Libléau, Lyse, Or 14
 Lilley, Patricia, Inv 18
 Lindberg, Matthias, Or 2
 Lulka, Hubert, Or 17
- Macintosh, Jenny, Inv 18
 Madeira, Alexandra Mendes, Or 14
 Magnan, Antoine, P 26
 Mailly, Philippe, P 18
 Małeck, Maciej, P 34
 Manchév, Vladimir, P 8
 Mannioui, Cécile Schiffer, P 4
 Maouche-Chrétien, Leila, P 11
 Marais, Thibault, Or 5
 Marcelin, Anne-Geneviève, Or 9
 Marie, Corinne, Inv 15
 Marini, Frank, Inv 14
 Marodon, Gilles, Or 9, P 28
 Matous, Etienne, P 31
 Matuska, Stanislav, Inv 19
 Mccoy, Aaron, P 1
 Melendez, Matias, E, Or 10
 Mérillon, Nathalie, Inv 13
 Mevel, Mathieu, P 6, P 26
 Midoux, Patrick, P 13
 Miller, Andrew D., Inv 20
 Miller, Andrew David, Inv 5
 Mingozi, Federico, Inv 18
 Mithieux, Gilles, P 32
 Monjaret, François, P 22
 Montier, Tristan, Or 2, P 10
 Montus, Marie, Or 19, P 17
 Moreau, Stéphanie, P 24
 Moullier, Philippe, Or 5, Or 14, Or 18,
 Or 19, P 17, P 24, P 27
 Mouly, Vincent, P 20
 Mousazadeh, Mohammad, P 9
 Muscari, Fabrice, Or 7
 Mutel, Elodie, P 32
- Nathwani, Amit C., Inv 18
 Neildez-Nguyen, Thi my anh, Or 6
- Ng, Catherine Y.C., Inv 18
 Nguyen, Tuan Huy, P 2, P 7, P 32
 Nienhuis, Arthur W., Inv 18
 Norol, Françoise, P 14
 Novell, Anthony, P 12
- Paganoni, Giorgio, Inv 19
 Paldi, Andras, Or 6
 Paques, Frédéric, P 4, P 8
 Pellegrini, Graziella, Inv 19
 Pengam, Sabrina, P 6
 Pequignot, Marie, P 16
 Perea, Javier, Or 1
 Peron, Jean-Marie, Or 7
 Petit, Nicolas, Or 9
 Picarda, Gaelle, P 31
 Pichard, Virginie, P 7
 Pichon, Chantal, P 13
 Pie, Jun, Inv 18
 Piguét, Françoise, Or 18, P 18
 Pilleul, Frank, P 32
 Ping, Yuan, Or 4
 Pion, Delphine, P 21
 Piron, Julien, P 12
 Pitard, Bruno, P 6, P 26, P 31
 Pitoiset, Fabien, Or 13
 Planel, Pierre, P 27
 Ploquin, Aurélie, Or 12
 Pointreau, Adeline, P 33
 Poirot, Laurent, P 4
 Pontes, Cécilia Abarrategui, P 2
 Poupiot, Jérôme, P 23
 Pourcher, Guillaume, Or 8
 Pradier, Laurent, Or 11
 Preat, Véronique, P 5
 Provost, Nathalie, Or 14
 Pucéat, Michel, P 11
- Quesniaux, Valerie J.F., Inv 11
 Quiviger, Mickaël, Inv 15
- Ragelle, Héloïse, P 5
 Ragon, Isabelle, P 28
 Rajas, Fabienne, P 32
 Rama, Paolo, Inv 19
 Raoul, Sylvie, Or 18
 Raslova, Hana, P 14
 Redini, Françoise, Or 16, P 31
 Reiss, Ulrike M., Inv 18
 Richard, Isabelle, P 21, P 22, P 23
 Richardson, Jennifer, Inv 13
 Riddell, Anne, Inv 18
 Riva, Raphaël, P 5
 Robert, Lorenne, P 16
 Rolling, Fabienne, Or 14
 Rommelaere, Jean, Inv 16
 Rosales, Cecilia, Inv 18
 Rouger, Karl, Inv 9
 Roujeau, Thomas, Or 18
 Rousseau, Julie, Or 16
 Ryffel, Bernhard, Inv 11, Inv 12

- Saiagh, Soraya, P 15
Salaün, Jean-Yves, P 10
Salehi, Rasoul, P 9
Salveti, Anna, Or 12
Sanatine, Peggy, Or 6
Santos, Christine Dos, P 3
Sarabayrouse, Guillaume, Or 15
Scherman, Daniel, Inv 15, Or 16
Schlegel, Anne, Inv 15
Schmitt, Françoise, P 2
Servais, Laurent, Or 19, P 17
Sevin, Caroline, Or 18, P 3
Sicard, Flavie, Or 7, Or 17, P 33
Silva, Georges H, P 8
Si-Tayeb, Karim, P 11
Sleep, Susan, Inv 18
Smith, Julianne, P 4
Sondhi, Dolan, Or 18
Spaeth, Erika, Inv 14
Spinelli, Alessandra, Inv 19
Stachurska, Anna, P 34
Stefanutti, Anne, P 32
- Sudres, Muriel, Inv 12
Suleman, Muhammad, Inv 13
Szecsi, Judit, Or 12
- Taleb, Kahina, P 28
Tartour, Eric, Inv 13
Thanou, Maya, Inv 20
Tesson, Laurent, P 2
Tirode, Franck, P 31
Tissot, Adrien, P 26
Trichet, Valérie, Or 16, P 31
Troquier, Laeticia, Or 11
Tubsawan, Alisa, P 11
Tuddenham, Edward G.D., Inv 18
- Vainchenker, William, P 14
Vallier, Ludovic, P 11
Vandermeulen, Gaëlle, P 5
Vanier, Marie-Thérèse, Or 18
Vassaux, Georges, Inv 4
- Verhoeyen, Els, Inv 3
Vidaud, Michel, P 19
Voit, Thomas, Or 5, Or 19, P 17
- Wakeling, Erin, Or 5
Weber, Anne, P 11
Weber, Michel, Or 14
Wein, Nicolas, P 20
Weinstein, Edward, P 1
Wilmes, Cyril, Inv 12, P 25
- Yamagata, Yoshiaki, Or 6
Yee, Thynn, Inv 18
- Zeghimi, Aya, P 12
Zerah, Michel, Or 18
Zerbato, Madeleine, Or 1
Zhang, Wenwei, Or 8
Zhou, Junfang, Inv 18

