UNIVERSITA' VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA

INTERNAZIONALE IN MEDICINA MOLECOLARE

Curriculum in Basic and Applied Immunology and Oncology

Preclinical development of an IL-1RA-based gene therapy approach for the treatment of autoinflammatory diseases

Director of Studies: Dr Alessandra Mortellaro

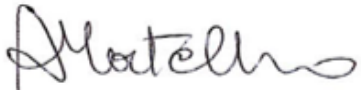
External Supervisor: Dr Greta Guarda

Tesi di DOTTORATO di RICERCA di Mariasilvia Colantuoni

Matriculation number: 013874

Ciclo di dottorato XXXIV SSD BIO/11 BIO/13

Anno Accademico 2020/2021



CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

Il/la sottoscritto/*I*	Mariasilvia Colantuoni
Matricola / *registration number*	013874
Nata a/ *born at*	Benevento
il/*on*	18/01/1995

autore della tesi di Dottorato di ricerca dal titolo / *author of the PhD Thesis titled*

Preclinical development of an IL-1RA-based gene therapy approach for the treatment of autoinflammatory diseases

 AUTORIZZA la Consultazione della tesi / AUTHORIZES *the public release of the thesis*

X NON AUTORIZZA la Consultazione della tesi per 12 mesi / DOES NOT AUTHORIZE *the public release of the thesis*

a partire dalla data di conseguimento del titolo e precisamente */ from the PhD thesis date, specifically*

Dal / *from* ……/……/…… Al / to ……/……/

Poiché /*because*:

□ l'intera ricerca o parti di essa sono potenzialmente soggette a brevettabilità/ *The whole project or part of it might be subject to patentability;*

X ci sono parti di tesi che sono già state sottoposte a un editore o sono in attesa di pubblicazione/ *Parts of the thesis have been or are being submitted to a publisher or are in press;*

□ la tesi è finanziata da enti esterni che vantano dei diritti su di esse e sulla loro pubblicazione/ *the thesis project is financed by external bodies that have rights over it and on its publication.*

Data /Date Firma/Signature

DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably.

All the results presented here were obtained by myself, except for:

1. **Collaborations**

- The purified LV.IL-1RA was produced by the SR-Tiget Processing Development Laboratory (PDL);
- The acquisition and analysis of the data shown in Figure 20, 21, 22 and 24 were performed in collaboration with Luca Basso-Ricci (SR-Tiget);
- Haematoxylin and eosin staining (Figure 21 and 22) was performed by Dr Francesca Sanvito (GLP Test Facility, SR-Tiget);
- Dr Marco Gattorno (IRCCS Istituto Giannina Gaslini, Genoa, Italy) provided the bone marrow samples of the $Nlrp3^{N475K/+}$ mice;
- The experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis was performed in collaboration with Dr Luca Muzio (IRCCS San Raffaele Scientific Institute).

2. **Fair Use Disclaimer**. The figures used in the introduction of this thesis are under fair use as defined under section 107 of the US Copyright Act of 1976 and according to Italian Law n. 68 of April 9 2003 «Attuazione della direttiva 2001/29/CE sull'armonizzazione di taluni aspetti del diritto d'autore e dei diritti connessi nella società dell'informazione». All rights and credit go directly to their rightful owners. No copyright infringement is intended.

All sources of information are acknowledged by means of reference.

Abstract

Systemic autoinflammatory diseases (SAIDs) delineate a group of diseases that manifest when the immune system is activated uncontrollably. One cardinal subgroup of SAIDs includes rare periodic fevers characterized by the dysregulated production of the proinflammatory cytokine interleukin-1 (IL-1). There is no cure for these conditions. Anakinra, the recombinant form of IL-1 receptor antagonist (IL-1RA), is the mainstay therapy for these patients. However, anakinra has a short half-life and poor tissue distribution. Severe patients respond inadequately and do not experience improvement in symptoms. Therefore, there is a need for a durable therapy that bypasses continuous drug administration and ensures a satisfactory resolution of tissue inflammation. In this PhD project, we addressed the urgency to develop an effective treatment for IL-1 induced SAIDs based on haematopoietic stem and progenitor cells (HSPCs) producing constitutively human IL-1RA using a lentiviral vector (LV)-mediated gene transfer approach. Human and mouse HSPCs transduced with an LV encoding human IL-1RA efficiently released this cytokine. Transduction procedure and IL-1RA over-expression did not alter HSPCs viability, clonogenic and differentiation potential *in vivo*. Next, we investigated whether, once transplanted in mice, IL-1RA-expressing HSPCs could ameliorate acute and chronic inflammation. Three mouse models were employed. The ectopic expression of IL-1RA by HSPC-derived immune cells suppressed neutrophil recruitment to the site of inflammation in mice with peritonitis induced by monosodium-urate crystals (MSU), well-known activators of the NLRP3-IL-1 axis. This protective effect was comparable to that obtained by anakinra. Next, we exploited an inducible mouse model of the cryopyrin-associated period syndrome (CAPS) carrying the dominant Nlrp3^{A350V} mutation. Syngeneic transplant of IL-1RA-transduced HSPCs in Nlrp3^{A350V+}CreT mice effectively prevented mice from disease onset and progression manifested as weight loss, leucocytosis, and high serum IL-6 level. Finally, preliminary data indicate that our gene therapy approach could improve mortality rate and disease severity in a mouse model of experimental autoimmune encephalomyelitis. Altogether, our results demonstrated that LV-mediated IL-1RA delivery in HSPCs is safe and efficient approach to controlling IL-1-mediated inflammation. These findings set the stage for future studies to evaluate the potential LV-mediated IL-1RA GT clinical application for IL-1-mediated systemic autoinflammatory diseases.

Table of Contents

ABBREVIATIONS AND ACRONYMS.....5

LIST OF FIGURES.....14

LIST OF TABLES.....16

CHAPTER 1: INTRODUCTION 17

1.1 IMMUNE SYSTEM AND INFLAMMATION..... 17

1.1.2 The inflammatory response 19

1.1.3 The inflammatory pathway..... 20

1.1.4 The inflammasome complex 25

1.1.5 The NLRP3 inflammasome..... 28

1.2 THE IL-1 FAMILY 31

1.2.1 Biological roles of IL-1 32

1.2.2 IL-1 receptors and downstream signalling 35

1.3 IL-1 RECEPTOR ANTAGONIST (IL-1RA) 37

1.3.1 Biological roles of IL-1RA 38

1.4 THE ROLE OF THE IL-1 IN HUMAN DISEASES 39

1.4.1 Systemic autoinflammatory diseases..... 40

1.4.2 IL-1 mediated systemic autoinflammatory diseases..... 41

1.4.3 Current treatments for SAIDs..... 46

1.4.4 IL-1 targeting biologics..... 48

1.5 MOUSE MODELS FOR NLRP3/IL-1 MEDIATED INFLAMMATION 51

1.5.1 MSU-induced peritonitis model..... 51

1.5.2 NLRP3 knock-in mouse models for hereditary NLRP3-mediated diseases52

1.5.3 Experimental autoimmune encephalomyelitis (EAE) as a mouse model of multiple sclerosis..... 53

1.6 HAEMATOPOIETIC STEM CELL TRANSPLANTATION AND GENE THERAPY 55

1.6.1 GT safety..... 58

1.6.2 Lentivirus and development of lentiviral vectors 59

1.6.3 Previous results on IL-1RA-mediated gene therapy..... 64

2. AIM OF THE WORK 67

3. RESULTS 68

3.1 PRECLINICAL SAFETY AND TOXICITY STUDIES 68

3.1.1 Generation of a transfer plasmid encoding human IL-1RA 68

3.1.2 LV.IL-1RA transduction ensured efficient IL-1RA production and secretion in mouse HSPCs with no evidence of toxicity 70

3.1.3 Efficient LV-mediated IL-1RA transfer in human HSPCs 72

3.1.4 Systemic IL-1RA production did not alter HSPC engraftment and immune reconstitution in vivo 75

3.2 EVALUATION OF THE EFFICACY OF *EX VIVO* IL-1RA GT IN CORRECTING IL-1-INDUCED ACUTE INFLAMMATION 79

3.2.1. Vector-derived IL-1RA effectively prevents neutrophilic inflammation in a mouse model of crystal-induced peritonitis 79

3.2.2. IL-1RA over-expression in human HSPCs partially suppress neutrophil' recruitment in a humanized mouse model of IL-1 mediated inflammation 81

3.2.3 Generation and characterisation of $Nlrp3^{N475K/+}$ mouse chimaeras 82

3.2.4 IL-1RA expression ameliorates CAPS associated inflammatory symptoms in the inducible $Nlrp3^{A350V/+}$CreT mouse model 85

3.2.5 Syngeneic transplant of IL-1RA-producing $Nlrp3^{A350V/+}$CreT HSPCs prevents CAPS disease progression 88

3.2.6 IL-1RA gene transfer in $Nlrp3^{A350V/+}$CreT BMDCs leads to the downregulation of proinflammatory cytokines expression 91

3.2.7 Evaluation of IL-1RA GT efficacy in the experimental autoimmune encephalomyelitis mouse model for multiple sclerosis 93

4. DISCUSSION 96

5. MATERIALS AND METHODS 103

5.1 CELL CULTURES 103

5.1.1 HEK293T cells 103

5.1.2 Mouse BM cells 103

5.1.3 Mouse Lin-negative HSPCs 103

5.1.4 Human $CD34^+$ HSPCs 103

5.2 PRODUCTION OF LV.IL-1RA 104
5.2.1 Generation of the IL-1RA transfer vector 104
5.2.2 Production of lab-grade vector preparation 104
5.2.3 Production of purified vector preparation 104
5.2.4 Measurement of viral titre and infectivity 105
5.3 LENTIVIRAL CELL TRANSDUCTION 105
5.3.1 Transduction of mouse haematopoietic cells 105
5.3.2 Lentiviral transduction of human $CD34^+$ HSPCs 106
5.3.3. BMDCs differentiation and LV-transduction 107
5.4 CFU ASSAY 108
5.5 MEASUREMENT OF VECTOR COPY NUMBER AND TRANSDUCTION EFFICIENCY . 108
5.6 IMMUNOBLOT ANALYSIS 109
5.7 ENZYME-LINKED IMMUNOSORBENT ASSAY 110
5.8 QUANTITATIVE REAL-TIME RT-PCR 110
5.9 MOUSE MODELS 110
5.9.1 C57BL/6N and C57BL/6-LY5.1 111
5.9.2 NOD scid gamma (NSG) mice 111
5.9.3 $Nlrp3^{N475K/+}$ mice 111
5.9.4 $Nlrp3^{A350V/+}$CreT mice 112
5.9.5 $Nlrp3A^{350V/+}$CreT mice genotyping 113
5.9.6 Tamoxifen treatment in vivo 115
5.10 TRANSPLANTATION EXPERIMENTS 115
5.10.1 Mouse-to-mouse BM chimaeras 115
5.10.2 Syngeneic HSPCs transplant in $Nlrp3^{A350V/+}$CreT mice 116
5.10.3 Xenotransplantation of human HSPCs in NSG mice 116
5.10.4 Haematological sampling 116
5.10.5 Histopathological analysis 116
5.11 MSU-INDUCED PERITONITIS MODEL 117
5.12 EAE MOUSE MODEL FOR MS 117
5.13 FLOW CYTOMETRY ANALYSES 118
5.13.1 Flow cytometry on mouse cells in chimeric mice 118
5.13.2 Flow cytometry analyses on human cells in NSG mice 119

5.14 STATISTICAL ANALYSIS 121

REFERENCES 122

ABBREVIATIONS AND ACRONYMS

AAV	Adeno-associated virus
AD	Alzheimer's disease
AD	Autosomal dominant
ADA-SCID	Adenosine deaminase severe combined immunodeficiency
AID	Autoinflammatory disease
AIM2	Absent in melanoma 2
AMI	Acute myocardial infarction
AML	Acute myeloid leukaemia
AOSD	Adult-Onset Still's Disease
AP-1	Activator protein-1
AR	Autosomal recessive
ASC	Apoptosis-associated speck-like protein
ATMPs	Advanced therapy medicinal products
ATP	Adenosine triphosphate
BB	Brilliant blue
BFU-E	Burst forming unit-erythroid
BM	Bone marrow
BMDCs	BM-derived dendritic cells
BMT	Bone marrow transplant
Bp	Base-pair
BUV	Brilliant™ Ultraviolet
CA	Capsid
cAMP	Cellular cyclic AMP
CAPS	Cryopyrin-associated periodic syndromes
CARD	Caspase activation and recruitment domain

CDA	Congenital dyserythropoietic anemia
CFA	Complete Freund's adjuvant
CFU	Colony forming unit
CFU-GEMM	Colony forming unit-granulocyte-erythrocyte-monocyte-megakaryocyte
CFU-GM	Colony forming unit-granulocyte-monocyte
CGD	Chronic granulomatous disease
CINCA	Chronic Infantile Neurological Cutaneous and Articular
CLRs	C-type lectin receptors
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CNS	Central Nervous System
Co	codon-optimized
COVID-19	Coronavirus disease 2019
cPPT/CTS	Polypurine tract/central terminal sequence
DAB	3,3′-diaminobenzidine
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
ddPCR	Droplet digital PCR
DIRA	Deficiency of IL-1 receptor antagonist
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
EMH	Extramedullary haematopoiesis
ENV	Envelope
FBS	Foetal Bovine Serum
FCAS	Familial cold autoinflammatory syndrome

FDA	Food and Drug Administration
FMF	Familial Mediterranean fever
G-CSF	Granulocyte colony-stimulating factor
G-CSF	Granulocyte colony-stimulating factor
gDNA	Genomic DNA
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMLP	Granulocyte-monocyte-lymphocyte progenitors
GMP	Granulocyte–macrophage progenitor
GSDMD	Gasdermin D
GSDMD-NT	N-terminal domain of GSDMD D
GT	Gene therapy
GvH	Graft-versus-host
HF	Heart failure
HIDS	Hyper IgD Syndrome
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1
HSC	Haematopoietic stem cell
hsCRP	High-sensitivity C-reactive protein
HSCT	Haematopoietic stem cell transplantation
HSPCs	Haematopoietic stem progenitor cells
HSPs	Heat shock proteins
HSV-1	Herpes simplex virus-1
i.c.	Intracisternal
i.v.	Intravenous/Intravenously

icIL-1RA	intracellular IL-1 receptor antagonist
IFN-γ	Interferon- γ
IKK	IκB kinase
IL-1	Interleukin-1
IL-10	Interleuikin-10
IL-11	Interleuikin-11
IL-12	Interleuikin-12
IL-13	Interleukin-13
IL-17	Interleukin-17
IL-1a	Interleukin-1a
IL-1R	Interleukin-1 receptor
IL-1RA	Interleuikin-1 receptor antagonist (IL-1RA)
IL-1RAcp	Interleukin-1 accessory protein
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-23	Interleukin-23
IL-33	Interleukin-33
IL-36α	Interleukin-36α
IL-36β	Interleukin-36β
IL-36γ	Interleukin-36γ
IL-37	Interleukin-37
IL-38	Interleukin-38
IL-6	Interleukin-6
ILCs	Innate lymphoid cells
IMDM	Iscove's Modified Dulbecco's Medium
IN	Integrase

IRAK	IL-1 receptor-activated protein kinase
IRAP	IL-1 receptor antagonist
IRF3	Interferon regulatory factor 3
JNK	Jun N-terminal kinase
KC	Neutrophil chemoattractant
KI	Knock-in
LC	Liquid culture
Lin-	Lineage negative
LPS	Lipopolysaccharide
LRR	leucine-rich repeats
LTR	Long terminal repeat
LV	Lentiviral vector
MA	Matrix
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MAS	Macrophage Activation Syndrome
M-CSF	Macrophage-colony stimulating factor
MDP	Monocyte–dendritic cell progenitor
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte–erythrocyte progenitor
miRNA	microRNA
miRTs	miRNA target sequences
MKD	Mevalonate Kinase Deficiency
MLD	Metachromatic leukodystrophy
MOI	Multiplicity of infection
mPB	Mobilized peripheral blood

MPP	Multipotent progenitor
MPS	Mucopolysaccharidosis
MS	Multiple sclerosis
MSU	Monosodium urate
mtDNA	mitochondrial DNA
MWS	Muckle-Wells syndrome
MYC	Mycobacterium tuberculosis
MyD88	Myeloid differentiation factor-88
NA	Not available
NACHT	Nucleotide-binding and oligomerisation domain
NBD	Nucleotide-binding domain
NC	Nucleocapsid
NEK7	NIMA-related kinase 7
NF-κB	Nuclear factor kappa B
NK	Natural killer
NK	Natural killer cell
NLRs	Nod-like receptors
NOD	Nucleotide-binding domain
NOMID	Neonatal-onset, multi-system inflammatory disease
NSAIDs	Non-steroidal antinflammatoy drugs
NSG	NOD scid gamma
O/N	Overnight
OA	Osteoarthritis
P/S	Penicillin/Streptomycin
PAMPs	Pathogen-associated molecular patterns
PAP	Phosphatidate phosphatase

PAPA	Pyogenic arthritis, pyoderma gangrenous, and acne
PBS	Phosphate-buffered saline
PDL	Processing Development Laboratory
PFAPA	Aphthous stomatitis, pharyngitis and cervical adenitis
PGE2	Prostaglandin E2
PGK	Phosphoglycerate kinase
PI	Propidium iodide
PKN	Protein kinase
PL	Piperlongumine
PR	Protease
PRR	Pathogen-recognition receptor
PTMs	Post-translational modifications
PYD	Pyrin domain
RCLs	Replication-competent lentiviruses
Real-time	RT-PCR Real-time reverse-transcriptase polymerase chain reaction
rhFlt3L	Recombinant human Fms-like tyrosine kinase receptor 3 ligand
rhIL-3	Recombinant human Interleukin-3
Rho	Ras homolog
rhSCF	Recombinant human stem cell factor
rhTPO	Recombinant human thrombopoietin
RIG	Retinoic acid-inducible gene
RLRs	RIG-like receptors
rmSCF	Recombinant mouse stem cell factor
rmTPO	Recombinant mouse thrombopoietin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

rpm	Revolutions per minute
SA	Splice acceptor
SAIDs	Systemic autoinflammatory diseases
SCF	Stem cell factor
SCID-X1	X-linked severe combined immunodeficiency
SD	Splice donor
SFFV	Spleen focus-forming virus
sIL-1R	Secreted IL-1 receptor
sIL-1RA	Secreted IL-1 receptor antagonist
SIN	Self-inactivating
SJIA	Juvenile Idiopathic Arthritis
StD	Standard deviation
SU	Surface
SV40	Simian vacuolating virus 40
T2D	Type 2 diabetes
TAM	Tyro3, Axl, and Mer,
TGF-β1	Transforming growth factor-β1
TGN	Trans-Golgi network
TH	T-helper
THL	Thiolutin
TIR	Toll IL-1 receptor
TIRAP	Domain Containing Adaptor Protein
TLR	Toll-Like Receptors
TM	Transmembrane
TR	Transduction
TRAF6	TNF receptor-associated factor 6

TRAPS	TNF receptor-associated periodic syndrome
TSS	Transcription start sites
uSAIDs	Undefined or undifferentiated SAIDs
UT	Untransduced
UT	Transduction units
VCN	Vector copy number
VP	Vector particles
VSV-G	Vesicular stomatitis virus
WBC	White blood count
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
γ-RVs	Gammaretroviral vectors

LIST OF FIGURES

Figure 1. Schematic representation of the inflammatory response' phases. 19
Figure 2. Representative scheme of the inflammatory pathway. 21
Figure 3. TRL signalling pathway. 23
Figure 4. Inflammasomes structure and mechanisms of activation. 27
Figure 5. The two-step NLRP3 Inflammasome activation model. 31
Figure 6. IL 1 functions. 35
Figure 7. Schematic illustration of the IL-1R complex. 37
Figure 8. Clinical manifestations in IL-1 mediates SAIDs. 43
Figure 9. Anti-IL-1 cytokine biological treatments. 49
Figure 10. Main stages in ex-vivo gene therapy protocol. 56
Figure 11. Illustration of the HIV-1 viral genome and virion structure. 61
Figure 12. Third-generation lentiviral vectors. 63
Figure 13. Generation and testing of the PGK.IL-1RA plasmid. 69
Figure 14. IL-1RA secretion in transduced HEK293T cells. 70
Figure 15. Efficient transduction and IL-1RA production in mouse HSPCs. 71
Figure 16. Evaluation of LV.IL1RA impact on mouse HSPCs functionality. 72
Figure 17. Successful human HSPCs transduction and IL-1RA production. 73
Figure 18. Evaluation of LV.IL1RA impact on human HSPCs functionality. 74
Figure 19. Successful engraftment of IL-1RA-transduced HSPCs in vivo. 75
Figure 20. Haematopoietic cell reconstitution capacity of IL-1RA-transduced HSPCs. 76
Figure 21. Histopathological and flow cytometry analysis of the spleen of IL-1RA chimeric mice. 77
Figure 22. BM composition of MOCK, GFP and IL-1RA chimaeras 78
Figure 23. Efficient suppression of neutrophil' recruitment in mice treated by IL-1RA GT. 80
Figure 24. Efficacy of IL-1RA GT in the humanised NSG mouse model. 82
Figure 25. Generation and characterization of $Nlrp3^{N475K/+}$ chimaeras. 84
Figure 26. Generation of $Nlrp3^{A350V/+}$CreT chimaeras expressing IL-1RA by immune cells. 86

Figure 27. IL-1RA GT ameliorated dermatitis and reduced serum IL-6 levels in Nlrp3$^{A350V/+}$CreT mice 87
Figure 28. Immune reconstitution and IL-1RA expression in Nlrp3$^{A350V/+}$CreT mice 89
Figure 29. Transplant of IL-1RA-expressing CreT^{+} cells in Nlrp3$^{A350V/+}$CreT^{+} prevents weight loss, blood leucocytosis and plasma IL-6 overproduction. 90
Figure 30. Effect of IL-1RA gene transfer on proinflammatory cytokines production by LPS-stimulated Nlrp3$^{A350V/+}$CreT BMDCs 92
Figure 31. Immune reconstitution and IL-1RA expression in BM chimaeras before EAE induction. 94
Figure 32. IL-1RA GT ameliorates mortality and disease' severity in the EAE mouse model of MS 95
Figure 33. Breeding strategy used to generate the conditional Nlrp3$^{A350V/+}$CreT mouse strain. 113
Figure 34. B6.Cg-Tg(CAG-cre/Esr1*)5Amc/ and Nlrp3$^{A350V/+}$CreT mouse genotyping. 115

LIST OF TABLES

Table 1. Main cytokines and their roles in the inflammatory response. 24
Table 2. Genetic features of monogenic IL-1-mediated systemic auto inflammatory diseases 42
Table 3. Pathology report showing morphological features of inflammation in the spleen, liver and bone marrow of MOCK and IL-1RA CreT^{+} mice following tamoxifen treatment. 88
Table 4. Summary of the different mice' groups. 89
Table 5. Summary of main parameters in the EAE progression and severity; incidence, mortality, cumulative score, onset and maximum score. 95
Table 6. Culture media for mouse haematopoietic cells. 105
Table 7. Culture media for human CD34^{+} cells. 107
Table 8. PCR Protocol for mouse genotyping. 114
Table 9. List of fluorescent antibodies for mouse haematopoietic cells phenotyping 118
Table 10. Mouse haematopoietic populations. 119
Table 11. List of fluorescent antibodies for WBD phenotyping 120
Table 12. Human haematopoietic populations identified by WBD analysis. 121

Chapter 1: Introduction

1.1 Immune system and Inflammation

The immune system has a vital role in host defence against harmful external agents and perturbations of homeostasis. It comprises the innate and the adaptive immune systems, which work together on numerous tasks. The innate immune system represents the body's first line of defence against germs surpassing the protection offered by the natural barriers, such as the skin and mucosal membranes. It acts very rapidly to counteract the spread of foreign pathogens throughout the body immediately. Conversely, the adaptive immune response eliminates the pathogen and protects the organism from reinfection with the same pathogen, given the presence of memory cells. It comprises highly specialized cells, namely the T and B lymphocytes, and blood-circulating antibodies (Parkin & Cohen, 2001).

The discovery of inflammation dates back to the ancient era of Egyptians and Greeks. The first description of inflammation comes from the observations of Aulus Cornelius Celsus, who defined four cardinal symptoms of inflammation: redness, swelling, warmth and pain. Inflammation is defined as the body's immune system response to damage and infection and acts by removing harmful stimuli and initiating the healing process, a defence mechanism indispensable for life (Nathan & Ding, 2010).

Ancient scientists based their studies not only on empiric observations but performed basic experiments uncovering the dynamics of the inflammatory response. Augustus Waller (Jarcho, 1971) and Julius Cohnheim (Malkin, 1984) isolated blood leukocytes, pivotal cells initiating an inflammatory reaction, and described their extravasation from the blood vessels into the tissue. Cohnheim also observed vasodilation: the swelling phenomenon discovered by Celsus (Medzhitov, 2010). In 1858, the fifth cardinal sign, *functio laesa* (disturbance of function), a general sign in all the inflammatory processes, was described by Rudolph Virchow in the book *Cellularpathologie* (Majno, 1975).

A decisive moment in understanding the inflammation process was in 1892 when Elie Metchnikoff described for the first time the process known as phagocytosis and postulated the theory of cellular immunity. Metchnikoff also highlighted the beneficial aspects of inflammation and revealed the critical role of macrophages and neutrophils in

host defence and restore of tissue homeostasis (Tauber, 2003). At the same time, Paul Ehrlich laid the foundation for the humoral theory of immunity, based on the discovery made by Emil von Behring and Shibasaburo Kitasato about serum therapy against diphtheria and tetanus toxins. In the late 19th century, Robert Koch, inspired by the work of Louis Pasteur, posited the germ theory of disease, which established that microbial agents are the primary triggers of an inflammatory response.

Inflammation can be classified as infectious or non-infectious as it can be caused by different factors, including infection, tissue and organ damage (Chen *et al*, 2018). In response to harmful stimuli, the body orchestrates a signalling cascade leading to cellular and molecular responses which result in tissue repairing and homeostasis. This process is defined as the inflammatory response, and it combines soluble mediators and blood-derived cells residing in all tissues (Takeuchi & Akira, 2010). A proper inflammatory reaction consists of three main stages (Figure 1):

1. Initiation;
2. Amplification;
3. Resolution and tissue repair.

The steps differ in intensity and duration and comprise different players as described in detail in the next session (Iqbal *et al*, 2017).

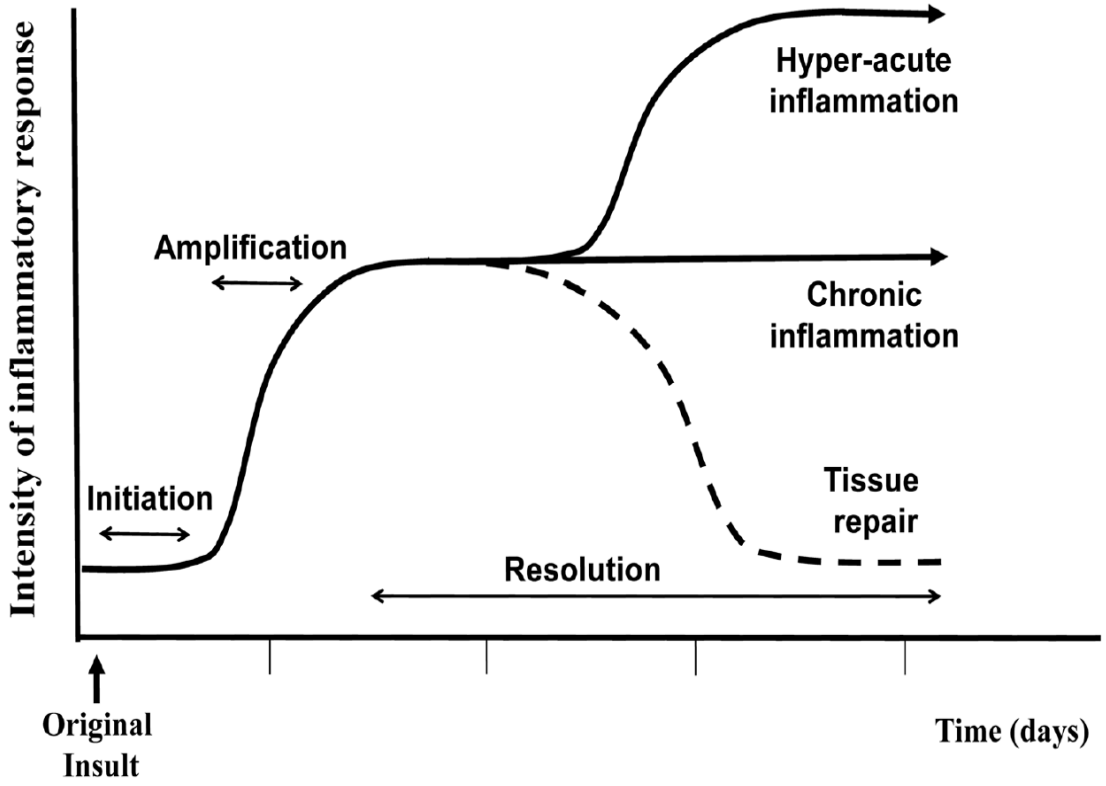


Figure 1. Schematic representation of the inflammatory response' phases.
When an insult invades the body, the initiation phase takes place, followed by the amplification phase. Once the harmful agents (tissue injury, bacteria, viruses) are removed, the damage is repaired during the resolution phase, ensuring tissue healing and returning to initial homeostasis. Adapted from (Iqbal et al, 2017).

1.1.2 The inflammatory response

The initiation step of an inflammatory response involves the pathogen recognition by tissue-resident cells of the innate immunity [*i.e.,* macrophages, dendritic cells (DCs), and mast cells] via specialised receptors. Upon activation, innate cells produce different soluble mediators, including chemokines and cytokines, which amplify the host response by evoking from the bloodstream an inflammatory cell exudate (in particular neutrophils) at the site of infection or injury (Stramer *et al*, 2007). Neutrophils eliminate the invading pathogens by releasing toxic components stored within intracellular granules, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). However, the uncontrolled release of these and others inflammatory mediators can damage host cells and tissues as neutrophils cannot discriminate between microbes and host cells (Nathan, 2006).

The acute inflammatory response is successfully resolved when the infectious agent is eliminated and the repair phase starts (Headland & Norling, 2015). At this stage, if the inflammatory stimulus persists, the long-lasting response leads to chronic inflammation. In this new risky condition, the neutrophils are replaced by macrophages and T cells. If the synergistic action of these cells is still not enough to eradicate the pathogen or the inflammatory trigger, a chronic inflammatory state propagates in specialised structures, such as granuloma and tertiary lymphoid tissues (Drayton *et al*, 2006). Moreover, the chronic inflammatory state can also originate from an immune response against self-antigens leading to autoimmune reactions.

Unravelling the signalling pathways and identifying mediators that maintain tissue homeostasis is vital as they may provide new targets for novel drugs and improved treatment approaches for inflammatory diseases.

1.1.3 The inflammatory pathway

Different cellular and molecular players are involved in each phase of an inflammatory response process. Although they exert different roles depending on the nature of the stimulus and where it is sensed throughout the body, they all share a common pathway, which can be summarized in Figure 2, as follows:

- Recognition of detrimental stimuli by specific **sensors**;
- Activation of a set of inflammatory **mediators**;
- Recruitment of inflammatory cells at **target tissue and organs**.

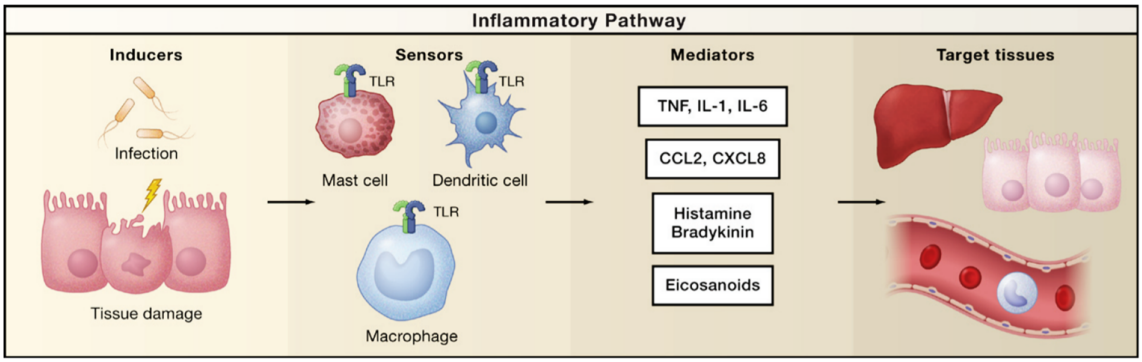


Figure 2. Representative scheme of the inflammatory pathway.
The inflammatory pathway is orchestrated by inducers, sensors, mediators, and target tissue wich are the effectors. Inducers are different types of stimuli which are detected by specialized sensors, such as Toll-like Receptors (TLRs). These receptors are normally expressed on sentinel cells of the immune system (i.e., tissue-resident macrophages, dendritic cells, and mast cells). The amplification of the inflammatory process occurs upon production of various mediators, including cytokines, chemokines, and eicosanoids, which, in turn, act on various target tissues (the effectors). Tissue-resident cells change their functional states and properties to adapt to the new harmful conditions (e.g., infection or tissue injury) and initiate the tissue-repair final stage. From (Medzhitov, 2010).

Exogenous inducers of inflammation are of microbial and non-microbial origins. The former are characterized by common molecular structures and are known as pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway, 1997). PAMPs are recognized by a set of receptors, known as pattern-recognition receptors (PRR), present in innate immune cells. Some PRRs can also recognize cellular components, known as danger-associated molecular patterns (DAMPS). They are usually sequestered intracellularly under homeostatic conditions but are released by dying cells triggering sterile inflammation in the absence of a pathogen (Gudkov & Komarova, 2016). Some examples of DAMPs are chromatin-associated protein high-mobility group box 1 (HMGB1), heat shock proteins (HSPs) and metabolites like adenosine triphosphate (ATP) and uric acid (Chen & Nuñez, 2010).

The PRRs comprise a variety of receptors: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi & Akira, 2010). In particular, TLRs are expressed by innate immune cells, such as macrophages and DCs, and non-immune cells, such as fibroblast and epithelial cells. Based on their localization, TLRs are classified in cell surface TLRs (TLR1, TLR2, TLR4, TLR5, TLR6) and intracellular/endosomal TLRs (TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13) (Kawai & Akira, 2010). The first class is specialized in recognizing microbial membrane elements like lipids,

proteins and lipoproteins. For example, TLR4 binds lipopolysaccharide (LPS) localized on the Gram-negative bacterial cell wall. This receptor can also recognize other signals, such as proinflammatory HMGB1. On the other hand, intracellular TLRs detect nucleic acid fragments of host, bacterial and viral origin (Blasius & Beutler, 2010). TLRs activation by PAMPs and DAMPs is determined by myeloid differentiation factor-88 (MyD88), which leads to the nuclear translocation of transcription factors activator protein-1 (AP-1), nuclear factor kappa B (NF-Kb), and interferon regulatory factor 3 (IRF3), resulting in the modulation of different intracellular pathways (Figure 3) (Kawai & Akira, 2010; Akira *et al*, 2001). Inducers-sensors interaction and subsequent activation of downstream signalling pathways lead to the production of many inflammatory mediators, which in turn act on tissue-resident cells, the effectors of an inflammatory pathway.

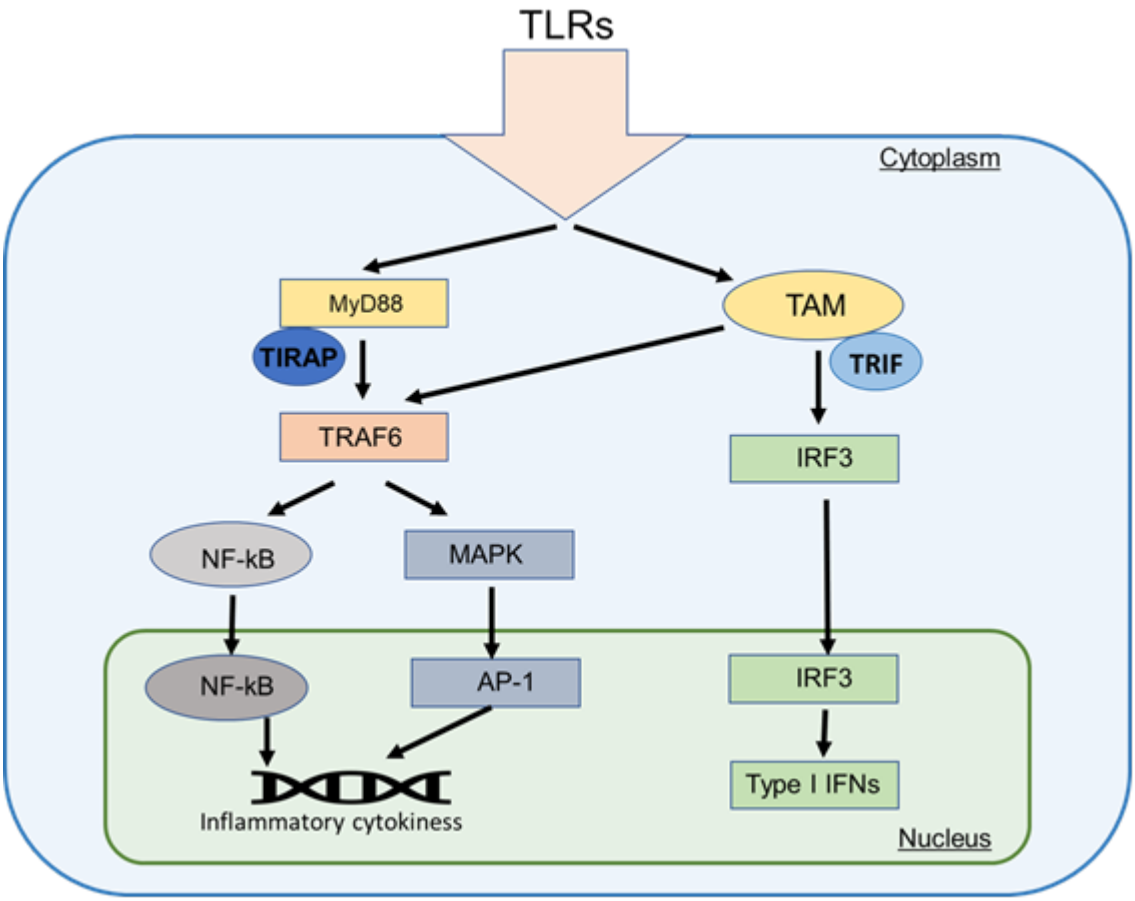


Figure 3. TRL signalling pathway.
Upon TLR engagement, MyD88- and TRIF-dependent TLR signalling is activated, resulting in AP-1, NF-κB, and IRF3 translocation in the nucleus, where these transcription factors modulate the gene expression of a variety of cytokines, chemokines and inflammatory mediators to amplify the inflammatory response. Abbreviations; IRF3; interferon regulatory factor 3, MyD88; Myeloid differentiation factor-88, TAM; Tyro3, Axl, and Mer, TIRAP; Domain Containing Adaptor Protein, TLRs; Toll-like receptors, TRAF6; TNF receptor-associated factor 6, MAPK; mitogen-activated protein kinase, NF-Kb; nuclear factor kappa B.
Adapted from (Chen et al, 2018). Created with Microsoft PowerPoint software.

Mediators of inflammation derive from plasma and secreted proteins. The formers are inactive in the plasma, but their levels can markedly increase during the acute-phase response. The latter are usually released by specialized myeloid cells, such as tissue-resident macrophages and mast cells. Inflammatory mediators are classified as vasoactive amines, peptides, fragments of complement components, lipid mediators, cytokines, chemokines, and proteolytic enzymes. In particular, cytokines are essential in regulating the immune response and inflammation via a complex network of interactions. Indeed, pro and antinflammatory cytokines promote and halt inflammation, respectively (Table 1). The most known proinflammatory cytokines are the tumour

necrosis factor (TNF), interleuikin-1 (IL-1), interleuikin-6 (IL-6), and many others. They are produced by different cells (in particular macrophages and mast cells) during the acute phase of an inflammatory response, with the biological function of promoting endothelium activation and favouring leukocytes recruitment at the site of infection or injury. Antinflammatory cytokines, such as IL-1 receptor antagonist (IL-1RA), IL-10, and IL-13 inhibit inflammation, counteracting proinflammatory cytokine activity and promoting the resolution phase.

Table 1. Main cytokines and their roles in the inflammatory response.
Adapted from (Chen et al, 2018). Abbreviations; NK, Natural killer.

Cytokine	***Main source***	***Function***
IL-1β	Macrophages, monocytes	Pro-inflammation, proliferation, apoptosis, differentiation
IL-1RA	Macrophages, monocytes, dendritic cells, epithelial cells	Anti-inflammation, inhibition of the proinflammatory cytokines
IL-4	T helper cells	Anti-inflammation, T cell and B cell proliferation, B cell differentiation
IL-6	Macrophages, T cells, adipocytes	Promotes inflammation, differentiation, cytokine production
IL-8	Macrophages, epithelial cells, endothelial cells	Pro-inflammation, chemotaxis, angiogenesis
IL-10	Monocytes, T cells, B cells	Anti-inflammation, inhibition of the proinflammatory cytokines
IL-12	Dendritic cells, macrophages, neutrophils	Promotes inflammation, cell differentiation, NK cell activation
IL-11	Fibroblasts, neurons, epithelial cells	Anti-inflammation, differentiation, acute phase protein induction
TNF	Macrophages, NK cells, $CD4^+$ T cells, adipocytes	Promotes cytokine production, cell proliferation, apoptosis, anti-infection
IFN-γ	T cells, NK cells, NKT cells	Pro-inflammation, innate, adaptive immunity, anti-viral

The complement is also a soluble mediator of inflammation and it includes a group of proteins that activate cells of the innate immune system. The key complement components for acute inflammation include C3a and C5a, also known as anaphylatoxins. C5a is a chemotactic factor for neutrophils, eosinophils, and macrophages, while C3a complement component has a pivotal role during opsonization for phagocytosis. The formation of the membrane attack complex (MAC) represents the final step during the activation process of the complement cascade. The MAC leads to the activation of neutrophils, monocyte, and mast cells (Haddad & Wilson, 2021; Bardhan & Kaushik, 2022).

The effectors of an inflammatory response are the cells and the tissues, which modify their functional state to adapt to the new harmful condition. They actively contribute to the resolution of inflammation, supervising key events of this final stage, such as inhibition of neutrophil influx and signalling pathways associated with leukocyte survival. Neutrophils elimination by phagocytosis is mediated by macrophages, and it occurs in a wide range of inflammatory sites (Savill, 1997). The phagocytosis process may also contribute to the initiation of signals that govern the inflammatory macrophage' migration alongside the inflamed tissues, reaching the lymphatics vessels (Bellingan *et al*, 1996). Moreover, upon phagocytosis of apoptotic cells, macrophages can also release mediators that stop the inflammatory response, such as the antinflammatoy cytokine transforming growth factor-β1 (TGF-β1), which can counteract proinflammatory signalling from TLRs (Fadok *et al*, 1998). Such antinflammatoy mediators can, in turn, promote the activity of enzymes needed for the production of other classes of eicosanoids such as lipoxins which are key components of this late stage (Levy *et al*, 2001).

In conclusion, cellular and molecular effectors are meant to activate dedicated signalling pathways to start the repair phase and properly preserve tissue integrity and restore homeostasis.

1.1.4 The inflammasome complex

Some intracellular PRRs, such as NLRs and absent in melanoma 2 (AIM2), can form multimeric complexes that Martinon and colleagues named inflammasome (Martinon *et al*, 2002). To date, different inflammasomes have been described based on the sensor protein involved in the formation of the inflammasome scaffold. They include members of the NLR (NLRP1b, NLRC4, and NLRP3 inflammasomes) (Zambetti *et al*, 2012), HIN200/AIM2-like receptor (AIM2 inflammasome), and Pyrin/TRIM20 (pyrin inflammasome). However, the inflammasome family is rapidly expanding, and other NLR-family proteins, such as NLRP2, NLRP6, NLRP7, NLRP9, and NLRP12, have been shown to form an inflammasome complex under certain circumstances (Xue *et al*, 2019). The inflammasome signalling has been extensively studied in macrophages, DCs, and neutrophils, but some sensor proteins are also expressed in non-immune cell types, such as epithelial and microglial cells (Martinon *et al*, 2009).

Diverse inflammasomes are assembled and controlled by mechanisms that depend on inflammasome protein structures and expression patterns. NLR family members typically share a standard structure which consists of three domains: 1) an N-terminal caspase activation and recruitment domain (CARD) or pyrin (PYD) domain; 2) a central nucleotide-binding and oligomerisation (NACHT) domain, common to all the NLRs members, which activates the signalling via ATP-dependent oligomerisation and 3) a variable number of C-terminal leucine-rich repeats (LRR), which sense stimuli (Schroder & Tschopp, 2010). NLRP1b, NLRC4/NAIP, and NLRP3 belong to this family of receptors.

Inflammasome aggregation is an organised process involving several steps: the upstream recognition of endogenous and exogenous stimuli via immune receptors, the activation of the intracellular sensors, the recruitment of adaptor proteins, and the activation of downstream effectors (Figure 4). Upon sensing specific inflammatory stimuli, the inflammasome sensor protein recruits the adaptor apoptosis-associated speck-like protein containing a CARD (ASC), which, through its CARD domain, recruits the precursor of caspase-1 (pro-caspase-1) via a CARD-CARD interaction (Man & Kanneganti, 2016). NLRs containing a CARD domain can directly interact with the CARD of caspase-1 without requiring the ASC protein. Upon inflammasome complex formation, pro-caspase-1 is activated by autocatalytic processing (Yang *et al*, 1998). Activated caspase-1 processes the precursor of the proinflammatory cytokines pro-IL-1β and pro-IL-18 into their bioactive forms (Martinon *et al*, 2002).Moreover, activated caspase-1 also processes the pore-forming protein gasdermin D (GSDMD), leading to the inflammatory cell death known as pyroptosis. Indeed, once processed, the N-terminal domain of GSDMD (GSDMD-NT) domain relocates to the plasma membrane and oligomerises to form a pore through which the mature form of IL-1β and IL-18 are released (He *et al*, 2015; Shi *et al*, 2015).

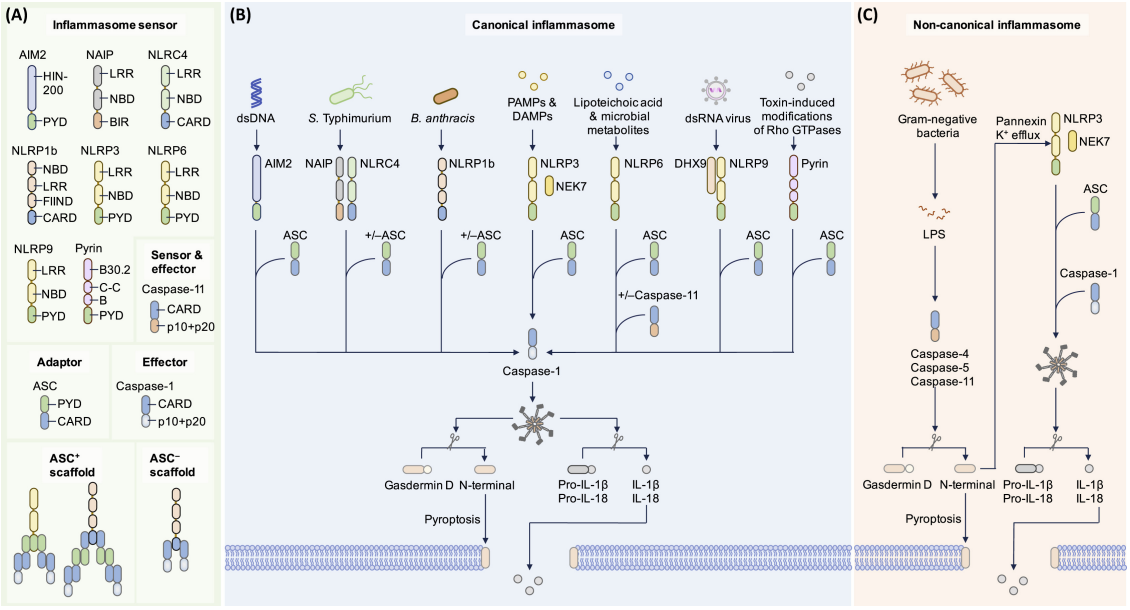


Figure 4. Inflammasomes structure and mechanisms of activation.
Abbreviations: LRR, leucine-rich-repeat domain; NBD, nucleotide-binding domain; B, B-box domain; FIND, function-to-find domain; BIR, baculovirus inhibitor of apoptosis repeat. AIM2, absent in melanoma 2; B, Bacillus; DHX9, DEAH-box helicase 9; NAIP, NOD-like receptor family apoptosis inhibitory protein; NEK7, NIMA-related kinase 7; S. Typhimurium, Salmonella enterica serovar Typhimurium. Figure from (Xue et al, 2019).

The NLRP1 inflammasome was the first inflammasome to be described. Three NLRP1 paralogs (NLRP1a–c) have been identified in mice. The human NLRP1 protein contains a CARD domain, and thus it can interact directly with pro-caspase-1 bypassing the ASC requirement. However, it has been shown that NLRP1 interaction with ASC improves inflammasome activity (Faustin *et al*, 2007). In contrast, ASC is dispensable for caspase-1 activation in the mouse setting since the NLRP1b orthologous lacks a functional PYD domain. Both rodent and human NLRP1 proteins are directly activated by the lethal anthrax toxin secreted by *B. anthracis* (Chavarría-Smith & Vance, 2013).

The NLRC4 inflammasome plays a pivotal role in antimicrobial defence via intracellular sensing of a set of bacterial components, including NAIP-mediated recognition of flagellin and components of type 3 secretion systems (TTSS) of the facultative intracellular pathogens *S. typhimurium*, *S. flexneri*, *P. aeruginosa*, *B. thailandensis*, and *L. pneumophila* (Kofoed *et al*, 2012). Since the NLRC4 receptor contains a CARD domain, it can directly bind pro-caspase-1.

The AIM2 inflammasome is triggered by double-stranded (ds) DNA of at least 70 bp long of microbial and host origin in a sequence-independent manner (Hornung *et al*, 2009). The flexibility in DNA sensing allows AIM2 to recognize genetic material from

a broad spectrum of pathogens and from damaged cells that accumulate endogenous DNA within the cytoplasm (Schroder *et al*, 2009).

The pyrin protein encoded by MEFV gene form the pyrin inflammasome, which is activated by Rho-modifying proteins, such as pathogens-toxins (glycosyltransferase TcdB of *Clostridium difficile*, adenyltransferase VopS of *Vibrio parahaemolyticus*, and the GTPase-activating protein YopE and cysteine protease YopT of *Yersinia pestis*) in murine and human macrophages (Xue *et al*, 2019). At steady state, active Ras homolog family member (Rho) A recruits the serine-threonine protein kinases (PKN) 1-2, which leads to pyrin phosphorylation at serine residues 208 and 242 in human cells (Park *et al*, 2016). These phosphorylated elements then attract pyrin 14-3-3 inhibitor proteins which block pyrin activation. Bacterial toxins remove these endogenous barriers by promoting RhoA inactivation, pyrin dephosphorylation and inhibition of 14-3-3 proteins binding, leading to inflammasome activation .(Xu *et al*, 2014).

1.1.5 The NLRP3 inflammasome

The NLRP3 inflammasome is the prototypic inflammasome. NLRP3 protein responds to various endogenous and exogenous stimuli, and its mechanisms of activation are by far the most characterized. NLRP3 is triggered by cellular stress induced by bacterial, viral, and fungal pathogens and endogenous DAMPs, such as particulates and environmental irritants. Understating how NLRP3 inflammasome activation takes place remains to be fully elucidated.

Since NLRP3 inflammasome plays a crucial role during an inflammatory process, its regulatory mechanisms need to be tightly controlled. There are two modalities by which the NLRP3 inflammasome is activated, defined as canonical and non-canonical pathways (Figure 5). The canonical NLRP3 activation is a two-step process in which a first signal from microbial molecules and endogenous cytokines associated to a second signal provided by extracellular molecules (ATP, pore-forming toxins, particulate matters) lead to NLRP3 inflammasome activation. In detail, the priming step involves the recognition of PAMPs and DAMPs by some PRRs (such as TLRs) as well as proinflammatory cytokines (*e.g.,* TNF). The mainstem function of the priming step is to upregulate NLRP3 expression and induce pro-IL-1β and pro-IL-18 (Bauernfeind *et al*, 2009). The additional function of this licensing step is the induction of different post-

translational modifications (PTMs) of NLRP3 through phosphorylation and ubiquitination to maintain the protein in an auto-suppressed state. Indeed, priming signals cause NLRP3 activation through the deubiquitinating enzyme BRCC3 (BRCC36 in humans) (Juliana *et al*, 2012; Lopez-Castejon *et al*, 2013). A further consequence of the priming step is the Jun N-terminal kinase (JNK1)-mediated NLRP3 phosphorylation, which represents a crucial step for NLRP3 self-assembly and inflammasome activation (Song *et al*, 2017). Thus, the licensing step prepares the cells to mount an appropriate inflammatory response. However, a second trigger is necessary for a full blow activation of the NLRP3 inflammasome (Kelley *et al*, 2019). Several different stimuli have been identified, including:

- ATP, K^+ ionophores (nigericin and maitotoxin);
- Heme;
- Particulate matter [monosodium urate (MSU) crystals, alum, silica, asbestos, amyloid-β, cholesterol crystals, and calcium crystals];
- pathogen-associated RNA and small antiviral components;
- Bacterial and fungal toxins and elements.

Since these triggers exhibit different biochemical structures, it has been hypothesised that NLRP3 does not directly interact with all these agonists. Indeed, it is unlikely that these inflammasome activators can induce a common cellular signal. However, the plethora of NLRP3 inflammasome-activating agents induce multiple cellular perturbations, including ionic flux, lysosomal disruption, mitochondrial DNA (mtDNA) release into the cytoplasm, mitochondrial dysfunction and production of ROS, metabolic changes and trans-Golgi disassembly (Swanson *et al*, 2019). K^+ efflux has long been identified as an ionic event shared among different NLRP3 stimuli, like nigericin, ATP and particulate matter (Perregaux & Gabel, 1994; Smith *et al*, 2013). Another critical signal for NLRP3 inflammasome activation is the cytosolic Ca^{2+} flux through the opening of plasma membrane channels and the endoplasmic reticulum (ER). Nigericin, alum and MSU crystals cause K^+ efflux, which, in turn, leads to the reverse Ca^{2+} influx (Triantafilou *et al*, 2013; Murakami *et al*, 2012). After phagocytosis, particulate components can hamper lysosomal integrity causing the cytosolic release of lysosomal content. Lysosomal damage appears as a critical signalling event. Indeed, it has been demonstrated that the lysosomotropic peptide Leu-Leu-OMe triggers NLRP3

inflammasome activation.(Hornung *et al*, 2008) Other lysosomal-derived enzymes, such as cathepsin-B, may act as NLRP3 inflammasome activating agents (Weber & Schilling, 2014).

Regarding the contribution of mitochondrial dysfunction and ROS production, pieces of evidence support the idea that ROS, accumulated through the inhibition of the mitochondrial respiratory chain can activate the NLRP3 inflammasome (Zhou *et al*, 2011). Finally, the Chen group reported that the trans-Golgi network (TGN) dispersion might be a common signalling event upstream of NLRP3 inflammasome activation (Chen & Chen, 2018). However, the canonical NLRP3 triggers and how they initiate the dispersion of the TGN still need to be elucidated (Chen *et al*, 2019). The NLRP3 inflammasome assembly is fully executed through the interaction of NLRP3 with ASC and pro-caspase-1. Additionally, the NEK7 protein is a critical component in this stage. In particular, NEK7 is fundamental for ASC specks formation, IL-1β release and pyroptosis (McKee & Coll, 2020).

The two-step canonical NLRP3 inflammasome activation was challenged by the discovery that mouse bone-marrow derived and splenic DCs can secrete mature IL-1β following LPS stimulation without the requirement of ATP (He *et al*, 2013). Moreover, human monocytes can also release IL-1β in response to LPS alone, primary through caspase 4-5 activation (Viganò *et al*, 2015) . Importantly, second-signal-independent NLRP3 activation was also observed *in vivo*. LPS intraperitoneal administration is sufficient for producing mature IL-1β in serum (He *et al*, 2013). Some reports proposed that this unconventional process might be mediated by complement activation. Indeed the complement component membrane attack complex has been shown to activate NLRP3 *in vivo* (Triantafilou *et al*, 2013; Laudisi *et al*, 2013).

Finally, it was demonstrated that cytoplasmic LPS sensing can induce endotoxic shock in mice, independently of TLR4 signalling (Hagar *et al*, 2013; Kayagaki *et al*, 2013). This pathway, defined as non-canonical NLRP3 inflammasome activation, represents a further layer of defence in response to Gram-negative. Intracellular LPS directly binds to human caspase-1, 4, and 5, and murine caspase-11, which process the GSDMD protein, leading to pyroptosis and K^+ efflux-mediated NLRP3 inflammasome (Xue *et al*, 2019).

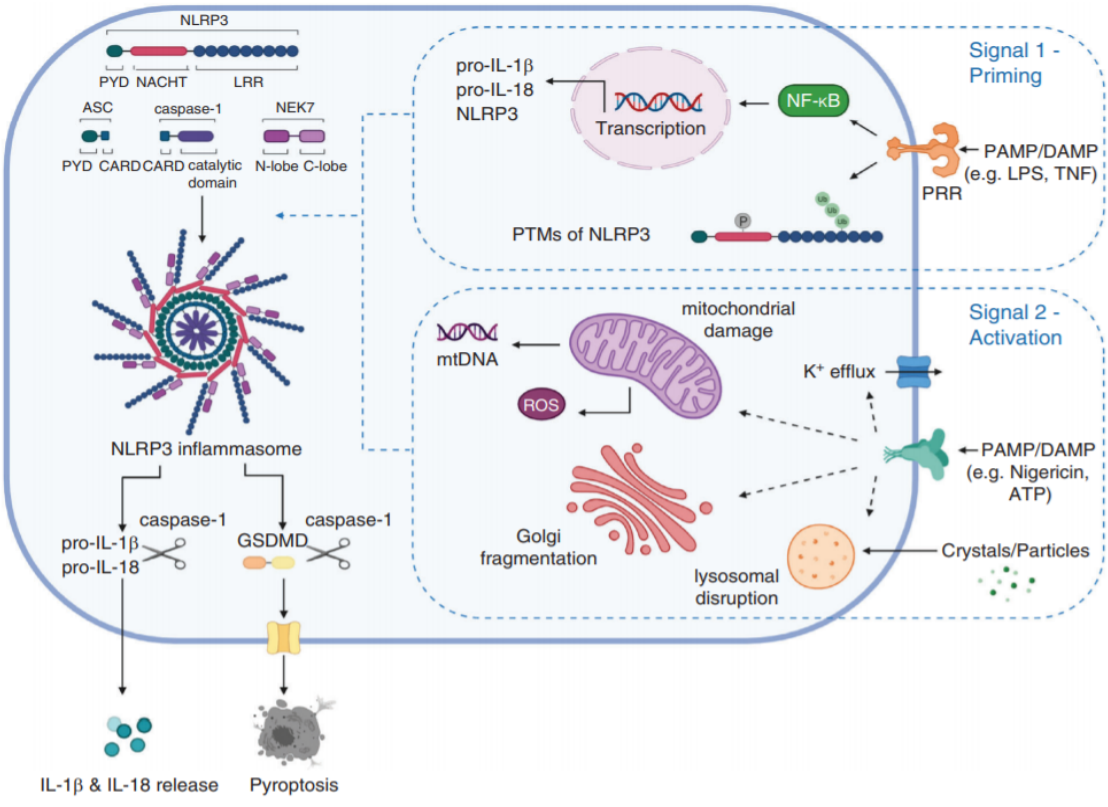


Figure 5. The two-step NLRP3 Inflammasome activation model.
Signal 1: the priming step involves the recognition of different PRRs, which promote the transcription of the inflammasome components NLRP3, pro-IL-1β and pro-IL-18. Signal 2: the activation step triggered by various extracellular agents (nigericin, ATP, and crystal particles) leads to different intracellular responses, including K^+ efflux, lysosomal disruption, Golgi fragmentation and mitochondrial damage. (McKee & Coll, 2020)

1.2 The IL-1 family

The IL-1 is the first cytokine to be discovered. It exists in two different isoforms: IL-1α and IL-1β. Before understanding their precise biological role, in 1974, Charles Dinarello described IL1α and IL-1β as two different "leukocytic pyrogens" thanks to their ability to induce fever in rabbits (Dinarello & Bernheim, 1981). A further step towards understanding how a single polypeptide could lead to various biological effects was made one decade later with the isolation of the human IL-1 cDNA (Auron *et al*, 1984). Since then, many studies have elucidated the role of this cytokine in the inflammation area and host defence.

Thereafter, other members of the IL-1 family have been discovered, and their pivotal roles in innate and adaptive immunity revealed. The IL-1 gene family consists of eleven

soluble proteins and ten IL-1 receptors. In the first group there are seven ligands with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ) and four with antagonistic activities [IL-1 receptor antagonist (IL-1RA), IL-36Ra, IL-37, IL-38). IL-1-like proteins are key in the innate immune response to infections, sterile tissue damage, and inflammation. In addition, their function in the activation and maturation of innate and adaptive compartments elucidated the broad spectrum of IL-1 system activity (Mantovani *et al*, 2019).

A crucial factor that links IL-1 family members to the innate response dates back to the discovery of the high homology between the cytoplasmic domain of the IL-1 receptor type I (IL-1RI) and the Toll protein of the fruit fly (Gay & Keith, 1991).The researchers postulated that IL-1 family members probably arose from a duplication of a common ancestral gene and named the cytosolic domain of the IL-1R1 as Toll IL-1 receptor (TIR), similarly to the TIR domains of the TLRs. In particular, these proteins share an amino acid sequence that folds in a two 6-stranded β-barrel (Taylor *et al*, 2002).

Based on the IL-1 consensus sequence and the ligand binding receptor, the IL-1 family members can be further stratified into 3 subgroups: IL-1, IL-18 and IL-36 subfamilies Garlanda *et al*, 2013). All the family proteins, except for IL-1RA, lack a signal peptide and are not constitutively secrete.

1.2.1 Biological roles of IL-1

IL-1α and IL-1β, the most investigated cytokines of the IL-1 superfamily; they are mainly produced by macrophages and dendritic cells of different lymphoid (thymus, spleen, lymph nodes, Peyer's patches, and bone marrow) and non-lymphoid organs (lungs, digestive tract, and liver). Furthermore, many other different cell types express both forms of IL-1, including neutrophils, keratinocytes, epithelial and endothelial cells (Takacs *et al*, 1988).

Although their biological functions are almost overlapping since they bind to the IL-1RI, the processes leading to their activation differ significantly. IL-1α and IL-1β are expressed as precursor proteins that require proteolytic cleavage to exert their cytokine activity (Afonina *et al*, 2015).

IL-1α was initially considered to act as a precursor protein without the requirement of proteolytic cleavage. However, more recent works demonstrated that mature IL-1α exerts a much higher activity than pro-IL-1α (Afonina *et al*, 2011). Indeed, IL-1α is described as a "dual-function cytokine", namely it can be found in the nucleus where it binds to DNA and as soluble cytokine that binds to the IL-1RI to initiate signal transduction. There are pieces of evidence that nuclear IL-1α acts as a transcription factor, promoting the expression of proinflammatory cytokines like IL-8 (Werman *et al*, 2004). Upon stress conditions, pro-IL-1α can be processed by proteases, including Ca^{2+}-dependent protease calpain, granzyme B (a cytotoxic T and NK cell protease), neutrophil elastase, and mast-cell chymase, reaching up a ~10-fold increase in activity over pro-IL-1α (Afonina *et al*, 2011). The IL-1α precursor is constitutively expressed by epithelial cells. Upon its release from necrotic cells, it acts as an endogenous DAMP that induces inflammation via binding to IL-1RI expressed by tissue-resident macrophages (Di Paolo & Shayakhmetov, 2016).

IL-1β is a pleiotropic cytokine whose processing, release and activation are tightly regulated. In contrast to IL-1α, IL-1β is not expressed under normal homeostatic conditions, and its precursor is inactive. Therefore, as previously mentioned, the inflammasome-activated mature form of IL-1β represents the primary source in macrophages and neutrophils during an inflammatory response. Nevertheless, it is worth mentioning that, besides caspase-1, other proteases can process and activate IL-1β in an inflammasome-independent fashion. This pathway involves IL-1β processing by neutrophil-derived enzymes, such as serine proteases, neutrophil elastases, cathepsin G, and granzyme A (Coeshott *et al*, 1999). This alternative pathway sustains IL-1β production in inflamed tissues, where neutrophils are abundant.

Despite its well-known nature of proinflammatory cytokine, IL-1β has other roles in cells and tissues. First, it is involved in haematopoiesis. Indeed, it was initially described as hemopoietin-1 for its ability to sustain haematopoiesis. Indeed, while acute IL-1β administration favours haematopoietic stem cell (HSC) regeneration after myeloablation and transplantation, long exposure to IL-1β promotes differentiation of stem cells into the myeloid cell-linage in *vivo* (Pietras *et al*, 2016). This phenomenon, known as “emergency haematopoiesis,” promotes uncontrolled HSC division and premature exhaustion (Manz & Boettcher, 2014).

Moreover, IL-1β has been investigated for its biological effects on T lymphocytes. More in detail, IL-1β, in combination with IL-23, activates a peculiar set of innate lymphoid cells (ILCs), which, similarly to IL-17 producing CD4+ T helper (TH) cells, release IL-17 and IL-22 (Spits *et al*, 2013). Indeed, IL-1β supports the differentiation and the cytokine response of TH17 cells, which favour the development of chronic inflammatory diseases in which the prostaglandin E2 (PGE2) is a crucial mediator (Zielinski *et al*, 2012). Finally, IL-1β activates γδ T cells, which are an additional source of IL-17 in tissues, and also a subset of TH1 cells expressing CD161, which are known as NKT cells (Sutton *et al*, 2009)(Cosmi *et al*, 2008).

There are other roles attributed to IL-1β that do not involve immune cell activation. For example, IL-1 acts on muscle catabolism, promoting synovial cell proliferation and the expression of extracellular matrix enzymes, such as collagenases. IL-1β can also promote bone-resorbing osteoclasts differentiation from precursors (Dayer, 2004). At a systemic level, IL-1β acts on the hypothalamus in the central nervous system to induce fever, lethargy, slow-wave sleep, and other signs of inflammation (Dinarello & Bernheim, 1981). Finally, IL-1β is also entailed in the apoptosis of pancreatic β-cells (Masters *et al*, 2010).

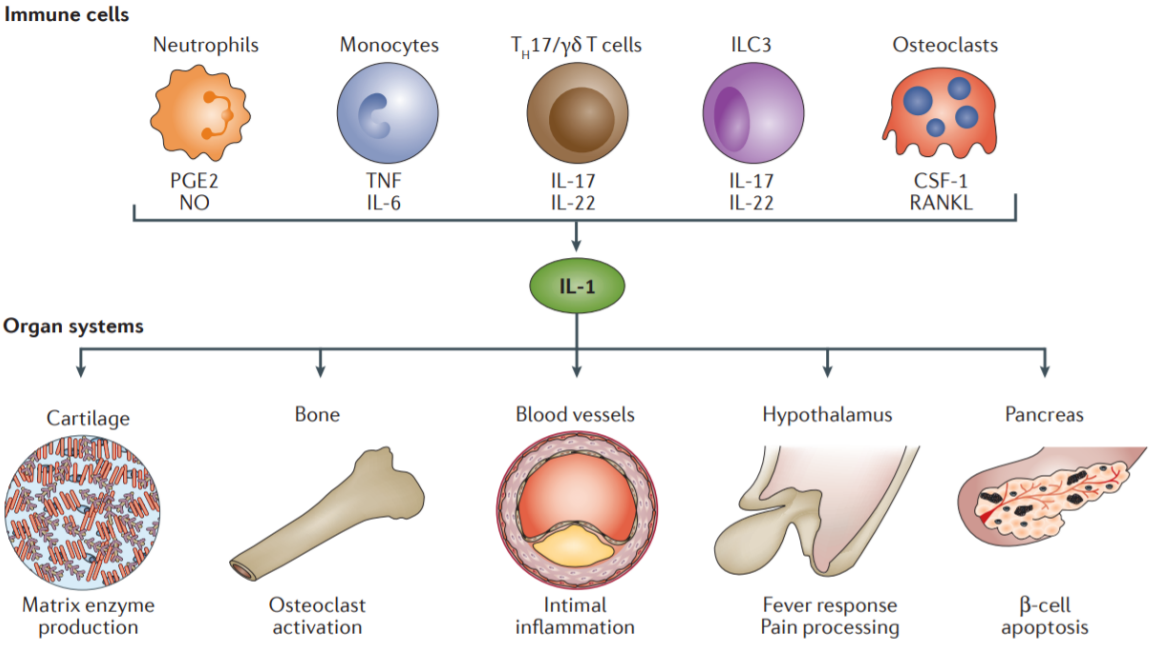


Figure 6. IL 1 functions.
IL-1β drives the activation of different innate immune cells, like neutrophils and monocytes. Many T-cell types, including T helper 17 (TH17) cells and γδ T cells, and innate lymphoid cells type 3 (ILC3), are stimulated by IL-1 activation. IL-1 also favours osteoclasts differentiation through the macrophage colony-stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL). IL-1β is a catabolic factor for the articular cartilage and bones, promotes blood-vessels inflammation and it is key for the fever response and pain processing in the hypothalamus. Finally IL-1β is also involved in the apoptosis of pancreatic-β cells. Adapted from (Schett et al, 2016).

1.2.2 IL-1 receptors and downstream signalling

The IL-1R complex is a critical component in the inflammatory response induced by IL-1. Many physiological mechanisms exist to negatively regulate its activation including either antagonist cytokines or decoy receptors.

The general protein architecture of the IL-1 receptors is based on an extracellular immunoglobulin-like domain responsible for ligand binding and an intracellular portion consisting of the TIR domain for signal transduction (Boraschi *et al*, 2018). The IL-1R complex is composed of the ligand-binding protein IL-1RI and the accessory protein IL-1R3 (also known as IL-1RAcP) (Figure 7). IL-1α or IL-1β binding leads to a conformational change of the IL-1RI, which allows its dimerization with IL-1R3. The homotypic interaction between IL-1RI and IL-1R3 leads to the signalling amplification via the two intracellular TIR domains and the recruitment of MYD88 and IL-1 receptor-activated protein kinase (IRAK) 4 (Thomas *et al*, 2012; Briskos *et al*, 2007). As a result,

this signalling machinery elicits the recruitment and oligomerization of TNF–associated factor (TRAF) 6 and the phosphorylation of IRAK1 and IRAK2. TRAF6 and IRAK proteins dissociate from the initial complex and activate the NF-κB and p38 transcription factors, JNKs, extracellular signal-regulated kinases (ERKs), and mitogen-activated protein kinases (MAPKs) (Dunne & Neill, 2003). The IL-1-mediated activation of NF-κB depends on the Iκ-B kinase (IKK) complex. Upon activation, the IKK system phosphorylates the NF-kB inhibitor I-kB leading to its degradation. P50 and p65 NF-κB subunits are then free to translocate into the nucleus, and bind to the consensus DNA sequences of genes encoding various inflammatory cytokines, such as IL-6, IL-8, monocyte chemoattractant protein 1 (MCP1), and cyclooxygenase 2 (COX2) (Weber *et al*, 2010).

A soluble form of IL-1RI has also been described. It binds to IL-1RI ligands thus preventing their interaction with the IL-1RI receptor complex and blocking the signalling activation (Svenson *et al*, 1993; Burger *et al*, 1995). Moreover, a second IL-1R isoform (IL-1R2) has also been discovered. It can properly bind to IL-1 ligands, but as it lacks the intracellular domain, when combined with the accessory protein IL-1R3, it cannot signal (Colotta *et al*, 1993) (Figure 7). Thus, IL-1R2 acts as a decoy receptor that downregulates the biological response to IL-1. Immune and non-immune cells differentially express the IL-1RI and IL-1R2 receptors. For example, neutrophils mainly express IL-1R2; therefore, higher concentrations of IL-1β are needed to activate neutrophils, while a low amount is enough to activate endothelial cells. The inhibition of the IL-1-mediated signalling relies on cytokines belonging to the IL-1 family exerting an inhibitory activity. One of these is the IL-1 receptor antagonist (IL-1RA), which has been discussed in the next paragraph in more detail.

In conclusion, the IL-1R complex is a critical component in the inflammatory response induced by IL-1. Its activation is tightly regulated either via the inhibition of receptor-binding site or interaction with inhibitory cytokines and decoy receptors.

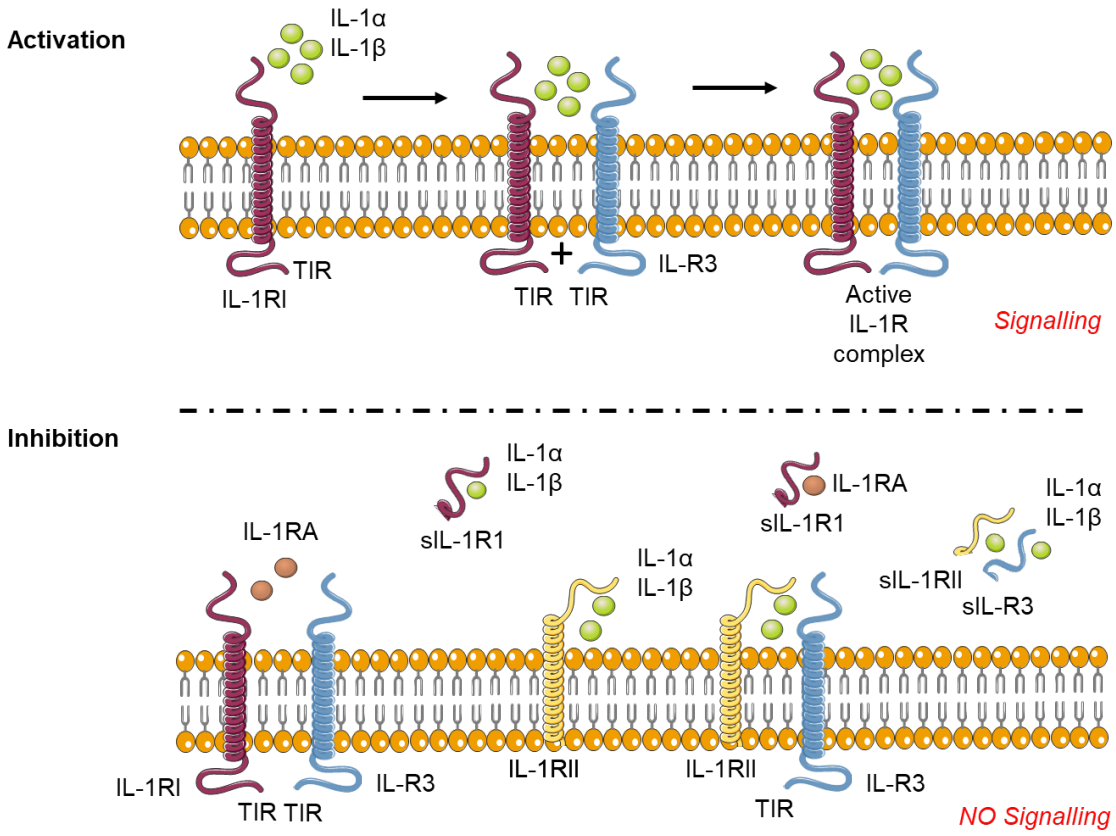


Figure 7. Schematic illustration of the IL-1R complex.
Activation Step. The IL-1α/β cytokines bind to the IL-1R1 inducing signalling. Inhibition Step. The interaction between IL-1R1 and the antagonist cytokine IL-1RA, IL-1R2 and the soluble receptors sIL-1R1, sIL-1R2, and sIL-1R3 can form inhibitory complexes to block IL- 1 binding to the receptor. Created with Microsoft PowerPoint website and modified from (Boraschi et al, 2018).

1.3 IL-1 Receptor Antagonist (IL-1RA)

IL-1RA (also called IL-1RN) was the first antagonist cytokine of a cytokine receptor to be discovered in the early 1980s in human fluids during endotoxin fever (Dinarello *et al*, 2021). A specific IL-1-blocking molecule was then isolated from human monocytes and urine of patients with juvenile arthritis (Arend *et al*, 1985). In 1987, this IL-1 inhibitor was described as a specific receptor antagonist for IL-1α and IL-1β binding to the IL-1R1 (Seckinger *et al*, 1987). The first cDNA coding for a secreted form of the molecule was cloned from a human monocyte library (Eisenberg *et al*, 1990). The similarity in exon-intron structure organization and the close proximity with the genes

encoding IL-1α and IL-1β suggested that the IL-1RA originated from gene duplication (Eisenberg *et al*, 1991).

Originally, IL-1RA was described as a protein mainly secreted by myeloid cells (monocytes/macrophages, DC, neutrophils), but it is also released by epithelial cells. Secreted IL-1RA (sIL-1RA) is synthesized as a pre-protein of 177 amino acids comprising a 25-amino acid leader sequence that is removed prior to the secretion of the 152 amino acid glycosylated protein. However, three additional intracellular isoforms of IL-1RA (icIL-1RA) have also been identified, although their biological roles remain unclear (Martin *et al*, 2020). The first identified intracellular isoform of IL-1RA (icIL-1RA1) is an alternative splicing form differing in the first exon from the sIL-1RA as it lacks the signal peptide and therefore it is retained in the cytoplasm (Haskill *et al*, 1991). IcIL-1RA isoform 1 is constitutively expressed by keratinocytes and other epithelial cells and is also produced with prolonged kinetics by stimulated monocytes and macrophages. The second and third intracellular protein sequences were further characterized. IcIL-1RA2 is encoded by an additional exon downstream of the icIL-1RA1–first exon, while icIL-1RA3 originates from an alternative translational initiation (Muzio *et al*, 1995; Malyak *et al*, 1998).

In summary, the IL-1RN gene encodes for four proteins, one secreted and three intracellular isoforms, which play a fundamental role in halting the stimulatory effects of IL-1.

1.3.1 Biological roles of IL-1RA

The main biological effect of sIL-1RA is to block the inflammatory effects of IL-1 by competitive binding to the IL-1R1 (Figure 7) (Arend *et al*, 1998). In spite of the similar binding affinity between IL-1RA and IL-1α/β, the former lacks a peptide loop between the β-strands 5 and 6. This structural difference block the conformational change that allows IL-1R1 to couple with IL-1R3, resulting in the inhibition of the signalling amplification (Schreuder *et al*, 1997). Consequently, IL-1RA-mediated occupancy of IL-1R1 prevents IL-1 binding and negatively regulates its proinflammatory activity. A 100-fold excess of IL-1RA over IL-1 is necessary to suppress an IL-1-mediated response efficiently (Arend, 1993).

The first evidence pointing to an antinflammatory property of IL-1RA derives from experiments performed in animal models of inflammatory arthritis. Neutralization of IL-1RA worsened LPS-induced arthritis in rabbits (Fukumoto *et al*, 1996). Conversely, IL-1RA treatment ameliorated the development of arthritis in various animal models (Arend *et al*, 1998). Mice genetically deficient for the IL-1RA gene developed a more premature and severe collagen-induced arthritis (CIA) than IL-1RA sufficient mice (Ma *et al*, 1998). In contrast, amelioration of CIA was showed in mice transgenic for sIL-1RA (Palmer *et al*, 2003). Similarly, IL-1RA knockout mice were more responsive to lethal endotoxemia and less to listeria infection than wild-type mice. These findings indicate that IL-1RA endogenously produced is a limiting factor that counteracts the aberrant effects of endotoxin, while IL-1 is pivotal in host defence to Listeria (Hirsch *et al*, 1996). Therefore, a balance between IL-1 and its antagonist IL-1RA should be maintained during a physiological response to infections. Concurrently, the antinflammatory role of endogenous IL-1RA might not be adequate to sustain a robust antinflammatory activity during inflammatory events (Arend & Gabay, 2000).

The biological role of the icIL-1RA isoforms remains largely under investigated. *In vitro* results using recombinant icIL-1RA proteins support the idea that, upon release, they can compete for IL-1R1 binding with sIL-1RA (Garat & Arend, 2003). In particular, Martin *et al.* demonstrated that the release of icIL-1RA type 1 by keratinocytes inhibits the IL-1-mediated signalling via the IL-1R1 complex (Martin *et al*, 2020).

1.4 The role of the IL-1 in human diseases

Inappropriate production of IL-1 has been linked to a broad spectrum of human diseases. In most cases, the dysregulated production of IL-1 can result either from an uncontrolled inflammasome activation or originate from an insufficient downstream antinflammatory activity. The pathological conditions in which IL-1 is involved are related to the basic biologic functions of this cytokine. As anticipated, IL-1β plays a pivotal role in haematopoiesis, as such its dysregulation could lead to some haematological diseases such as acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (Arranz *et al*, 2017).

Exaggerated levels of inflammatory cytokines are key features of chronic inflammation and have been found to promote initiation and progression of metabolic diseases (Robbins *et al*, 2014). For example, NLRP3 inflammasome can be hyperactivated upon chronic exposure to excessive levels of free fatty acids and glucose, leading to increased IL-1β-mediated apoptosis of β-cells in obese type 2 diabetes mellitus (T2D) condition (Maedler *et al*, 2002; Zhou *et al*, 2010). Furthermore, IL-1 is also a critial regulator of tissue response to injury and disease in the Central Nervous System (CNS) playing a pivotal role in neuroinflammation. Indeed, IL-1 levels were found elevated in patients following acute brain injury and those suffering from neurodegenerative diseases, such as Alzheimer's disease (AD) and multiple sclerosis (MS) (Liu & Quan, 2018).

IL-1β dysregulation also contributes to the spectrum of cardiovascular diseases. Indeed, it is central to the pathological response to injury and represents a critical factor in forming the atherosclerotic plaques and during the myocardial response to ischemic and non-ischemic injury. IL-1 and NLRP3 inflammasome are potential targets also for the risk of developing acute myocardial infarction (AMI), heart failure (HF), and recurrent pericarditis (Abbate *et al*, 2020).

Apart from all these conditions in which IL-1 may contribute to the complex clinical picture, this cytokine is historically linked to a group of systemic autoinflammatory diseases.

1.4.1 Systemic autoinflammatory diseases

Systemic autoinflammatory diseases (SAIDs) delineate a group of disorders caused by a dysregulated innate immune response. As anticipated in their name, a common aspect of SAIDs is their systemic pathophysiology, whose clinical manifestations can affect different tissues and organs. SAIDs can be considered an expansion of the autoimmune disease family characterized by a defective adaptive immune system. The primary cell types belonging to the innate immune system are monocytes, macrophages, and neutrophils, whereas it is generally accepted that autoimmune diseases are mediated by B and T lymphocytes. Nevertheless, different processes connect the innate and the adaptive immune system. Indeed, innate immune cells, like DCs, are required for the activation of B and T lymphocytes and contribute in some instance to autoimmune

diseases. Since IL-1β is also involved in adaptive immunity, this cytokine is considered to bridge both arms of the immune system (Waldner, 2009).

Autoinflammatory diseases are characterized by a broad spectrum of symptoms affecting multiple tissues and organs; the heterogeneous signs of inflammation may lack specificity and may impact a clear clinical diagnosis (Rowczenio *et al*, 2019).

The term "autoinflammatory diseases" was proposed for the first time in 1999 by Daniel Kastner, who coined this concept to describe the innate immune dysregulation identified in two disease conditions: familial Mediterranean fever (FMF) and TNF receptor-associated periodic syndrome (TRAPS) caused by mutations in the genes encoding MEFV/pyrin and the TNFRSF1A/TNF receptor type 1 (TNFR1) (McDermott *et al*, 1999). From that time, disease-based gene discovery and basic research have contributed to dissect the molecular mechanisms at the basis of excessive innate immune responses. Several mutations in more than 30 new genes have been identified and found to be responsible for autoinflammatory phenotypes. The number of patients showing clinical phenotypes resembling SAIDs is rapidly expanding. However, for at least 40–60% of them, no clear diagnosis can be made, leaving these conditions as undefined or undifferentiated SAIDs (uSAIDs). Thus, there is a strong need for a comprehensive classification that considers both genetic mutations and clinical criteria (Ter Haar *et al*, 2019). Although the central group of SAIDs has a genetic origin with mutations affecting single genes, polygenic or multifactorial disease also exist as it occurs for the autoimmune family of diseases (Hashkes & Laxer, 2019).

In conclusion, autoimmune and autoinflammatory diseases can be grouped in one single group of diseases characterized by different immunologic abnormalities with autoinflammatory syndromes at one end and autoimmune diseases at the opposite side. Whit in this model, immunological diseases are placed along a continuum line based on the degree of involvement of adaptive and innate immunity counterparts.

1.4.2 IL-1 mediated systemic autoinflammatory diseases

Excessive IL-1 signalling, production and release are responsible for the inflammatory conditions of one major group of monogenic SAIDs (De Jesus *et al*, 2015) (Table 2).

Table 2. Genetic features of monogenic IL-1-mediated systemic auto inflammatory diseases
Abbreviations: AD, autosomal dominant; AR, autosomal recessive; NA, not available. Adapted from (De Jesus et al, 2015).

IL-1 mediated systemic autoinflammatory diseases					
		OMIM NO.	***Inheritance***	***gene***	***protein***
CAPS (Cryopyrin-Associated Periodic Syndrome)	NOMID	607115	AD	*NLRP3*	NLRP3
	MWS	191900	AD	*NLRP3*	NLRP3
	FCAS	120100	AD	*NLRP3*	NLRP3
FMF (Familial Mediterranean fever)		249100	AR/AD	*MEFV*	MEFV /pyrin
NLRC4-MAS (NLRC4-related Macrophage Activation Syndrome)		NA	AD	*NLRC4*	NLRC4
HIDS/MKD (Hyper IgD Syndrome/ Mevalonate Kinase Deficiency)		260920	AR	*MVK*	MK
TRAPS (Tumour necrosis factor Receptor Associated Periodic Syndrome)		142680	AD	*TNFRSF1A*	TNFR1A
Majeed syndrome		609628	AR	*LPIN2*	Lipin-2
DIRA (Deficiency of Interleuikin-1 Receptor Antagonist)		612852	AR	*IL1RN*	IL-1RA

As mentioned previously, IL-1 is a potent endogenous fever-inducing pyrogen. Therefore, there are multiple checkpoints to control IL-1 release and biological effects, including the transcriptional regulation, the requirement of inflammasome-mediated activation, and the presence of protein inhibitors, like IL-1RA. When any of these surveillance mechanisms are lost, for instance, due to genetic mutations, a hyper-inflammatory state prevails. Clinically speaking, IL-1 mediated SAIDs are severe conditions that, in the majority of the cases, affect children and can be often fatal. The complex clinical picture shows organ-specific patterns of inflammation, and some of them are illustrated in Figure 8.

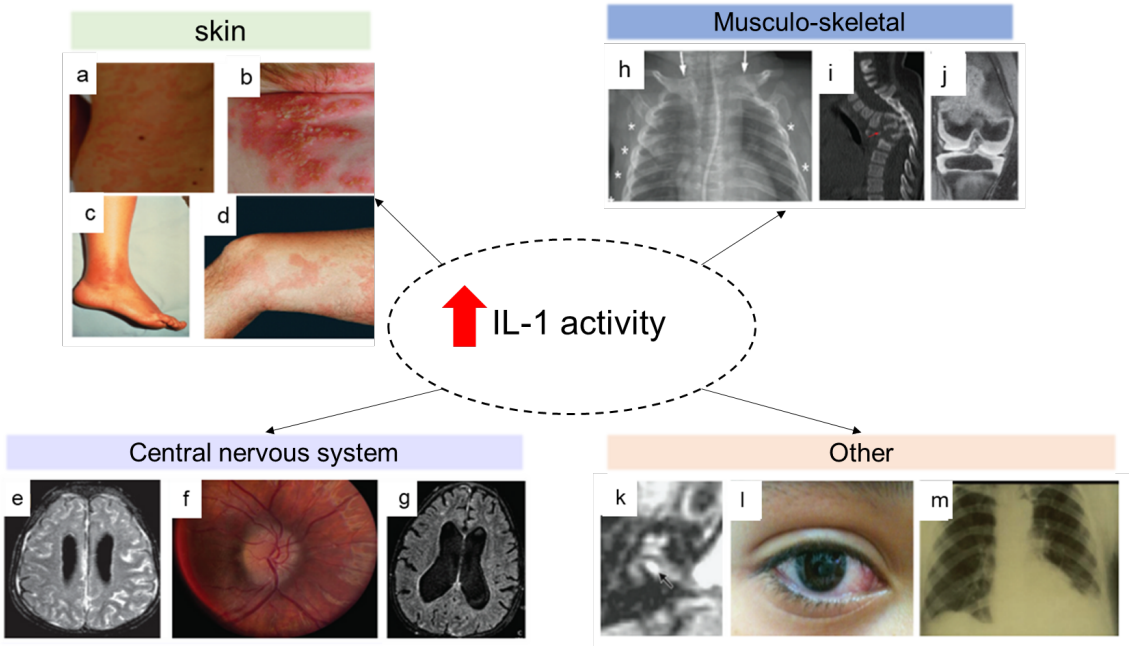


Figure 8. Clinical manifestations in IL-1 mediates SAIDs.
(a) Urticarial rash. (b) Pustular rash. (c) Erysipelas-like erythema. (d) Erythematous dermal macules during flares. (e) Leptomeningeal enhancement. (f) Inflammation-induced chronic papilledema. (g) Hydrocephalus and cerebral atrophy in NOMID. (h) Widening of multiple ribs (asterisks) and clavicles (arrows) osteomyelitis. (i) Destruction of vertebral bodies and severe kyphosis due to osteomyelitis (j) Metaphyseal bone overgrowth (k) Cochlear enhancement. (l) Conjunctival erythema (m) Pleural effusion. Adapted from (De Jesus et al, 2015)

Cryopyrin-associated periodic syndromes (CAPS) delineate a family of three autosomal dominant diseases caused by gain-of-function mutations in the NLRP3 gene (Agostini *et al*, 2004). They are rare diseases, with an estimated prevalence of 1–2 per million. Germline and somatic mutations can cause CAPS, most of which are localized in the NATCH domain of the NLRP3 receptor (Hoffman *et al*, 2001). As anticipated, the CAPS spectrum includes three distinct disorders: FCAS, MWS, and NOMID /CINCA syndromes. The three diseases have different grades of severity: FCAS is the milder form, NOMID/CINCA presents the most severe phenotype, and the MWS phenotype is in the middle of the spectrum. The clinical scenario of CAPS is characterized by chronic systemic inflammation, such as fever, fatigue, headache, and organ-specific pattern of inflammatory symptoms in the skin, musculoskeletal, ears, eyes and central nervous system.

Mutations in NLRP3 lead to a reduced binding affinity of cellular cyclic AMP (cAMP) and CARD8, which negatively regulates NLRP3. The resulting hyper-activation of NLRP3 inflammasome leads to increased IL-1β secretion and uncontrolled

inflammation (Ito *et al*, 2014). Indeed, blood monocytes from CAPS patients secretes higher levels of mature IL-1β compared to healthy controls, following LPS-stimulation. Indeed, patients' cells do not require second activator signal, and inflammasome priming with LPS alone results in maximal release of IL-1β (Gattorno *et al*, 2007). Therefore, IL-1β continuously activates its gene transcription and priming the inflammasome through the IL-1RI (Dinarello, 2009).

Familial Mediterranean fever (FMF) is the most widespread monogenic autoinflammatory disease, affecting more than 100,000 individuals worldwide, primarily in the Mediterranean region (Yilmaz *et al*, 2001). FMF is an autosomal recessive disease, although cases of autosomal dominant inheritance have been reported too (Stoffels *et al*, 2014). More than 80 distinct missense mutations in the MEFV gene have been described with a hot-spot region in exon 10, which encodes the B30.2 domain of the Pyrin protein, a critical inflammasome-mediated sensor upstream of IL-1β production (refer to paragraph 1.1.4) (Aksentijevich *et al*, 1997). Most patients experience the first attack in childhood, with long-lasting and recurrent flares episodes alternated by symptom-free intervals (Sönmezgöz *et al*, 2019). Flares include fever attacks, abdominal pain, generalized peritonitis, pleuritis, pericarditis, and, less frequently, aseptic meningitis. Along with persistent chronic inflammation, FMF patients may develop systemic amyloidosis, resulting in renal failure.

NLRC4-related macrophage activation syndrome (NLRC4-MAS) is caused by heterozygous mutations in the inflammasome-associated protein NLRC4 (Canna *et al*, 2014; Romberg *et al*, 2014). Macrophage activation syndrome (MAS) is a life-threatening inflammatory aggravation of many rheumatic diseases correlated with uncontrolled macrophage activation. The clinical picture includes fevers, pancytopenia, hepatitis, splenomegaly, and coagulopathy. In 2014, two independent research groups discovered de-novo gain-of-function mutations in the NATCH domain of NLRC4 in four patients from distinct families (Canna *et al*, 2014; Romberg *et al*, 2014). Three of these patients suffered from enterocolitis and recurrent MAS flares. Disease manifestations were homogenous across patients but differences in disease severity, ranging from intermittent, mild flares to mortality in early childhood. As already described, NLRC4 encodes an intracellular innate immune receptor that interacts with caspase-1, leading to IL-1β and IL-18 secretion (refers to paragraph 1.1.4). Similar to

CAPS, mutations in NLRC4-MAS cause spontaneous inflammasome formation and uncontrolled proinflammatory cytokines production. However, the symptoms of MAS differ from CAPS, underlying the different biological role of the two inflammasome components. In particular, mutations in the NLRC4 gene cause constitutive IL-18 hypersecretion, which is at the basis of enterocolitis and MAS, while IL- β secretion does not cause such detrimental effect.

Hyper-IgD syndrome (HIDS)/mevalonate kinase deficiency (MKD is a rare autosomal recessive disease originated from a mutation in the mevalonate kinase gene (MVK). The mevalonate pathway produces isoprenoids, which lead to the prenylation of RhoA and subsequent phosphorylation of Pyrin, which interact with the inhibitor protein 14-3-3. A mutated mevalonate pathway prevents this signalling, thus leading to hyper-activation of the Pyrin inflammasome (Park *et al*, 2016) (refers to paragraph 1.1.4). Depending on the degree of enzyme deficiency, MKD is considered a disease continuum of two phenotypic conditions: one mildest form Mevalonic Aciduria (MEVA) and Hyper-IgD (Immunoglobulin D) Syndrome (HIDS), being the most severe. The most common symptoms are intermittent fevers associated with abdominal pain, vomiting, diarrhoea, and different skin rashes. MEVA manifests with more severe complications, like dysmorphic features, growth retardation, ocular and neurological symptoms(Favier & Schulert, 2016).

TNF receptor-associated periodic syndrome (TRAPS) is an autosomal recessive disorder initially described as Hibernian fever (Williamson *et al*, 1982). It is caused by missense mutations in the *TNFRSF1A* gene encoding the TNF receptor 1 (TNFR1). The missense amino acid substitutions affect cysteine-cysteine disulphide bonds, vital for the protein structure. The resulting misfolded protein fails to bind TNF and instead accumulate in the cell cytoplasm. Consequently, increased NF-kB activation and inflammation developed (Nedjai *et al*, 2008). Clinical manifestations of the disease can be different among patients, and the most specific features include recurrent fever, abdominal pain, limb pain, pleuritis, arthralgia, periorbital oedema, and conjunctivitis. Neurological manifestations can manifest as headaches and, less frequently, aseptic meningitis, optic neuritis, and behavioural alterations (Hull *et al*, 2002).

In conclusion, IL-1 and TNF are both involved in this disease, suggesting no specific restrictions in regulating the production/secretion of these cytokines.

Majeed syndrome delineates an ultra-rare autosomal recessive disease driven by mutations in *LPIN2*. The protein lipin-2 has phosphatidate phosphatase (PAP) enzyme activity and catalyses the conversion of phosphatidate to diacylglycerol. This process is important for the maturation of membrane phospholipids and the formation of phosphatidylcholine, phosphatidylethanolamine, and other lipids that are mediators of an inflammatory response (refers to paragraph 1.1.3) (Reue, 2009).

Deficiency of IL-1 receptor antagonist (DIRA) represents a sporadic disease inherited in a recessive manner. Loss-of-function mutations causing DIRA lead to an absent or inactive IL-1RA, which translates into unbalanced signalling between IL-1 and its negative regulator IL-1RA through the IL-1R1 (refers to paragraph 1.3) (Aksentijevich *et al*, 2009). As a result, cells are hyper-responsive to IL-1α/β stimulation, leading to uncontrolled production of proinflammatory cytokines, chemokines, and other signalling molecules. Symptoms arise near birth with multisystem inflammation, pustular skin rashes and osteomyelitis involving musculoskeletal apparatus such as ribs, clavicles, and vertebral bodies.

A partial IL-1 dysregulation is also associated with several complex autoinflammatory/autoimmune diseases, in which there is no clear genetic origin. Different environmental factors may provide the basis for the clinical phenotype. Some examples are: gout, rheumatoid arthritis, Still's disease, pyogenic arthritis, pyoderma gangrenous, and acne (PAPA), and Bechet's disease.

1.4.3 Current treatments for SAIDs

There is no definitive cure for IL-1 mediated autoinflammatory diseases, but some treatments can ameliorate the clinical outcome. So far, the treatment of patients with SAIDs aims at controlling the recurrent episodes of systemic inflammation.

Colchicine is one of the first drugs that have been used to treat patients affected by rheumatologic diseases, such as gout, and it is the current mainstay treatment for FMF patients (Dasgeb *et al*, 2018). Colchicine is an alkaloid extracted from the meadow saffron whose efficacy in preventing attacks and developing amyloidosis was proved in several clinical trials with FMF patients (Demirkaya *et al*, 2016). In 2009, colchicine, sold under the name of Colcrys® received the final approval by the US Food and Drug Administration (FDA) for the treatment of acute flares in gout and FMF. The

antinflammatory mechanism of action of colchicine relies on the inhibition of microtubule assembly, which blocks proinflammatory pathways, such as inflammasome assembly, cytokine secretion, phagocytosis, cell development and chemotaxis. The potential therapeutic use of colchicine can be extended to other monogenic diseases, such as TRAPS and PFAPA, and also complex immune-mediated conditions, such as Bechet's disease, primary biliary cirrhosis, psoriasis, and comorbidities arising with gout, such as osteoarthritis and pericarditis (Dalbeth *et al*, 2014). Although colchicine is currently the treatment of choice for FMF, a consistent cohort of patients is refractory to this treatment due to partial remission or intolerance.

Within the spectrum of conventional drugs, non-steroidal antinflammatory drugs (NSAIDs) alone or in combination with glucocorticoids are used in 70–80% of all patients with monogenic IL-1-mediated inflammatory disease. The main therapeutic benefits of NSAIDs derive from the inhibition of cyclooxygenases, enzymes able to convert arachidonic acid into prostaglandins and thromboxane. Some of these eicosanoids commonly act as potent mediators during an inflammatory response (refers to paragraph 1.1.3). However, because NSAIDs do not act upstream in the catalytic cascade originating arachidonic acid, these drugs do not directly block the cause of the disease (Haar *et al*, 2013). On the same line, glucocorticoids reduce the activity of many proinflammatory mediators, counteracting leukocytes extravasation and controlling either acute manifestations (*i.e.,* pain, fever, oedema) or secondary inflammatory stages (*e.g.,* reparative processes, such as cell proliferation and wound repair) (Rhen & Cidlowski, 2005). Glucocorticoids represent the preferred treatment for patients with PFAPA syndrome, as their sustained administration interrupts the recurrent flares (Feder & Salazar, 2010).

Finally, other conventional antinflammatory, immune-modulatory, and immunosuppressive agents, such as azathioprine, leflunomide, methotrexate, cyclosporine and antihistamines, have been tested in monogenic autoinflammatory diseases mostly with a poor clinical outcome (Haar *et al*, 2013).

The involvement of the NLRP3 inflammasome in several diseases and the vital role of this protein in the production of IL-1β prompted the scientific community to discover new molecules that can selectively inhibit NLRP3 inflammasome activation. In these recent years, various small molecule inhibitors targeting NLRP3 inflammasome have

been developed, and some are currently under preclinical and clinical investigation. Two main modalities have been envisaged: direct inhibition of NLRP3 or inhibition of upstream or downstream events to indirectly suppress inflammasome complex formation or inflammasome execution mechanisms. In particular, the second modality is the best exploited, taking advantage of the diverse range of targets involved in the inflammasome assembly process (Gritsenko *et al*, 2020). For instance, Glyburide, a sulfonylurea drug commonly used in the United States for the treatment of T2D, specifically inhibits ATP- induced K+ efflux in pancreatic β cells (Ashcroft & Ashcroft, 2005). Another key event for NLRP3 inflammasome activation is NLRP3-ASC interaction. Proof-of-concept results showed that targeting the PYD of the adaptor protein ASC by specific peptides can effectively reduce cytokines-release from human monocytes following NLRP3 activation. Indeed, α-helical stapled peptides are stable and able to enter into the cells, disrupting ASC-specks formation and caspase-1 processing (Pal *et al*, 2019).

On the other hand, the diary sulfonylurea compound MCC950 is the most potent and direct NLRP3 inflammasome inhibitor. It has been in phase II clinical trials for the treatment of rheumatoid arthritis (RA), but the trial was interrupted because of liver toxicity (Chen *et al*, 2021; Mullard, 2019).

Nevertheless, all the significant discoveries came from studies performed *in vitro* or in mouse models of human diseases. There are still very few studies in humans on NLRP3 and the inflammasome complex. The lack of a comprehensive understating of how NLRP3 inflammasome gets activated makes developing products that selectively inhibit NLRP3 hard to achieve. Inhibiting NLRP3 activity could be helpful in IL-1 mediated inflammatory diseases with an evident upregulation of this type of inflammasome due to genetic mutations. However, it would not represent a solution for all the other conditions in which IL-1β dysregulation originates from different sources.

1.4.4 IL-1 targeting biologics

Given the detrimental role of IL-1 in many SAIDs, the standard of care for these patients is represented by the pharmacological blocking of the IL-1 pathway. The FDA approved three biologics for inflammatory diseases (Figure 9): Anakinra (Sobi™), the non-glycosylated form of IL-1RA produced in *Escherichia coli*, Canakinumab (Ilaris), a

human monoclonal antibody neutralizing IL-1β, and Rilonacept (Arcalyst), a fusion protein that binds IL-1β and IL-1α (Dinarello *et al*, 2012). Anakinra and Canakinumab also received approval from the European medicines agency (EMA).

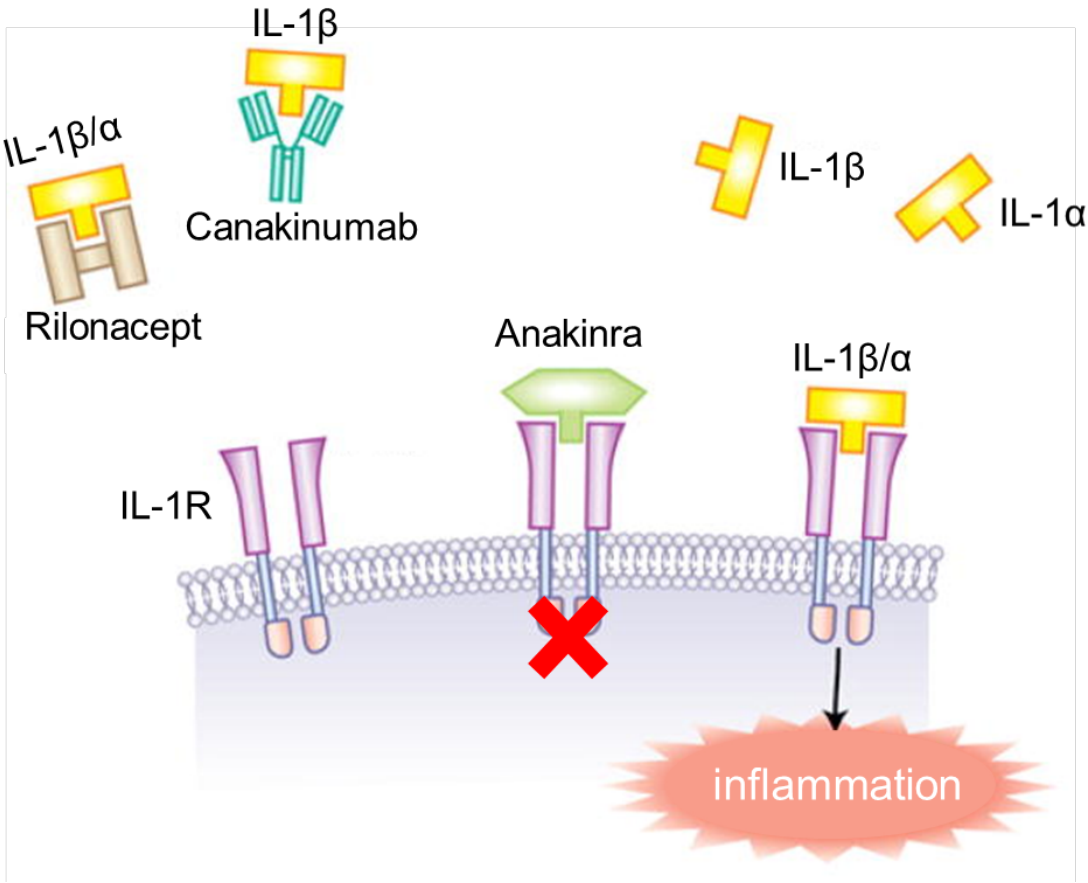


Figure 9. Anti-IL-1 cytokine biological treatments.
Rilonacept, a chimeric protein composed by IL-1R1 and IL-R3. Canakinumab, a human monoclonal antibody neutralizing IL-1β activity. Anakinra, the recombinant non-glycosylated form of IL-1RA, counteracting both IL-1α IL-1β. Adapted from (Hoffman, 2009).

Anakinra was the first IL-1 blocker developed and is currently the most used drug for autoinflammatory diseases. It suppresses IL-1-mediated signalling by competing with IL-1α/β for binding to IL-1RI. Anakinra was first approved in 2001 for rheumatoid arthritis (Mertens & Singh, 2009). To date, it received marketing authorization also for CAPS and DIRA. In Europe, it is also approved for Still's disease, including Systemic Juvenile Idiopathic Arthritis (SJIA) and Adult-Onset Still's Disease (AOSD) (Lyseng-Williamson, 2018; Oelkers, 2012). In 2021, EMA started evaluating the use of Anakinra for the treatment of coronavirus disease 2019 (COVID-19) based on the positive results obtained in adult patients with severe pneumonia (Kyriazopoulou *et al*, 2021).

Anakinra has demonstrated a remarkable level of safety and efficacy in a broad spectrum of autoinflammatory diseases. However, there are critical issues that limit its use. Anakinra has poor tissue distribution and a very short half-life. Therefore, patients require daily injections and lifelong treatment. Moreover, Anakinra has only partial efficacy in treating secondary AID-induced amyloidosis and articular involvement in FMF patients (Özçakar *et al*, 2016). In severe NOMID patients, IL-1 inhibition does not entirely revert hearing loss and vision impairment (Sibley *et al*, 2012). It can cause severe side effects, including injection-site reactions and serious infections such as skin/bone/joint infections and pneumonia. Other mild consequences are worsening arthritis symptoms, nausea, vomiting, diarrhoea, stomach pain, headache, and general joint pain, all of which reduce patients' quality of life, and also constitute an economic burden on healthcare systems. Finally it is not approved in all countries, for example it is not available in Brazil.

Canakinumab is a human monoclonal antibody that selectively binds to human IL-1β. Its efficacy depends on the blockade of IL-1β, thus preventing IL-1R1/IL-1β interaction and activating the inflammatory signalling cascade. It is currently approved for the treatment of CAPS, TRAPS, MKD, FMF, and active Still's disease, including AOSD and SJIA (Dhimolea, 2010). Recently, Canakinumab entered into a clinical trial to prevent adverse cardiac events in patients with a history of myocardial infarction and elevated high-sensitivity C-reactive protein (hsCRP), and the results are favourable (Aday & Ridker, 2018). One of the principal limits of this treatment is the cost. In addition, Canakinumab does not block completely IL-1-mediated response since also IL-1α activates IL-1RI.

Rilonacept, also known as IL-1 trap, is a dimeric fusion protein made up of the ligand-binding domains of IL-1R1 and IL-1RAcP linked to the Fc region of the human IgG1 that, acting as a soluble decoy receptor, neutralizes IL-1. While Anakinra and Canakinumab are approved in Europe, Rilonacept is only available in the United States to treat CAPS and recurrent pericarditis and maintain remission of DIRA.

In conclusion, anti-IL-1 cytokine drugs showed good clinical outcomes and a safety profile. However, they are not curative; the poor-tissue distribution, loss of efficacy and side effects limit their application. Thus, a new alternative curative approach for these patients still need to be developed. A case report study of a patient with FMF shed light

on allogeneic bone marrow transplantation (BMT) as a good option for the treatment of this disease (Milledge *et al*, 2002). The patient was paediatric and diagnosed with both congenital dyserythropoietic anemia (CDA) and FMF. The CDA treatment relied on repeated transfusions, which resulted in iron overload and a high ferritin level. An iron chelation therapy was not applicable due to her relapsing FMF condition. For these reasons, she underwent sibling BMT. During conditioning, FMF symptoms, such as arthritis, splenomegaly and periodic abdominal pain started to disappear, making the child off of colchicine treatment. The authors hypothesized that the newly produced patient's granulocytes were the cells responsible for FMF-phenotype correction after BMT. Thus, this was the first evidence that BMT can be curative for FMF.

1.5 Mouse models for NLRP3/IL-1 mediated inflammation

Several mouse models have been established to investigate the NLRP3/IL-1 role in the pathology of autoinflammatory and autoimmune diseases. We reported in the introduction those that have been used in our *in vivo* experimentations.

1.5.1 MSU-induced peritonitis model

Constitutive NLRP3 inflammasome activation leads to excessive IL-1β production resulting in a hyper-inflammatory state typical of several autoinflammatory conditions. One example is gout, the most common cause of inflammatory arthritis, driven by an overactive immune system. Gout is caused by joint deposition of MSU crystals in patients with hyperuricemia. The pathology of the disease includes discontinuous episodes of joint inflammation with symptom-free periods. If untreated, the disease severity can progress, leading to the formation of urate deposits (tophi) in soft tissues and progressive joint destruction (So & Martinon, 2017).

NLRP3/IL-1β–mediated inflammation is crucial in gouty inflammatory reactions that constitute the hallmark of the disease. In gout, IL-1β is produced by macrophages which, upon MSU-crystals sensing, activates NLRP3 inflammasome (Martinon *et al*, 2006). Intracellular MSU particles trigger NLRP3 inflammasome assembly through lysosomal destabilization (Guo *et al*, 2015), ROS production, ATP release and

purinergic signalling activation (Riteau *et al*, 2012; Gicquel *et al*, 2015). IL-1β release initiates a complex inflammatory cascade through the IL-1 receptor complex, resulting in neutrophil-recruitment from the bloodstream to the site of infection (So & Martinon, 2017). A similar biological process can be easily mimicked *in vivo* by administering MSU crystals in the peritoneal cavity or knee joints. NLRP3 activation in tissue-resident macrophages leads to IL-1β production, which, in a few hours, recruited neutrophils ($CD11b^{+}Ly6G^{+}$) quantified by flow cytometry (Martinon *et al*, 2006). This model has been used to test the therapeutic effect of some NLRP3 inhibitors such as Thiolutin (THL), which inhibits deubiquitinating of NLRP3 and Piperlongumine (PL) an alkaloid with antinflammatoy propertiers (Ren *et al*, 2021; Shi *et al*, 2022) Moreover, the MSU-peritonitis mouse model has also been exploited to test the antinflammatory role of the IL-1 family- related cytokines IL-37 and IL-33 (Liu *et al*, 2016; Shang *et al*, 2019).

1.5.2 NLRP3 knock-in mouse models for hereditary NLRP3-mediated diseases

Genetic mouse models harbouring gain of function mutations in the NLRP3 gene have been developed to investigate the chronic inflammatory manifestations derived by IL-1 overproduction and test new medications. As discussed in paragraph 1.4.2, dominant mutations in the NLRP3 gene are associated with CAPS disease, comprising three distinct autoinflammatory syndromes caused by uncontrolled IL-1 production: FCAS, MWS and NOMID.

Various mouse models mimicking CAPS manifestations have been developed. Two examples are the conditional $Nlrp3^{A352V/+}$ and $Nlrp3^{L353P/+}$ mice harbouring the alanine 352 to valine (A352V) and leucine 353 to proline (L353P) mutations identified in patients with MWS and FCAS, respectively (Brydges *et al*, 2009). Moreover, these amino acids are shared between mouse and human NLRP3 genes. Taking advantage of various mouse lines expressing the Cre recombinase under cell/tissue-specific promoters, $Nlrp3^{A352V/+}$ and $Nlrp3^{L353P/+}$ mice expressing constitutively or tamoxifen-inducible (CreT) NLRP3 mutant proteins have been established. The $Nlrp3^{A350V/+}$CreT and $Nlrp3^{L351P/+}$CreT exhibit a CAPS-like phenotype characterized systemically by IL-1-mediated inflammation, including weight loss, dermal neutrophilic infiltration and upregulation of proinflammatory cytokines in the serum such as IL-6. Moreover, BM-derived DCs (BMDCs) of $Nlrp3^{A350V/+}$CreT showed hyper production of inflammasome-

mediated IL-1β *in vitro,* which is released even in the absence of a second signal. Although T cells do not have a pathogenic role in CAPS, an increase in T helper cells expressing high levels of Th17-associated cytokines such as neutrophil chemoattractant (KC), IL-6, and granulocyte colony-stimulating factor (G-CSF) is also evident.

The main features of NOMID, the most severe form of CAPS, have been recently recapitulated in the $Nlrp3^{N475K/+}$ KI model, which constitutively expressed the NLRP3 N475K mutant protein (Bertoni *et al*, 2020). These mice show signs of systemic inflammation like rash, failure to thrive, increased serum proinflammatory cytokines (IL-1β, IL-1α and IL-18), splenomegaly, inflammatory cell infiltrates in several tissues (lungs, spleen and liver) and high mortality rate. In contrast to other models, these mice develop multi-organ amyloidosis, one of the primary morbidities of chronic inflammation in CAPS patients. Moreover, myeloid-derived cells from these mice displayed a pattern of cytokine expression similar to that of patients' monocytes characterised by hyper-responsiveness to LPS stimulation and elevated proinflammatory cytokine release (Tassi *et al*, 2010; Carta *et al*, 2015). Overall, this model well recapitulates disease symptoms.

1.5.3 Experimental autoimmune encephalomyelitis (EAE) as a mouse model of multiple sclerosis

IL-1 has a proinflammatory role also in the context of neuroinflammation associated with multiple sclerosis (MS). The experimental autoimmune encephalomyelitis (EAE) mouse model represents the most commonly used animal model for MS (Lin & Edelson, 2017).

Multiple sclerosis is a chronic inflammatory disease affecting the CNS, in which a genetic component and environmental factors contribute to the heterogeneous phenotype. MS manifests with neurologic symptoms ranging from mild impairment of key motor functions to serious cognitive decline. In the majority of the cases, the disease manifests with a relapsing and a remitting pattern (RRMS), although some patients are affected by a progressive form of MS (Musella *et al*, 2020). The key players in MS progression are the T cells, which recognize CNS antigens, spread through the brain and spinal cord, resulting in demyelination and axon loss (Kaskow & Baecher-Allan, 2018). Autoreactive $CD4^+$ T cells are the major drivers of MS pathology,

although γδ T cells, $CD8^+$ T cells, and B cells also contribute to disease (Kaskow & Baecher-Allan, 2018). The available disease-modifying therapies (DMTs) dampen the inflammatory stage of the disease, targeting different inflammatory cells such as Th1/Th17 and impairing immune cells' such as B cells infiltration to the central nervous system.

Cytokines are also attractive targets for new therapies. Several studies reported the involvement of IL-1 in MS pathogenesis (Lin & Edelson, 2017; Musella *et al*, 2020). Cerebrospinal fluid (CSF) and blood of MS patients showed high IL-1β levels and impaired IL-1β/IL-1ra ratio (Hauser *et al*, 1990; Dujmovic *et al*, 2009). Moreover, NLRP3 and Caspase-1 transcripts were found upregulated in patients' mononuclear cells compared to healthy donors' cells (Furlan *et al*, 1999; Heidary *et al*, 2014). Finally, at the time of remission, the RRMS patients with detectable levels of IL-1β in the CSF experienced a worst disease outcome (Rossi *et al*, 2014). To better elucidate the mechanisms elicited by IL-1β in MS, the experimental autoimmune encephalomyelitis (EAE) mouse model have been explored. The EAE model delineates the most used mouse model for MS and can be performed through active immunization with a myelin antigen. In the most popular version, female C57BL/6 mice are immunized with a complete Freund's adjuvant (CFA)-based suspension containing a peptide derived from murine myelin oligodendrocyte glycoprotein (MOG35-55) and mycobacterium tuberculosis to elicit peptide-specific Th cells. Mice are then treated with pertussis toxin (PTX) as a co-adjuvant to induce a sustained, disabling clinical disease (Constantinescu *et al*, 2011). Five to eight days post-immunization, IL-1β is produced primarily by monocyte-derived DCs/macrophages (moDCs/Macs) in the peripheral draining lymph nodes (DLNs). $CD4^+$ T constitutes also a cellular source for IL-1β. Once produced, IL-1β enhances M-CSF production by $CD4^+$ T cells promoting T-cell pathogenicity. Moreover, different myeloid cells in the CNS respond to IL-1β stimulation through the IL-1RI, but their contribution to the MS pathogenesis is still debatable. Thus, IL-1β appear to be a critical mediator of the EAE phenotype produced by myeloid cells, mast cells, and T cells, acting on both immune and non-immune cells (Lin & Edelson, 2017). Indeed, IL-1β- and IL-1RI-deficient mice are resistant to EAE-induction (Sutton *et al*, 2006). Alongside, EAE severity is augmented in mice lacking the inflammasome components NLRP3, ASC, caspase-1 and caspase-11 (Govindarajan

et al, 2020; Hou *et al*, 2020). Finally, IL-1RA treatment positively impacts EAE development (Furlan *et al*, 2007). Indeed, a clinical trial with anakinra for the treatment of chronically inflamed white matter lesions in MS (ClinicalTrials.gov Identifier: NCT04025554) has recently started. All these pieces of evidences point to a detrimental role of IL-1 in MS/EAE disease.

1.6 Haematopoietic stem cell transplantation and gene therapy

Haematopoietic stem cell transplantation (HSCT) has been successfully applied as a standard procedure for the treatment of inborn errors of the immune system and metabolism for more than 50 years (Tan *et al*, 2019). The first successful transplantation using allogeneic (from a compatible donor) haematopoietic stem and progenitor cells (HSPCs) was conducted in patients affected by X-linked severe combined immunodeficiency (SCID-X1), adenosine deaminase severe combined immunodeficiency (ADA-SCID), and Wiskott–Aldrich syndrome (Gatti *et al*, 1968; Bach *et al*, 1968). Extraordinary progress has been made in improving donor matching, graft-versus-host (GvH) reactions, conditioning' type, toxicity and infections management. However, the availability of an immune-compatible donor with suitable human leukocyte antigen (HLA) genotype matching limits HSCT application. Gene therapy (GT) approaches based on the genetic manipulation of autologous (from patient) HSPCs, might overcome this limitation and represent a powerful alternative treatment (Styczyński *et al*, 2020).

GT modifies the genetic material to add therapeutic genes or repair the defective ones. The *ex-vivo* autologous GT approach uses the patient's stem cells that, once genetically corrected, can be re-infused back into the patient. Toward this approach, sustained and durable production of the therapeutic gene can be achieved since the newly HSPC-derived immune cells can migrate to all body's districts in the form of tissue-repopulating cells.

From 1995 to 2020, 406 patients with primary immunodeficiencies (55.2%), metabolic diseases (17.0%), haemoglobinopathies (24.4%), and bone marrow failures (3.4%) were treated with different types of vectors-based strategies. Three advanced therapy medicinal products (ATMPs) based on HSPC-GT received marketing

authorization in the EU: Strimvelis® and Libmeldy™ for ADA-SCID and metachromatic leukodystrophy (MLD), respectively by Orchard Therapeutics, and Zynteglo™ for beta-thalassemia by Bluebird (Tucci *et al*, 2021) . Other products are currently under evaluation (Bueren *et al*, 2020). The clinical protocol is based on the following main steps (Figure 10).

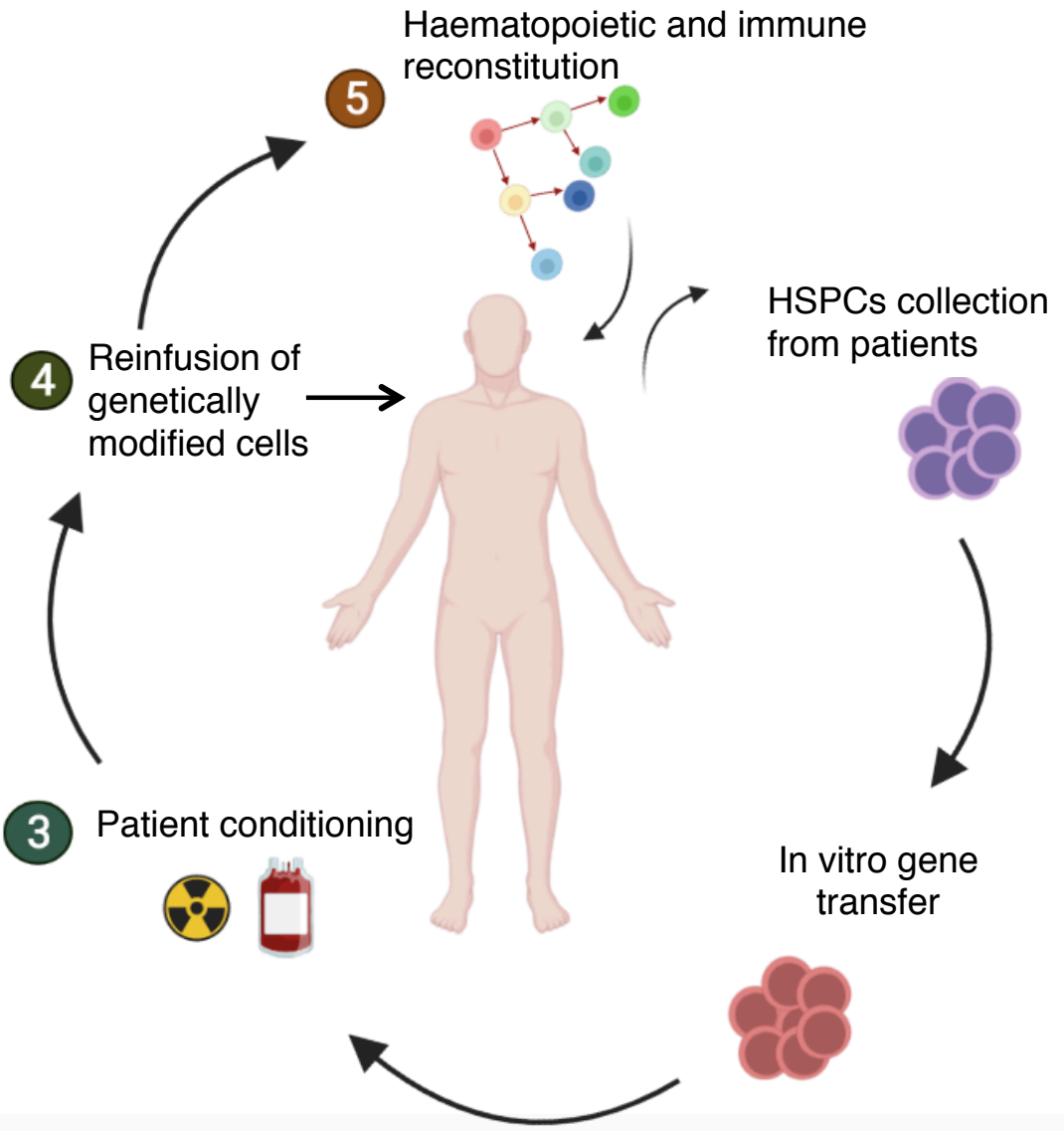


Figure 10. Main stages in ex-vivo gene therapy protocol.
1) Collection of patients' HSPCs. 2) Genetic modifications through vector-based gene transfer strategies. 3) Patient conditioning via chemotherapeutic agents. 4) Re-infusion of genetically modified autologous HSPCs. 5) Lon-term haematopoietic and immune reconstitution of newly corrected HSPCs. Created with Microsoft PowerPoint web-site.

1) HSPC collection. Patients HSPCs are collected from the iliac crests or by leukapheresis through the use of mobilization agents. The collected material is enriched for $CD34^+$ cells fraction, which comprise primitive haematopoietic stem cells (HSCs)

and lineage-committed HSPCs. The relative composition of the purified cells can vary based on disease background, age, and the collection method used (Thornley *et al*, 2001; Basso-Ricci *et al*, 2017). Since mobilized peripheral blood contains more HSPCs and ensures faster haematopoietic reconstitution than bone marrow (BM), it has become the preferred procedure (Gertz, 2010). The first mobilizing agent used for transplantation purposes was the G-CSF even though some patients can exhibit a poor mobilization response. Thus, mobilization procedures in the context of GT have been implemented through the additional use of the CXCR4 antagonist Plerixafor (Aiuti *et al*, 2013; Thompson *et al*, 2018; Río *et al*, 2019).

2) Vector-based HSPCs modification and in vitro culture. Following HSPC collection, cells are genetically modified through gene transfer technologies. Most of these approaches exploit viral vectors, such as gamma-retroviruses and lentiviruses, to allow therapeutic gene integration into the genome of target cells. The *in vitro* culture of genetically modified HSPCs requires specific protocols that preserve the most primitive cell populations. Indeed, efficient viral-mediated correction of HSPCs involves cells' exit from quiescence achieved by incubation with a cocktail of cytokines during *ex vivo* culture. Optimizing cell culture conditions in terms of duration, cytokine concentration, and vector amount is fundamental to retain HSPCs clonogenic output. Moreover, the efficiency of transduction can be limited by the presence of antiviral restriction factors expressed by HSPCs (Colomer-Lluch *et al*, 2018). To overcome these limitations, promising results have been obtained using transduction enhancers *ex vivo*. For instance, compounds, such as cyclosporine H (Petrillo *et al*, 2018) and prostaglandin E2 (Zonari *et al*, 2017; Heffner *et al*, 2018) allow the degradation of these antiviral factors, thus leading to high HSPC transduction.

The overall efficacy of a GT drug product currently relies on the use of specific parameters that can be tested in culture. They include the measure of the average number of vector copies (VCN) per genome detected in cells exposed to the vector, the maintenance of HSPCs' clonogenic potential and the levels of transgene expression/function. Humanized mouse models, such as immunodeficient NOD scid gamma (NSG) mice, have been exploited *in vivo* for xenotransplant and GT purposes (Ferrari *et al*, 2021; Carrillo *et al*, 2018).

3) Patients conditioning and re-infusion of genetically modified cells. Patients receive conditioning through the use of chemotherapeutic or immunosuppressant drugs to deplete the endogenous HSPC pool and prepare the BM niche to receive the gene-corrected cells. To achieve this goal, a deep knowledge of the disease-specific background and the level of engraftment of corrected cells are pivotal. Indeed, reduced-intensity non-myeloablative conditioning is sufficient to establish mixed chimerism when replenishing cells are granted with a natural selective advantage, such as in the case of deficiencies affecting the adaptive immune compartment, such as T and B lymphocytes (Aiuti *et al*, 2002), and Fanconi anaemia (Río *et al*, 2019). In contrast, myeloablative doses are needed when a high degree of engraftment is required, like in lysosomal storage disorders and beta-thalassemia (Biffi *et al*, 2013; Marktel *et al*, 2019). Therefore, the intensity and type of conditioning regimen administered to patients enrolled in a GT protocol should be fine-tuned to reach a minimal therapeutic benefit with a low level of toxicity. Regarding this aspect, future strategies based on monoclonal antibodies that can selectively deplete BM cells, avoiding off-target effects, should be implemented for their use in the GT arena (Bernardo & Aiuti, 2016).

4) Engraftment and immune reconstitution of modified HSPCs. One week after infusion, vector-transduced haematopoietic cells are already present in the blood. The haematopoietic reconstitution is first supported by the myeloid compartment, like granulocytes and monocytes, followed by NK, B and T cells (Aiuti *et al*, 2002). In most GT trials with lentiviral vectors (LVs), patients showed long-term engraftment of transduced cells up to 8 years post-treatment. Cells transduced with a gamma-retroviral vector encoding ADA persisted for at least 15 years in ADA-SCID patients (Aiuti *et al*, 2013; Cicalese *et al*, 2016). Of note, the high level of cell persistence is associated with sustained expression of the therapeutic transgene, leading to disease correction (Miller *et al*, 1993).

1.6.1 GT safety

The first clinical trials of HSPC GT for immunodeficiencies, including ADA-SCID, were based on the Moloney murine leukaemia virus-derived gammaretroviral vectors (γ-RVs). While starting to demonstrate the extraordinary clinical benefits of this approach, these clinical trials also highlighted the major limitations of the first

generation γ-RVs. First, these initial approaches have only a partial ability to transfer genes into the most primitive cell compartment. Moreover, it was reported that the preferred γRVs' integration pattern was within transcription start sites (TSS) and CpG islands on the contrary of LVs, which usually integrate into actively transcribed genes or transcription units. Consistently, γRV integration in the genome of host cells results in several cases of insertional mutagenesis (Naldini, 2015). The random integration of γRV can lead to the loss of expression of a tumour suppressor gene or upregulation of proto-oncogenes, causing haematological malignancies (Hacein-Bey-Abina *et al*, 2003; Naldini, 2015). Indeed, episodes of T-cell mediated leukaemia were reported in five out of twenty SCID-X1 patients enrolled in two independent clinical trials with γRVs. In these cases, γRV insertion in the promoter region of the proto-oncogenes *LMO2* and *IL2RG* caused the transactivation of the genes under the control of the long terminal repeat (LTR) regions of the viral vector, which are enhancers located up to 100 kilobases apart (Howe *et al*, 2008). Moreover, a myelodysplastic syndrome caused by insertional activation of growth-promoting genes PRDM16 and MSD1/EVI1102-103 occurred in chronic granulomatous disease (CGD) patients treated with γRVs (Grez *et al*, 2011; Reis *et al*, 2021).

Adeno-associated virus (AAV) vectors have gained interest in the field, although they mostly persist within cells as episomes and only few vector integration has been observed (Deyle & Russell, 2009). Therefore LVs with an improved safety profile have become the most commonly used vectors for delivery purposes. Indeed, many genetic modification such as the use of self-inactivating (SIN) LTRs, the exploitation of micro RNA target sequences, the changing form endogenous to weak-to-moderate promoters, the use of insulators, the refinement of the viral envelope are prevailing (Sauer *et al*, 2014; Naldini, 2011).

1.6.2 Lentivirus and development of lentiviral vectors

The retroviridae family of viruses are characterized by genome consisting of single-stranded positive-sense RNA which is converted into double-stranded DNA during replication. The lentivirus belong to this family, and the most used for GT applications is the Human immunodeficiency virus type-1 (HIV-1) (Klimatcheva *et al*, 1999). HIV-1 has an RNA genome, including three significant structural genes necessary for

integrating into the host genome: *gag, pol*, and *env* (Figure 11). The *gag* element encodes for the three major viral proteins: matrix proteins, capsid proteins and nucleocapsid proteins responsible for virion assembly, building up of hydrophobic core, and protection of the viral genome from interaction with RNA, respectively. The *pol* gene is uncharged for proteins' production essential during viral replication, such as viral protease, reverse transcriptase and integrase. Finally, the *env* gene produces the surface protein gp120 and the transmembrane protein gp41 surface proteins, which, by mediating virus entry upon interaction with cellular receptors and fusion with cellular membranes, determine the viral tropism (Fan & Johnson, 2011). Moreover, *tat* and *rev* genes also cover important roles as they are the first proteins synthesized after viral integration, and they are involved in transcription, splicing, and transport of nuclear mRNA to the cytoplasm (Sakuma et al, 2012). Other critical elements are the supplement genes *vif, vpr, vpu*, and *nef*.

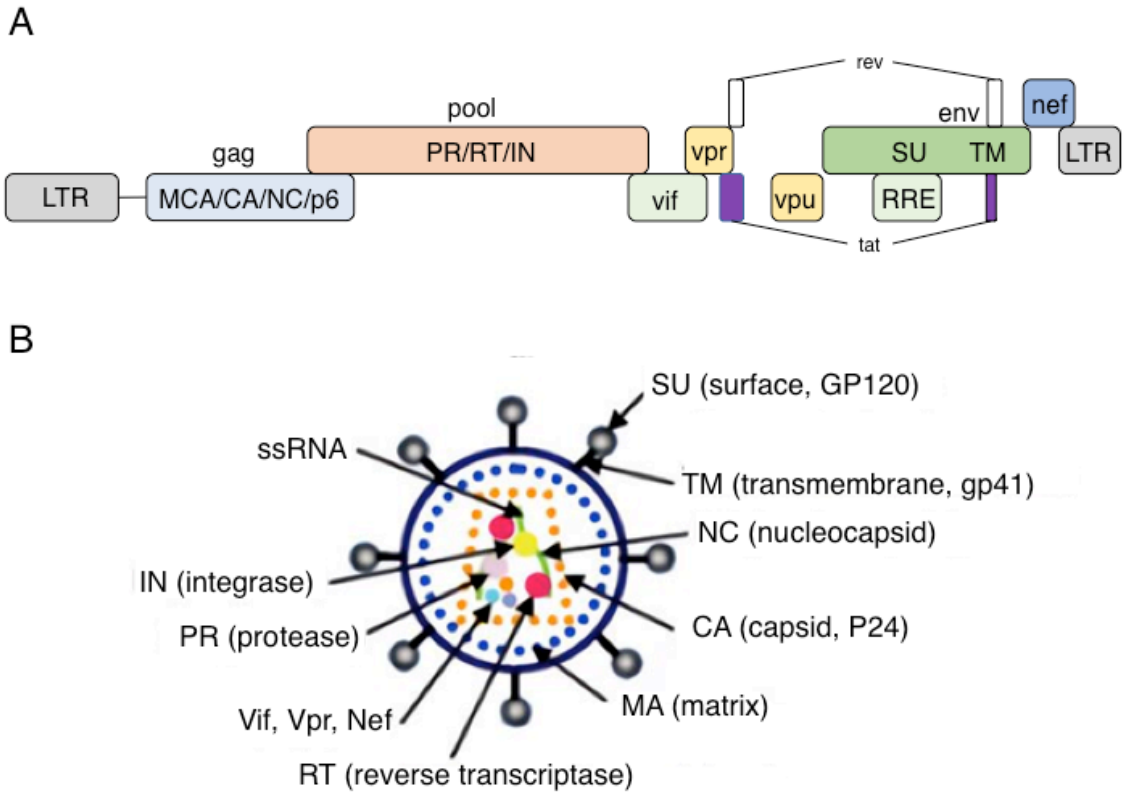


Figure 11. Illustration of the HIV-1 viral genome and virion structure.
*A. Long terminal repeats (LTRs) surround the three structural (*gag, pol, *and* env*), regulatory (*rev *and* tat*) and accessory (*vif, vpr, vpu, *and* nef*) proteins. The gag gene includes matrix (MA), capsid (CA) and nucleocapsid (NC). Three essential replication enzymes: reverse transcriptase (RT), integrase (IN), and protease (PR). The env protein produces viral surface (SU) and transmembrane (TM) domains. B. Lentivirus virion structure with two single-stranded viral RNAs, RT, IN, PR, and CA, accessory proteins protected by CA, and two viral membranes surrounded by MA. Created with Microsoft PowerPoint software and modified from (Sakuma et al, 2012)*

Two LTR sequences flank the LV genome are essential for reverse transcription and integration. The LTR sequences are structured into three regions: 1) U3 sequence containing genetic elements to regulate viral transcription such as enhancers, basal promoter, and att sites. Through this sequence the 5'-LTR allows transcription of the viral genome by the host cell's RNA polymerase II. 2) R repeated sequence with cleavage polyadenylation sites; and 3) U5 sequences. The 3' LTR binding of R-U5 stabilizes newly synthesized transcripts by regulating their polyadenylation. At the beginning, the transcription step is only partially active, allowing the production of few viral mRNAs which are spliced into transcripts encoding tat, rev, and nef proteins. These proteins in turn allow the production of singly spliced transcripts or non-spliced viral RNA leading to lifecycle progression (Wei *et al*, 1998; Malim *et al*, 1989).

The latter constitute the source for gag and pol production or progeny viruses, while spliced transcripts encode env and accessory proteins. In the last budding stage, full-length viral RNA and proteins are ready to be assembled at the plasma membrane and released into the extracellular space from the host cell.

LVs are genetically modified lentiviruses capable of carrying exogenous genes acting as DNA transfer vehicle. They can efficiently infect target cells, but they are deprived of their replication capacity. Hence, they are unable to produce viral particles (VP) and propagate to other cells. This unique feature makes LVs very attractive for GT approaches. Furthermore, there are other advantages in the preferential use of LVs, including (Zheng *et al*, 2018):

- Long-term transgene expression through stable integration;
- Infection of both dividing and non-replicating cells;
- Very high tropism for stem cells targeted by GT;
- Defective of immunogenic viral proteins after vector transduction;
- Large gene carrying capacity (such as intron-containing sequences);
- To some extent, easy handling for vector manipulation and high-scale production.

In the attempt to increase the safety of LVs and avoid the accidental generation of replication-competent lentiviruses (RCLs) during the laboratory-scale production, the first-generation of lentiviral systems separated the genome necessary for packaging and LVs production. This was achieved by co-transfecting a packaging cell line with three different plasmids:

- The packaging plasmid encoding gag, pol and regulatory/accessory proteins.
- The envelope (env) expressing a viral glycoprotein providing the vector particles enter into the cells.
- The transfer plasmid encoding the transgene and other cis-elements necessary for packaging, reverse transcription and integration.

The LTRs and the ψ packaging signal are included in the transfer vector and not in the packaging and env plasmids to avoid VP transmission. Therefore, as the genomic components responsible for the packaging are divided from the genomic elements that activate them, packaging sequences cannot be included into the viral genome, and the LV cannot be propagated after the infection of host cells (Kafri *et al*, 1997; Parolin *et*

al, 1994). The maintenance of four of the nine HIV genes such as *gag, pol, tat,* and *rev* associated to the genetic depletion of the accessory *vpr, vpu, nef*, and *vif* genes from the LV backbone lead to a second generation optimized systems.

The third-generation LVs emerged afterwards (Figure 12). Rev was provided from a cis-element in another separate plasmid. To increase the safety profile, Tat was removed from the packaging plasmid, but to preserve its function a promoter was inserted in the U3 region of the 5'-LTR in the transfer plasmid (Dull *et al*, 1998). Another critical step made to reduce the genotoxicity in the third-generation method regarded the LTR sequences. Since LTRs have an enhancer and promoter region, integration of LTRs into the genome can enhance adjacent proto-oncogenes. SIN-LVs were developed by depleting the viral promoter and the TATA box (U3 region) in the 3' LTR. Consequently, if the U3 element is deleted, the same modification would be reflected into the 5'-LTR region of the integrated genome during the reverse transcription step, thus resulting in transcriptional silencing of the enhancer/promoter region (Zufferey *et al*, 1998). The use of SIN required the employment of different promoters that drives transgene expression, such as the cytomegalovirus (CMV), or the spleen focus-forming virus (SFFV) strong promoter, the VAV and the phosphoglycerate kinase (PGK) gene derived weak/moderate promoters (Modlich *et al*, 2009), or the lineage-specific promoters such as the Cathepsin G/c-Fes Chimeric promoter (Santilli *et al*, 2011).

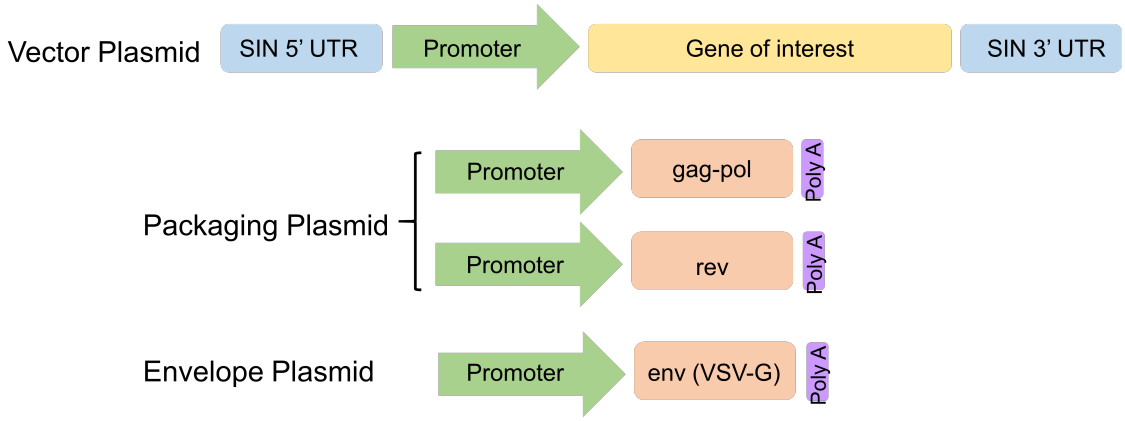


Figure 12. Third-generation lentiviral vectors.
Third-generation lentiviral vectors are made up of a SIN vector/transfer plasmid, two packaging plasmids, one encoding gag and pol and the other one encoding rev. A third envelope plasmid implemented with the vesicular stomatitis virus (VSV-G) G glycoprotein is also used. Created with PowerPoint software and modified from (Milone & O'Doherty, 2018).

Moreover, many modifications have been made to increase transduction efficiency and protein expression. For example, the HIV-1 env glycoproteins (SU and TM) included in the envelope plasmid have been substituted or integrated with other proteins of other viruses, thus allowing increased LV target host-cell tropism. The vesicular stomatitis virus (VSV-G) G glycoprotein is the most used thanks to its high stability, titre concentration, and extensive tropism (Coil & Miller, 2005). Indeed it has been demonstrated that VSV-G pseudotyped HIV-derived vectors efficiently transduce into long-term repopulating HSCs (Akkina *et al*, 1996).

Some modifications have been implemented to increase transgene expression and viral titre concentration. They include the use of the regulatory element of the woodchuck hepatitis B virus (WPRE), which increases nuclear transcript levels (Zufferey *et al*, 1999), and the central polypurine tract/central terminal sequence (cPPT/CTS), which enhances the nuclear translocation of the pre-integration complex (Follenzi *et al*, 2000). Other elements have also been added to increase the vector uptake, such as primer binding sites required for the retrotranscription and Rev-response elements (RRE) (Sakuma *et al*, 2012). Finally, to improve cell-type specificity of transgene expression, microRNA (miRNA) post-transcriptional elements were exploited. These small regulatory elements allow gene suppression in a specific cell-type by destabilizing mRNA transcription phase. Thus, miRNA target sequences (miRTs) which are complementary to the desired miRNAs, can be added in the LV cassette, increasing cell-type specificity and avoiding the transgene expression in specific subpopulations (Merlin & Follenzi, 2019).

1.6.3 Previous results on IL-1RA-mediated gene therapy

There is an urgent medical need for SAIDs since IL-1-targeting agents have some limitations and are not curative. Some initial attempts unveiled the potential application of IL-1RA-based GT to treat IL-1-mediated inflammation. The first shreds of evidence came in the early 90s when an MFG-IRAP retroviral vector was used to target the expression of human IL-1RA in synovial cells. IL-1RA expressing cells were subsequently transferred in rabbits by intraarticular injection. Hung and colleagues challenged the animals with recombinant IL-1β to test the efficacy of vector-derived IL-1RA in counteracting inflammation. They showed that IL-1RA expressing cells in the

knees successfully reduced neutrophils infiltrated in the joint space and blocked the pathophysiological sequelae caused by IL-1β injection (Hung *et al*, 1994). The same research group also used the same retroviral system to transduce mouse' HSPCs to test IL-1RA delivery after transplantation (Boggs *et al*, 1995). Although mice receiving IL-1RA-transduced cells showed sustained engraftment of donor cells and IL-1RA in the plasma, the authors did not provide results on the efficacy of vector-derived IL-1RA in reducing IL-1-mediated inflammation. These first results suggest the possibility to use IL-1RA-mediated *ex vivo* gene delivery in the context of autoinflammatory diseases such as rheumatoid arthritis. Indeed, this strategy was tested in preclinical studies with different animal models of RA and in two small human trials. Although the data were encouraging, the use of retrovirus ran the risk of insertional mutagenesis (Evans *et al*, 2005; Wehling *et al*, 2009).

More recently, Nixon *et al.* explored a novel vector-based approach using AAV as an IL-1RA vehicle for delivery into joints of small and large animal models of osteoarthritis (OA) (Nixon *et al*, 2018). AAV improved intra-articular IL-1RA delivery since it efficiently targets synoviocytes (Goossens *et al*, 1999). On the other hand, limitations related to the low efficiency of AAV in individuals who have pre-existing neutralising antibodies to the serotypes used for vector development, together with the fact that chondrocytes transduction may be hard to achieve in specific settings, presently delay the clinical translation of the protocol (Evans, 2018).

IL-1RA GT has also been exploited in the context of MS/EAE disease. In 2007, Furlan *et al.*, used a non-replicative Herpes simplex virus-1 (HSV-1) to deliver IL-1RA in the CNS by intracisternal (i.c.) injection. They reported that HSV-1 vector led to efficient IL-1RA production and EAE-treated mice showed a delayed disease progression by decreasing the number of inflammatory macrophages in the CNS. Nevertheless, the short-term expression of the therapeutic gene by this type of HSV-1-derived vectors currently limit the therapeutic exploitation of this approach (Furlan *et al*, 2007).

In 2020, it was reported that HSPC-based GT using an LV encoding IL-1RA ameliorates the cognitive decline and prevented memory deficits and hyperactivity in a mouse model of MPSIIIA, a lysosomal storage disease characterised by the accumulation in the brain of heparan sulphate and other substrates (Parker *et al*, 2020).

However, there was no indication of long-term engraftment and sustained IL-1RA production of transduced HSPCs.

In conclusion, all these works pointed to the potential therapeutic role of IL-1RA in diseases contexts in which a dysregulated production of IL-1 play a role, opening a new winning GT approach based on IL-1RA LV.

2. Aim of the work

SAIDs are a group of diseases distinguished by recurrent episodes of systemic inflammation. A central subgroup of SAIDs comprises rare periodic syndromes characterized by dysregulated proinflammatory IL-1 activity. Patients experience severe and recurrent inflammation ranging from fever to gradual hearing loss, blindness, and organ failure due to amyloid accumulation. Anti-IL-1 therapy is the conventional treatment for these patients, and the recombinant form of IL-1RA, known as anakinra and sold under the brand name Kineret®, is the most commonly used medication. However, the short plasma half-life (~4 hours) and poor tissue distribution of anakinra limit its application. Efficient IL-1RA delivery into the tissues is necessary to resolve inflammation in patients completely. A single long-lasting therapy could overcome the daily drug administration, often associated with injection site reactions and severe side effects. This PhD project addressed the urgent need to find a new curative treatment for these diseases.

GT is highly effective in delivering or editing the missing or malfunctioning gene found in inherited life-threatening diseases like PIDs. More recently, GT has been successfully applied to deliver immunomodulatory molecules against cancer and infections. Therefore, we aimed to develop a preclinical GT approach based on infusions of HSPCs transduced with a third-generation LV encoding human IL-1RA as a potential definitive treatment for autoinflammatory diseases characterized by exaggerated IL-1 activity.

In particular, the specific aims of the study were to:

1. Characterize the engraftment and clonogenic potential of mouse and human HSPCs transduced with an LV encoding human IL-1RA.
2. Establish mouse models of IL-1-mediated inflammation.
3. Evaluate the efficacy of IL-1RA GT in preventing acute inflammation induced by monosodium urate crystals.
4. Assess the efficacy of IL-1RA GT in modulating inflammation in a mouse model of CAPS disease and multiple sclerosis.

3. Results

3.1 Preclinical safety and toxicity studies

3.1.1 Generation of a transfer plasmid encoding human IL-1RA

To develop a lentiviral vector expressing human IL-1RA, we generated a transfer plasmid encoding the codon-optimized sequence of the secreted human IL-1RA isoform (co-IL-1RA). Codon-optimization maximizes protein translation by removing unfavourable mRNA regions and reducing local GC content. The co-IL-1RA cDNA was cloned into the backbone of the pCCL.sin.cPPT.PGK.GFP.WPRE plasmid at the 3' of the ubiquitous human phosphoglycerate kinase (PGK) promoter sequence, using the BamHI and SalI restriction sites (Figure 13A). Moreover, a Kozak consensus sequence was inserted between the PGK and co-IL-1RA sequence to enhance the translation of the therapeutic transgene (McClements *et al*, 2021). We used the PGK promoter to allow high and stable transgene expression in HSPCs and myeloid cells (Salmon *et al*, 2000; Tucci *et al*, 2021). PGK.LVs have been successfully used in our Institute for *ex-vivo* GT clinical trials for the treatment of Metachromatic Leukodystrophy (MLD) and Mucopolysaccharidosis Type I (MPS-I) (Orchard Therapeutics) (Fumagalli *et al*, 2022; Gentner *et al*, 2021).

Initially, to test whether IL-1RA is efficiently expressed and secreted, HEK293T cells were transfected with increasing amounts of the PGK.IL-1RA plasmid (0.2, 0.5, 1.0, 2.0 μg per well) in the presence of lipofectamine 2000 as transfection agent. Transfection with lipofectamine 2000 alone was used as negative control (Lipo) (Figura 13B). Upon transfection with the PGK.IL-1RA plasmid, HEK293T expressed and secreted IL-1RA in a dose-dependent manner, indicating that PGK.IL-1RA plasmid was functional even at low plasmid-concentration.

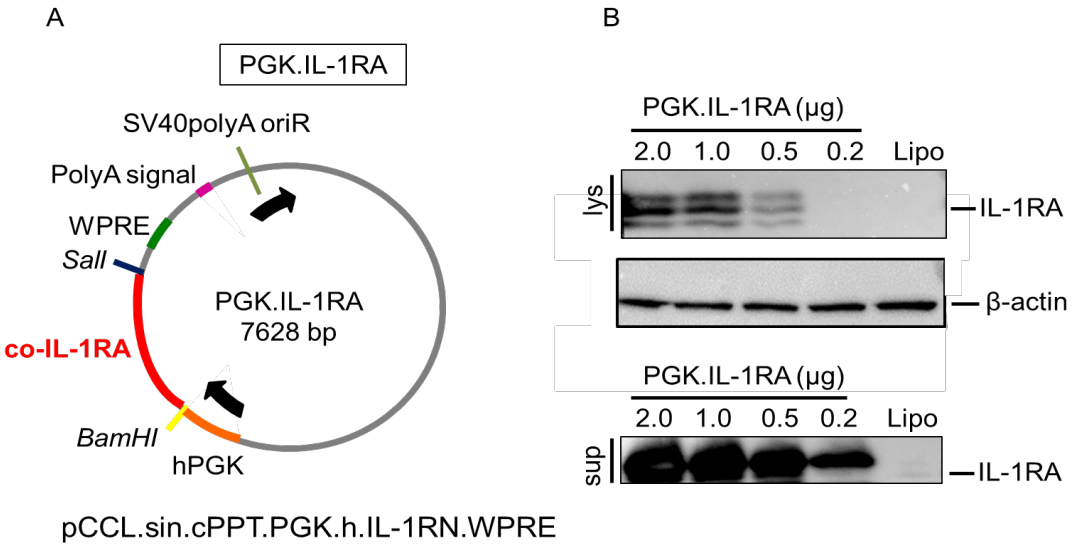


Figure 13. Generation and testing of the PGK.IL-1RA plasmid.
(A) Map of the PGK.IL-1RA transfer plasmid. (B) Intracellular co-IL-1RA expression in total cell lysates and protein' release in supernatants collected from HEK293T cells transfected with the PGK.IL-1RA plasmid at the indicated plasmid concentrations. Lipofectamine-treated cells are shown as negative controls. Abbreviations: PGK, phosphoglycerate kinase gene; WPRE, the post-transcriptional regulatory element of the woodchuck hepatitis B virus; co, codon-optimized.

Next, the PGK.IL-1RA plasmid was used to produce a lab-grade LV.IL-RA by co-transfecting HEK293T cells with three packaging plasmids (refers to section 1.6.2), using a standard protocol developed at SR-Tiget (Aiuti *et al*, 2013; Biffi *et al*, 2013). The LV.IL1RA titre was 2.20E+10 TU/mL with an infectivity of 7.56E+07 TU/ng P24. An LV expressing the GFP protein (LV.GFP) was also produced and used as a control vector in some experiments (Figure 14A). HEK293T cells were transduced with the LV.IL-RA at different multiplicity of infection (MOI) to test the efficiency of IL-RA production. Cells transduced with LV.IL-RA secreted human IL-1RA in a dose-dependent fashion, while untransduced (UT) and GFP-transduced cells did not (Figure 14B and 14C).

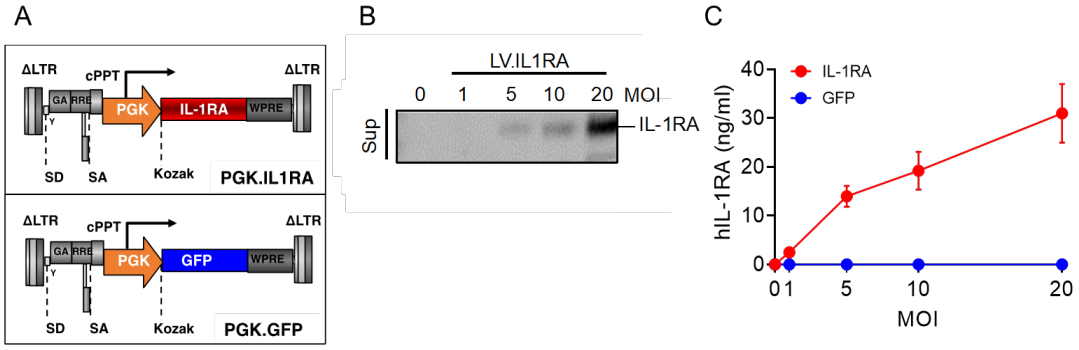


Figure 14. IL-1RA secretion in transduced HEK293T cells.
(A) Schematic representation of LV.IL-1RA and LV.GFP. (B, C) Secretion of IL-1RA from HEK293T cells transduced with LV.IL-1RA and LV.GFP at increasing vector dose (MOI). Untransduced cells (MOI 0) and GFP cells are shown as negative controls. Abbreviations: cPPT, central polypurine tract; GA, truncated gag sequence; LTR, long terminal repeat; MOI; multiplicity of infection; PGK, phosphoglycerate kinase gene-derived; RRE, Rev Response Element; SA, splice acceptor site; SD, splice donor site; WPRE, post-transcriptional regulatory element of the woodchuck hepatitis B virus.

3.1.2 LV.IL-1RA transduction ensured efficient IL-1RA production and secretion in mouse HSPCs with no evidence of toxicity

To set up a preclinical gene therapy approach is fundamental to preserve the "stemness" of HSPCs upon LV exposure. A deep characterization of hIL-1RA ectopic expression in mouse HSPCs was necessary to rule out possible detrimental effects derived from both the transduction procedure and the constitutive expression of the transgene in the most primitive cells. During LV preparations, transgene products can accumulate in producer cells and their medium. Vector-encoded cytokines in particular, might negatively impact transduction efficiency and target cells' phenotype. In this regard, it is important to reduce the concentration of LV-encoded cytokines in the LV stocks, preserving transgene expression. In house purified vector voided of plasmid DNA and soluble proteins from transfected HEK293T cell can overcome this limit, improving the quality of lentiviral vectors in research-grade preclinical studies (Soldi *et al*, 2020). In collaboration with the SR-Tiget Processing Development Laboratory, purified preparations of LV.IL-1RA and LV.GFP were produced as discussed in paragraph 5.2.3 in Methods. Mouse HSPCs (identified as lineages negative cell fraction) were isolated from the BM of C57BL/6 mice at a cell purity of 90% (Figure 15A) and transduced with the purified LV.IL-1RA and LV.GFP at MOI of 10, 50 and 100 in the presence of HSPC supporting cytokines for 14 +/- 2 hours. Transduction

efficiency was evaluated as the percentage of vector-positive colony forming units (CFU) derived from HSPCs by droplet digital PCR (ddPCR). A vector dose per cell of 10 was sufficient to transduce more than 80% of cells, and nearly 100% of CFUs were transduced with the LV.IL-1RA and LV.GFP at the 50 MOI (Figure 15B). The mean vector-copy number (VCN) per cell increased with increasing MOI's from 2.3 to 5.3 and 1.8 to 10.5 in HSPCs transduced with LV.IL-1RA and LV.GFP, respectively (Figure 15C). Moreover, substantial amounts of IL-1RA were measured in the culture supernatant collected from *in vitro* expanded HSPCs at day 14 post-transduction. In contrast, IL-1RA was absent in culture supernatants collected from untransduced and LV.GFP-transduced cells (Figure 15D). There was a positive correlation between IL-1RA levels and LV.IL-1RA dose (Figure 15E), indicating that LV.IL-1RA transduction directed the release of human IL-1RA in mouse HSPCs in a dose-dependent manner.

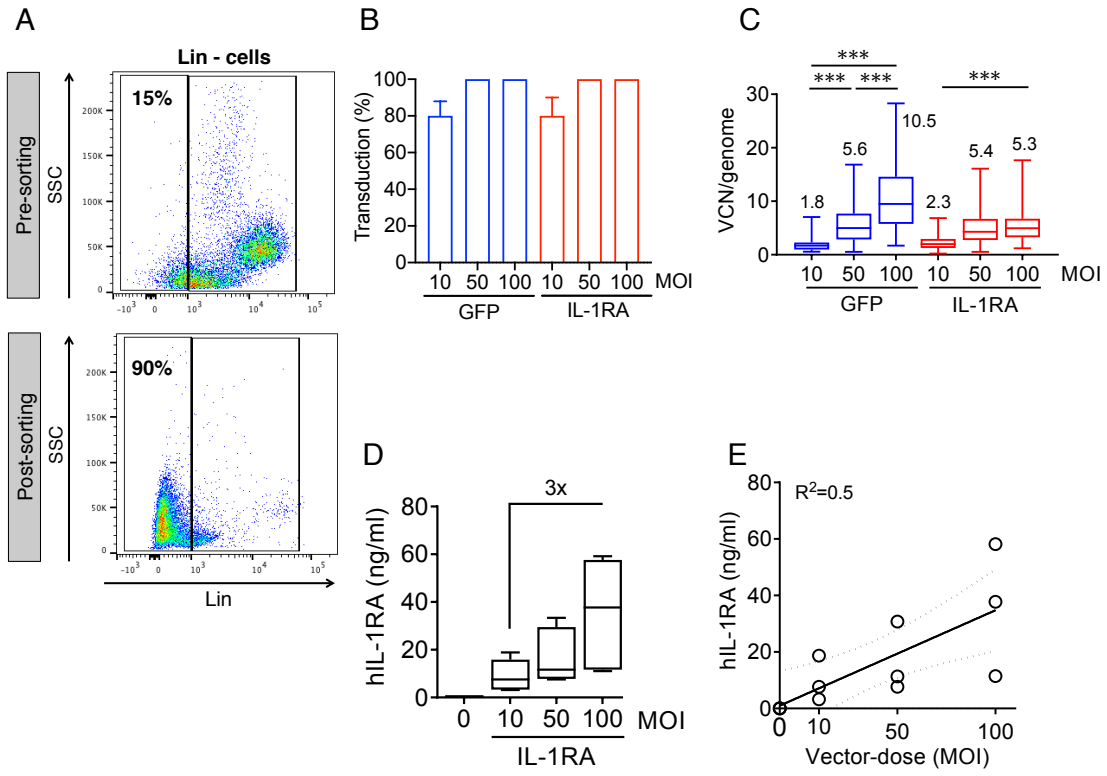


Figure 15. Efficient transduction and IL-1RA production in mouse HSPCs.
(A) Representative flow cytometry analysis of mouse BM cells before and after isolating lineage negative (Lin-) cells by immunomagnetic beads. (B, C) Percentage of vector-positive CFUs and quantification of VCN per genome in individual colony derived from HSPCs transduced with LV.IL-1RA and LV.GFP at the indicated vector-dose obtained from three independent experiments, as assessed by ddPCR. Means +/- StD of 3 biological replicates are reported in panel B. In panel C, the median and interquartile range of the VCN in single CFUs scored with a VCN>0.5 out of 32 CFUs are shown per each condition as a pool obtained from three biological replicates. (D, E) Human IL-1RA secretion measured in the supernatants of

*transduced HSPCs after 14 days of culture at increasing MOI. Three biological replicates. Abbreviations: SSC, side scatter. Mann-Whitney t-test: *, p<0.05; **, p<0.005; ***, p<0.0005.*

We also tested whether the transduction procedure and the ectopic expression of IL-1RA in HSPCs might alter the survival and the clonogenic potential of these cells. The total cell number of HSPCs collected before and after transduction with LV.IL-1RA and LV.GFP was similar to MOCK cells even at high vector dose (Figure 16A). Moreover, when plated in semi-solid medium, MOCK, LV.IL-1RA- and LV.GFP-transduced HSPCs generated an equal percentage of erythroid burst-forming units (BFU-E) and granulocyte-macrophage colony-forming units (CFU-GM) in all conditions (Figure 16B).

Our data collectively indicate that the transduction procedure and ectopic IL-1RA expression preserve the clonogenic potential of HSPCs.

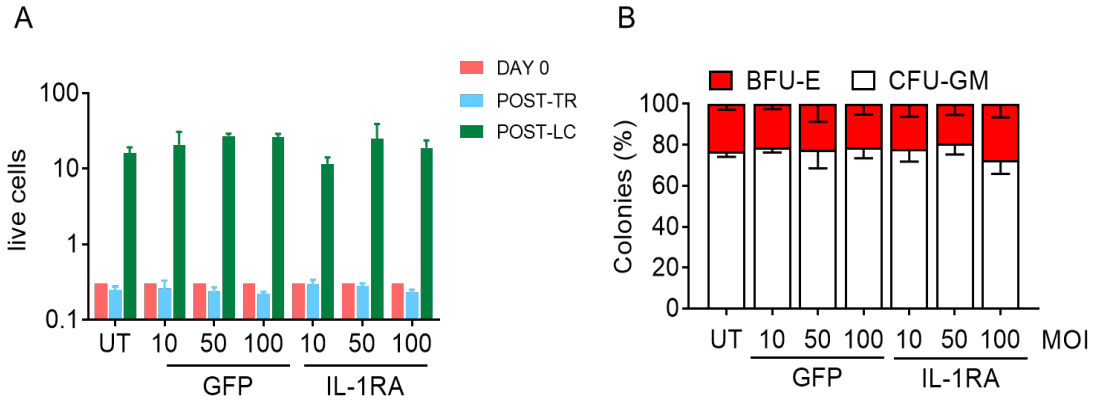


Figure 16. Evaluation of LV.IL1RA impact on mouse HSPCs functionality.
(A) Absolute count of mouse HSPCs pre- and post- exposure to the IL-1RA and GFP vector (B)Percentages of single colonies derived from myeloid (CFU-GM) and erythroid (BFU-E) progenitors in untransduced (UT), IL-1RA-, and GFP-transduced conditions. Means +/- StD of 3 biological replicates.

3.1.3 Efficient LV-mediated IL-1RA transfer in human HSPCs

Next, we examined the effect of lentiviral-mediated IL-1RA expression on human HSPCs. We isolated $CD34^+$ cells from mobilized peripheral blood, and we transduced them with LV.IL-1RA at MOI 10 and 50, in the presence of prostaglandin E2 as a transduction enhancer (Zonari *et al*, 2017; Heffner *et al*, 2018). The average transduction efficiency within transduced CFU-GM and BFU-E ranged between 80% and 100% in all conditions (Figure 17A). The mean VCN values were 7.6-9.4, and 3.9-

3.5 in BFU-E and 3.9-4.8 and 5.4-2.8 in CFU-GM, derived from HSPCs transduced with LV.IL-1RA or LV-GFP (Figure 17B). The VCN per cell in the BFU-E colonies was generally higher than in CFU-GM, as previously observed for other LVs (Charrier *et al*, 2011). Untransduced human HSPCs secreted IL-1RA, whereas LV.IL-1RA transduction led to the over-production of IL-1RA three times above the basal level (Figure 17C). A vector-dependent increased IL-1RA release was evident also in CD34-derived cells after 14 days of culture (Figure 17D).

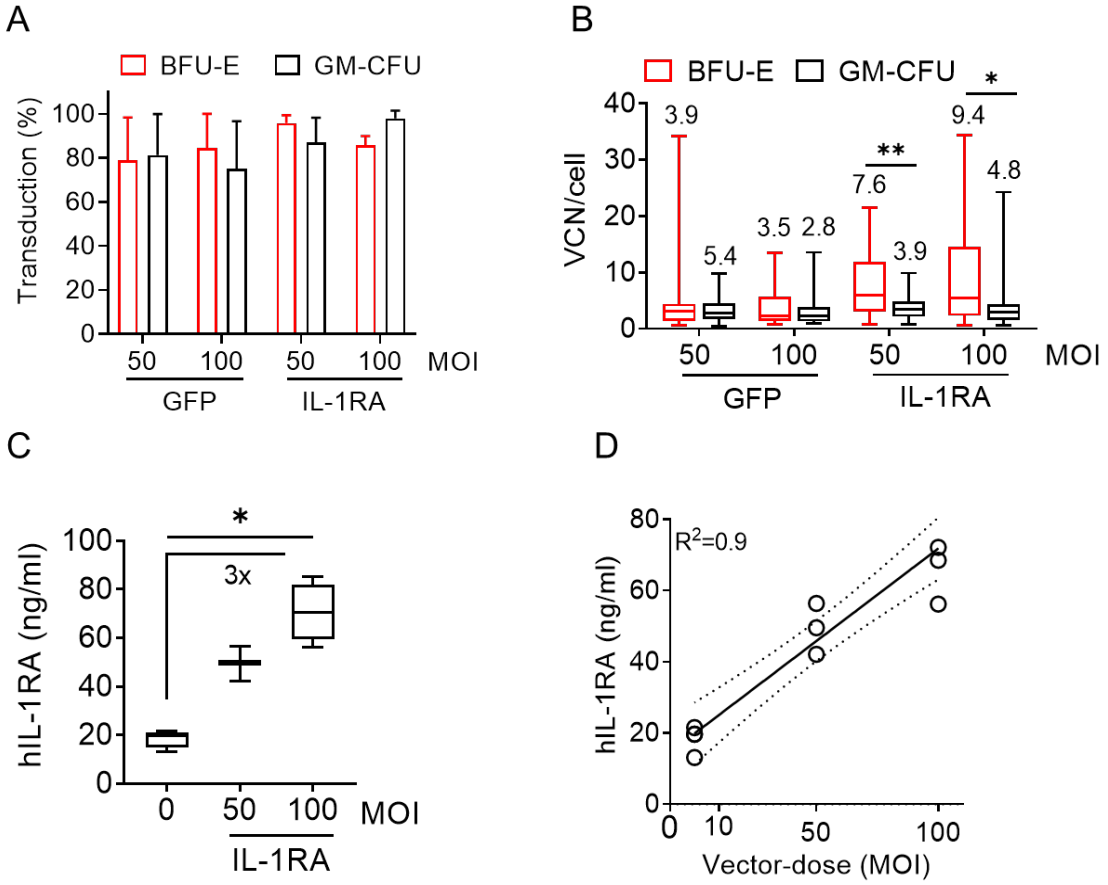


Figure 17. Successful human HSPCs transduction and IL-1RA production.
*(A,B) Percentage of transduction and mean VCN per cell were calculated on single vector-positive BFU-E and GM-CFU from three different CD34⁺ transduction experiments with LV.IL-1RA and LV.GFP. (C,D) IL-1RA production was measured in the supernatants of transduced human HSPCs after 14 days of culture at increasing MOI. (A) Mean +/- StD of 3 biological replicates. (B) Three biological replicates; median and interquartile range of single CFU scored with VCN>0.5 out of 24 CFUs per each condition. (C,D) 3-4 biological replicates. Mann Whitney test: *, p<0.05, **, p<0.005.*

Moreover it was worth to assess whether lentiviral-mediated IL-1RA expression could alter CD34$^+$ cells' functional status. Similar numbers of untransduced and transduced cells were recovered, indicating no toxicity of transduction and IL-1RA expression (Figure 18A). IL-1RA transduced CD34$^+$ cells maintained their self-renewing potential as they generated granulocyte, monocyte, and erythroid progenitors even at high VCN. (Figure 18B and 18C).

Overall, these results indicate that LV-based strategy successfully directed human IL-1RA production by both mice and human HSPCs without significantly impacting on self-renewal and clonogenic ability of IL-1RA modified HSPCs.

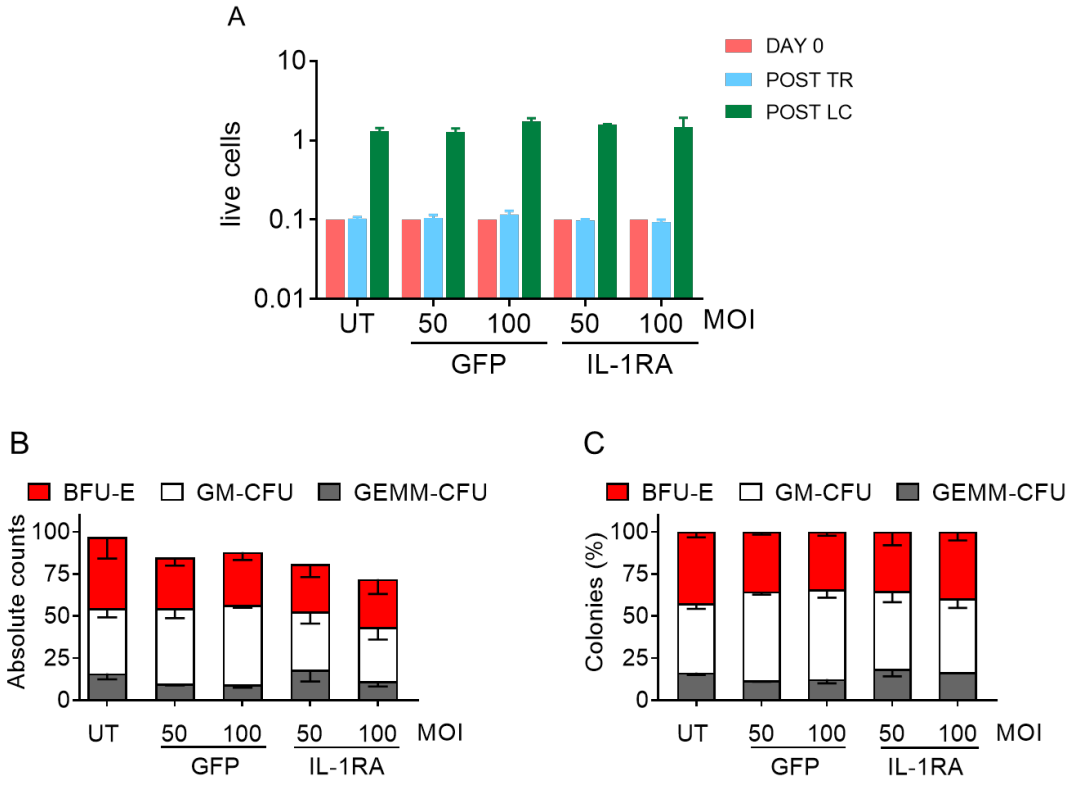


Figure 18. Evaluation of LV.IL1RA impact on human HSPCs functionality.
(A) Absolute counts of CD34$^+$ cells pre- and post- exposure to the IL-1RA and GFP vector.
(B,C) Absolute counts and percentages of single colonies derived from erythroid (BFU-E), myeloid (CFU-GM) and granulocyte, erythroid, macrophage, megakaryocyte colony-forming units (GEMM-CFU) progenitors in untransduced (UT), IL-1RA-, and GFP-transduced conditions. Mean +/- StD of 3 biological replicates.

3.1.4 Systemic IL-1RA production did not alter HSPC engraftment and immune reconstitution in vivo

We next investigated the feasibility of the IL-1RA gene transfer approach *in vivo* with the final goal to assess whether the overexpression of human IL-1RA might impact HSPC engraftment and immune reconstitution. Thus, HSPCs were isolated from the BM of CD45.1 C57BL/6 mice, transduced with purified LV.IL-1RA and LV.GFP at a vector dose of 100 and adoptively transferred into lethally irradiated congenic CD45.2 C57BL/6 recipient mice (Figure 19A). Mice receiving untransduced MOCK HSPCs were used as a control group. Vector-positive leukocytes of donor origin were present in the peripheral blood with a VCN ranging between 2 and 6 already at five weeks and remained stable over time. Transduced cells with similar VCN range were also detected in the BM and spleen (Figure 19B). GFP mice showed a higher VCN than IL-1RA mice, probably due to the higher infectivity of the vector. However, we noticed that VCN in the IL-1RA group was in the lower range than previous experiments, thus suspected that viral preparation lost its infectivity and decided to re-titre it. Indeed, we found that the actual vector dose was 25 instead of 100 vector particles per cell. Alongside, IL-1RA chimeric mice showed increasing and sustained levels of IL-1RA derived from immune cells differentiated from HSPC (Figure 19C).

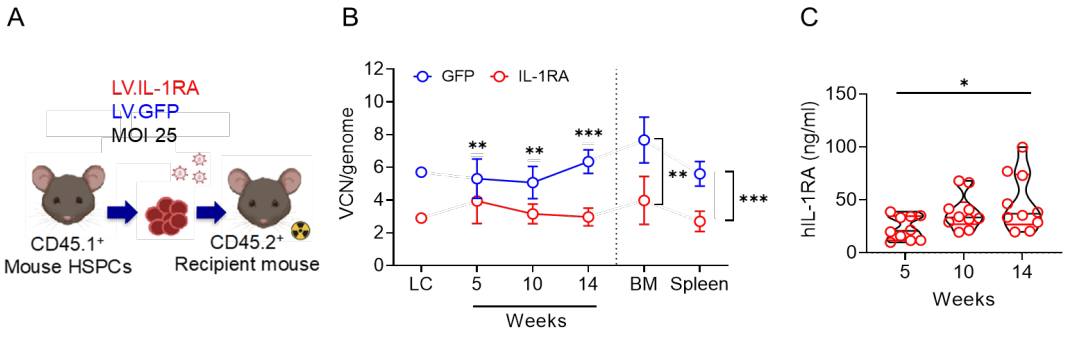


Figure 19. Successful engraftment of IL-1RA-transduced HSPCs in vivo.
*(A) Schematic representation of the BM transplant set-up. CD45.1⁺ HSPCs transduced with the LV.IL-1RA were transferred i.v. into lethally irradiated congenic CD45.2⁺ C%/BL/6 mice. (B) The mean VCN/genome was evaluated in transplanted cells expanded in vitro for 14 days, total blood leukocytes, BM, and spleen. Data are represented as mean +/- StD of GFP (n=6) and IL-1RA (n=10) mice. Mann-Whitney test: **, p<0.005; ***, p<0.0005. (C) Longitudinal analysis of plasma IL-1RA levels measured at 5, 10, 14 weeks post-transplant. Data are represented as the median and interquartile range of 10 IL-1RA mice. Paired t-test: *p<0.05.*

The absolute count of donor cells in the peripheral blood was comparable among the three groups and expanded from week 10 to 14 (Figure 20A). Engrafted HSPCs produced all the major immune cell populations, including innate (*i.e.,* granulocytes, monocytes, dendritic and NK cells) and adaptive (T and B cells) immune cells, as assessed in the peripheral blood of recipient mice by flow cytometry (Figure 20). As expected, HSPC engraftment led initially to innate cell reconstitution followed by adaptive cells. No significant difference in the absolute counts and reconstitution kinetics of all cell lineage tested were observed in the peripheral blood of MOCK, GFP, and IL-1RA mice (Figure 20).

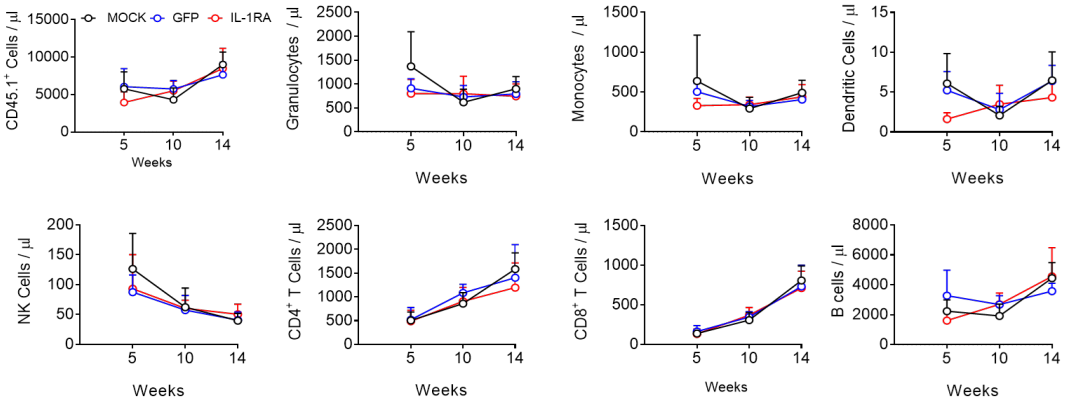


Figure 20. Haematopoietic cell reconstitution capacity of IL-1RA-transduced HSPCs.
Time-course analysis of donor cells identified as CD45.1$^+$ cells in the peripheral blood of mice at 5, 10 and 14 weeks post-transplant. Longitudinal analysis of the haematopoietic reconstitution of the major myeloid and lymphoid cell lineages in peripheral blood of MOCK (n=9), GFP (n=6) and IL-1RA (n=10) mice at 5, 10, and 14 weeks post-transplant. Data are represented a mean +/- StD in a time-course analysis.

IL-1RA-transduced donor cells repopulate the spleen and the relative frequency was comparable with mice receiving mock or GFP transduced HSPCs (Figure 21A and 21B). At termination, the presence of microscopic and macroscopic abnormalities was evaluated in immune and non-immune organs by an expert pathologist of the SR-Tiget GLP Test Facility. The tissue architecture of the spleen of IL-1RA mice was comparable to MOCK and GFP-chimeras in terms of cell distribution and cellularity (Figure 21C).

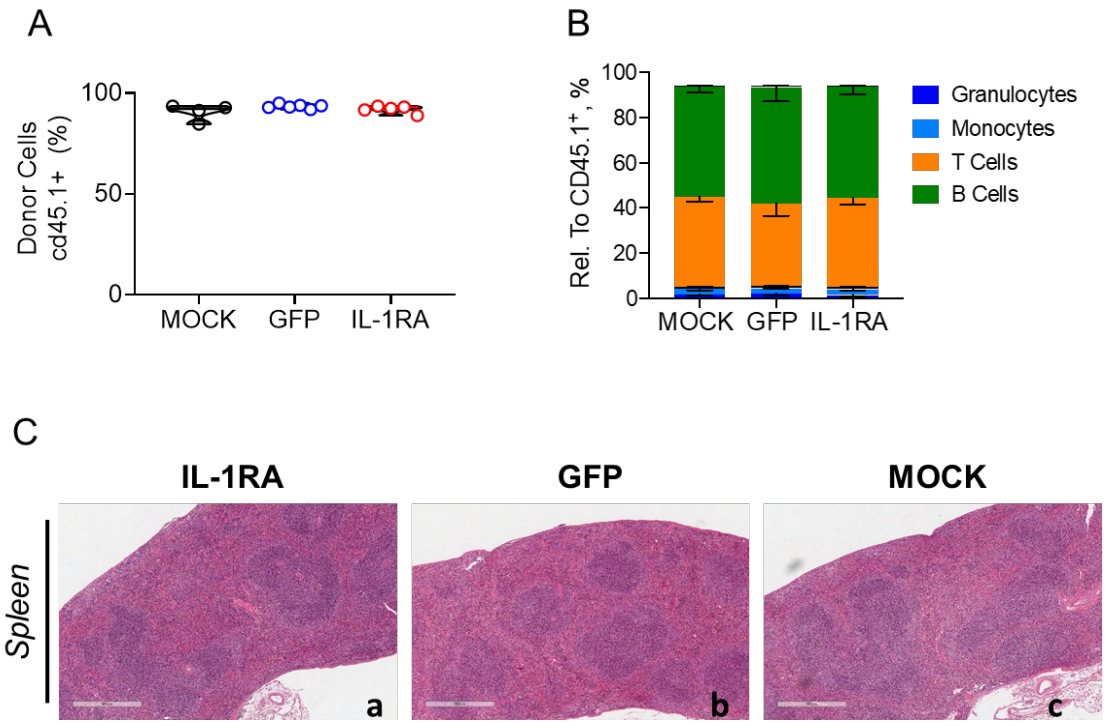


Figure 21. Histopathological and flow cytometry analysis of the spleen of IL-1RA chimeric mice.
(A, B) Percentage of total CD45.1$^+$ donor cells and various immune cells (i.e., granulocytes, monocytes, T and B cells) evaluated in the spleen by flow cytometry. (C) Haematoxylin and eosin staining of representative section of spleen isolated from MOCK, GPF and IL-1RA mice.

Concurrently, the histopathological analyses of the BM unveiled no differences in terms of tissue integrity among IL-1RA, MOCK and GFP-chimeras (Figure 22A). We next evaluated the engraftment of donor cells in the BM of transplanted mice at 14-weeks post-transplant. The percentage and the absolute counts of CD45.1$^+$ cells were comparable between MOCK, GFP-, and IL-1RA-chimaeras (Figure 22B and 22C). The relative frequency of donor-derived granulocytes, monocytes, B and T cells did not differ among the different groups (Figure 22D). We also assessed the impact of constitutive IL-1RA expression on the different progenitors by flow cytometry. The absolute counts of primitive HSCs, multipotent progenitors (MPPs), granulocyte-myeloid-lymphoid progenitors (GMLPs), granulocyte-monocyte progenitors (GMPs),

and megakaryocyte-erythroid progenitors (MEPs) were similar in mice receiving untransduced, GFP- and IL-1RA-transduced HSPCs (Figure 21E).

Altogether, these results demonstrate that IL-1RA-expressing HSPCs maintain their ability to home to the BM, self-renew, and differentiate into all immune cell types without causing any toxic effect.

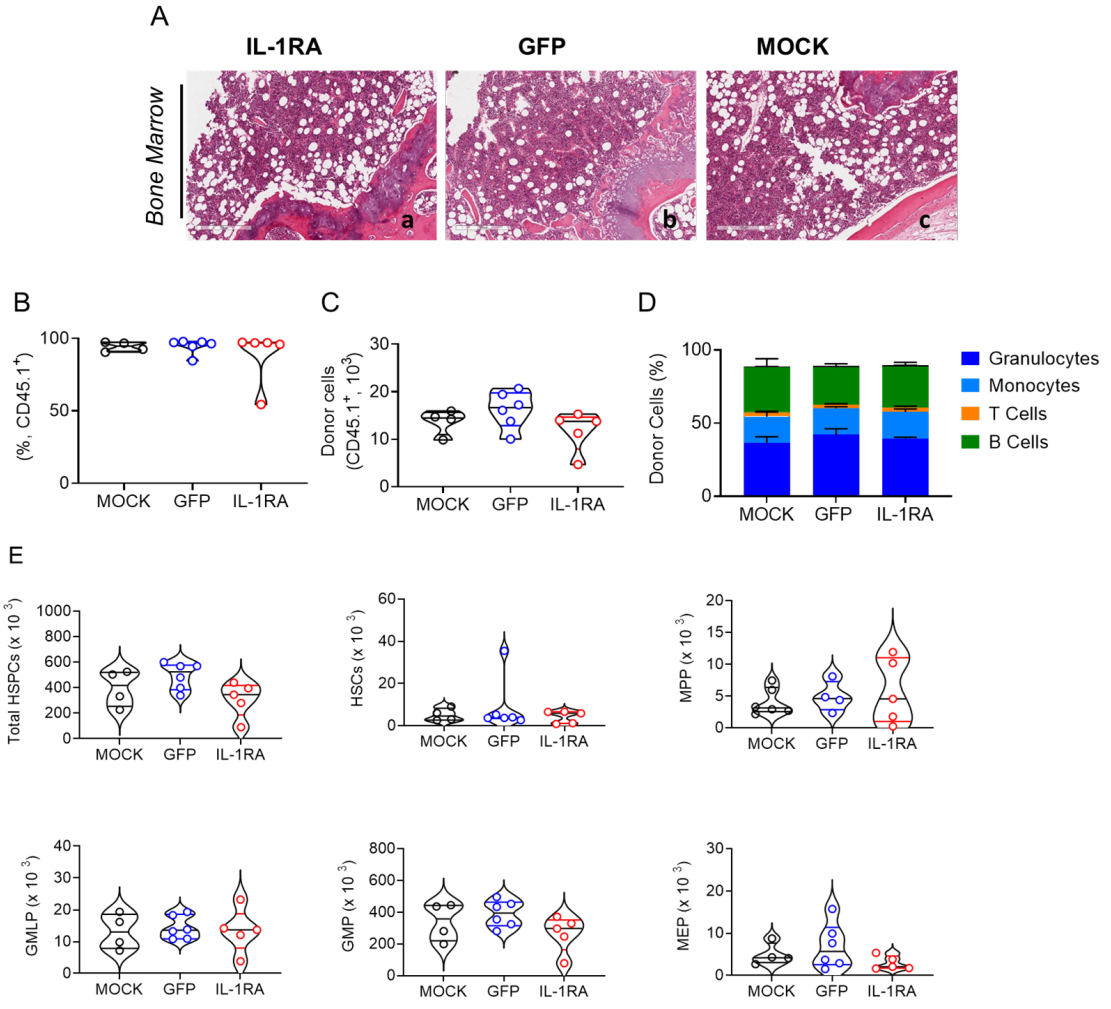


Figure 22. BM composition of MOCK, GFP and IL-1RA chimaeras
(A) Haematoxylin and eosin staining of representative section of BM isolated from MOCK, GPF and IL-1RA mice. (B,C) Relative BM frequency and absolute count of CD45.1⁺ donor cells in MOCK, GFP and IL-1RA groups at 14 weeks post-transplant. (D) Percentage of granulocytes, monocytes, T and B cells relative to CD45.1⁺ donor cells in the BM. (E) Total cell counts of the various progenitors in the HSPC population. Abbreviation: HSC, haematopoietic stem cells; MPP, multipotent progenitors; GMLP, granulocyte-myeloid-lymphoid progenitors; MEP, megakaryocyte-erythroid progenitors; GMP, granulocyte-monocyte progenitors. Median and interquartile range of MOCK (n=4), GFP (n=6) and IL-1RA (n=5) mice

3.2 Evaluation of the efficacy of *ex vivo* IL-1RA GT in correcting IL-1-induced acute inflammation

3.2.1. Vector-derived IL-1RA effectively prevents neutrophilic inflammation in a mouse model of crystal-induced peritonitis

One of the major effects of IL-1β production is to induce an inflammatory response characterised by rapid recruitment of neutrophils at the site of the affected tissue. IL-1-induced acute inflammation can be properly recapitulated *in vivo* through an intraperitoneal injection of MSU crystals, well-known activators of the NLRP3 inflammasome (Martinon *et al*, 2006). We, therefore, tested whether IL-1RA GT could effectively suppress neutrophilic inflammation in an MSU-induced peritonitis model (Figure 23A). Following MSU treatment, the peritoneal macrophages activate the NLRP3 inflammasome which, in turn, triggers IL-1β release. This cytokine acts as a potent pro-inflammatory mediator attracting circulating blood neutrophils into the peritoneal cavity, thus amplifying the inflammatory response pathway. MOCK and IL-1RA chimaeras were treated with MSU crystals and, after 6 hours, peritoneal exudates were collected. Peritoneal recruitment of CD11b+Ly6G+ neutrophils was measured by flow cytometry. The challenge with MSU crystals led to robust recruitment of neutrophils in MOCK mice (Figure 23B, C). In contrast, the percentage and the absolute number of neutrophils were significantly reduced in IL-1RA-chimeras (Figure 23C). The efficacy of IL-1RA GT in suppressing neutrophil's recruitment was comparable to that obtained in C57BL/6 mice receiving an administration of anakinra (Figure 23D).

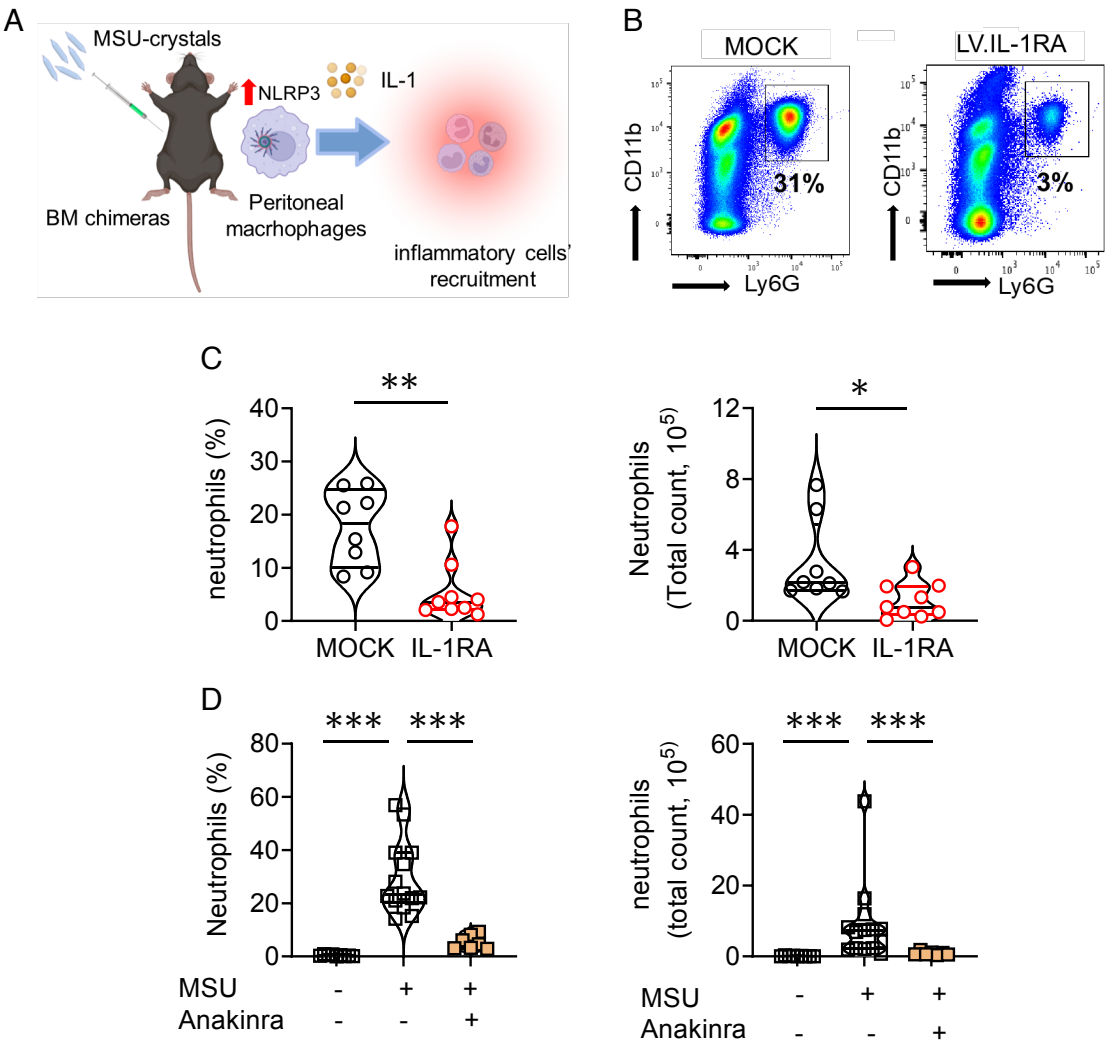


Figure 23. Efficient suppression of neutrophil' recruitment in mice treated by IL-1RA GT. *(A) Illustration of MSU-induced peritonitis mouse model. MOCK and IL-1RA chimaeras were treated i.p. with MSU crystals activating the NLRP3 inflammasome in tissue-resident macrophages. (B) Representative FACS dot plots (C), percentage and absolute count of CD11b+Ly6G+ neutrophils collected from peritoneal exudates of MOCK and IL-1RA mice. (D) Percentage and absolute count of neutrophils in C57BL/6 mice pre-treated with anakinra receiving MSU crystals. Median and interquartile range of 3-4 different experiments of MSU-treated MOCK (n= 8) and IL-1RA (n=9) groups, untreated (n= 9), MSU-treated (n=14), anakinra and MSU-treated (n=7) C57BL/6. Mann Whitney test: *, p<0.05, **, p<0.005, ***, p<0.005.*

3.2.2. IL-1RA over-expression in human HSPCs partially suppress neutrophil' recruitment in a humanized mouse model of IL-1 mediated inflammation

To study the efficacy of IL-1RA GT in a human setting, we exploited the NSG immunocompromised mouse model, which is ideal for studying long-term the engraftment and multilineage differentiation of human HSPCs *in vivo* (Carrillo *et al*, 2018). NSG mice lacking the IL-2R common γ chain have no B, T, and NK cells, allowing high and stable human $CD34^+$ engraftment, thereby permitting long-term studies. Based on these premises, NSG mice have been extensively used to study autoimmune diseases, such as RA, and test new therapeutic approaches (Koboziev *et al*, 2015; Schinnerling *et al*, 2019). Therefore, we exploited the NSG mouse model to test the efficacy of IL-1RA GT in suppressing IL-1-mediated inflammation.

IL-1RA-transduced $CD34^+$ cells isolated from the PB of a healthy donor were transduced with LV.IL-1RA at vector-dose of 100 and transplanted intravenously into sub-lethally irradiated NSG mice. A group of NSG mice transplanted with untransduced $CD34^+$ cells (MOCK) was prepared as a control. After 10 weeks from transplant, MOCK and IL-1RA NSG chimaeras were treated intraperitoneally with MSU particles to elicit an acute inflammatory response (Figure 24A). The percentage of human cells in the peripheral blood ranged between 20% and 40 % and was comparable between MOCK and IL-1RA mice (Figure 24B). Human B cells represented the most abundant circulating cell types, as already reported in similar experimental set-ups with this mouse strain (Figure 24C). T cell counts were very low (1-3 cells/μl) and was reduced in the IL-1RA mice compared to mice in the MOCK group (Figure 24C). As expected, low human myeloid cells ($CD45^+CD33^+$) engraftment' level was observed in the peripheral blood with no differences between the two groups. A substantial IL-1RA level (15 ng/ml) was measured in the plasma of NSG mice reconstituted with IL-1RA-transduced $CD34^+$ cells (Figure 24D). Upon challenge with MSU crystals, the percentage of human $CD45^+$ in the peritoneal cavity ranged between 0.5% and 2.5%. No major difference was observed among mice of the MOCK and IL-1RA groups, independently of the type of treatment (Figure 24E). In contrast, the MSU challenge led to a significant increase of human myeloid cells in the peritoneal cavity of MOCK mice compared to the MOCK-untreated group, suggesting that circulating-human cells can be efficiently recruited at the site of injury in response to an inflammatory stimulus (Figure

24F). Although not significant, myeloid cell counts showed a tendency toward a decrease in IL-1RA mice compared with MOCK mice, suggesting a partial beneficial effect of vector-derived IL-1RA on an acute inflammatory response.

These preliminary results suggest that NSG mice might be a valuable model to study the efficacy of IL-1RA GT in the context of human inflammation. However, it is well known that the NSG model allows a poor myeloid cell differentiation, thus limiting its use in the field of autoinflammatory diseases, which are primarily mediated by innate immune cell types (Coughlan et al, 2016; Mian et al, 2021).

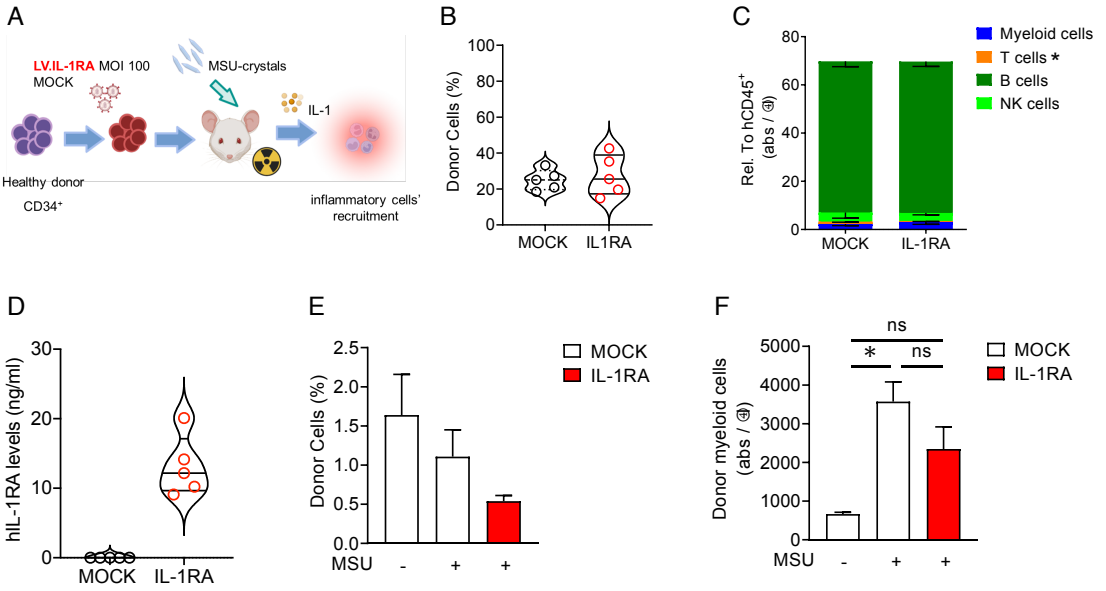


Figure 24. Efficacy of IL-1RA GT in the humanised NSG mouse model.
*(A) Experimental design: healthy donor' CD34⁺ cells transduced with LV.IL-1RA were transplanted in sub-lethally irradiated NSG mice. After 10 weeks, mice were treated i.p. with MSU crystals to induce an IL-1-mediated inflammatory response. (B, C) Percentage of human CD45⁺ cells and absolute number of various donor-derived immune cell types present in the peripheral blood of MOCK and IL-1RA mice. (D) Human IL-1RA levels in the plasma of MOCK and IL-1RA mice. (E) Percentage of human CD45⁺ cells evaluated in the peritoneal cavity of MOCK and IL-1RA mice treated i.p. or not with MSU crystals. (F) Absolute counts of CD45+CD33+ neutrophils quantified in peritoneal exudates of MOCK and IL-1RA mice by flow cytometry. Mean +/- StD of MOCK-MSU (n=3), MOCK MSU-treated (n=6) IL-1RA MSU-treated (n=4). Mann Whitney test: *, p<0.05.*

3.2.3 Generation and characterisation of Nlrp3$^{N475K/+}$ mouse chimaeras

Dominant mutations in the NLRP3 gene resulting in uncontrolled IL-1 production are associated with a class of systemic autoinflammatory diseases named CAPS (refer to paragraph 1.4.2). Different mouse models mimicking human CAPS disease have been developed. One example is the newly developed NLRP3 knock-in mouse model

harbouring a single N475K mutation in the mouse Nlrp3 gene locus which has been found also in humans associated to NOMID disease (Bertoni *et al*, 2020). $Nlrp3^{N475K/+}$ mice develop a phenotype resembling the human pathology. Indeed, they exhibit systemic inflammation like rash, failure to thrive, increased serum proinflammatory cytokines (IL-1β, IL-1α, and IL-18), splenomegaly, inflammatory cell infiltrates in the lungs, spleen and liver, and a high mortality rate. We obtained this strain of mice from our collaborator Dr Marco Gattorno at the IRCCS Istituto Giannina Gaslini (Genoa, Italy). We initially tested the possibility to induce a CAPS-like phenotype in a healthy mouse by transplanting BM cells isolated from $Nlrp3^{N475K/+}$ mice. Thus, we adoptively transferred BM cells from $Nlrp3^{N475K/+}$ and $Nlrp3^{+/+}$ mice (CD45.2/CD45.1) into lethally irradiated CD45.1 C57BL/6 mice (Figure 25A). Two out of four $Nlrp3^{+/+}$ mice and one out of six $Nlrp3^{N475K/+}$ mice were found dead between 10 and 20 days post-transplantation, likely due to irradiation, whereas at six weeks post-transplant, one $Nlrp3^{N475K/+}$ mouse died. $CD45.2^{+}$ donor cells were present significant proportion (>60%) in the peripheral blood, BM and spleen in both groups (Figure 25B). $Nlrp3^{N475K/+}$ chimaeras showed a CAPS-like phenotype characterised by severe dermatitis, splenomegaly, a tendency toward a higher expression of IL-1β, Cxcl2 and Ccl2 cytokines in the spleen and white blood cell count (WBC) than $Nlrp3^{+/+}$ chimaeras (Figure 25C-F). These results demonstrate the pathogenic role of $Nlrp3^{N475K/+}$ mutation and that the disease can be transferred to naive hosts by transplanting BM cells from an affected mouse.

Although the original objective of establishing a chimeric mouse model for CAPS was successfully achieved, we encountered unforeseen difficulties. The breeding capacity of the $Nlrp3^{N475K/+}$ colony was very low and the amount of BM cells was insufficient to transplant a significant number of recipient mice. In addition, we also considered that for GT, even more cells would have been needed as cells die during the transduction procedure. Finally, disease' severity in $Nlrp3^{N475K/+}$ chimaeras seemed to be too strong and pathological manifestations developed so rapidly that it was hard to predict whether IL-1RA-producing cells were in sufficient amounts to stop disease progression. For all the above considerations, we chose to withdraw this spontaneous model.

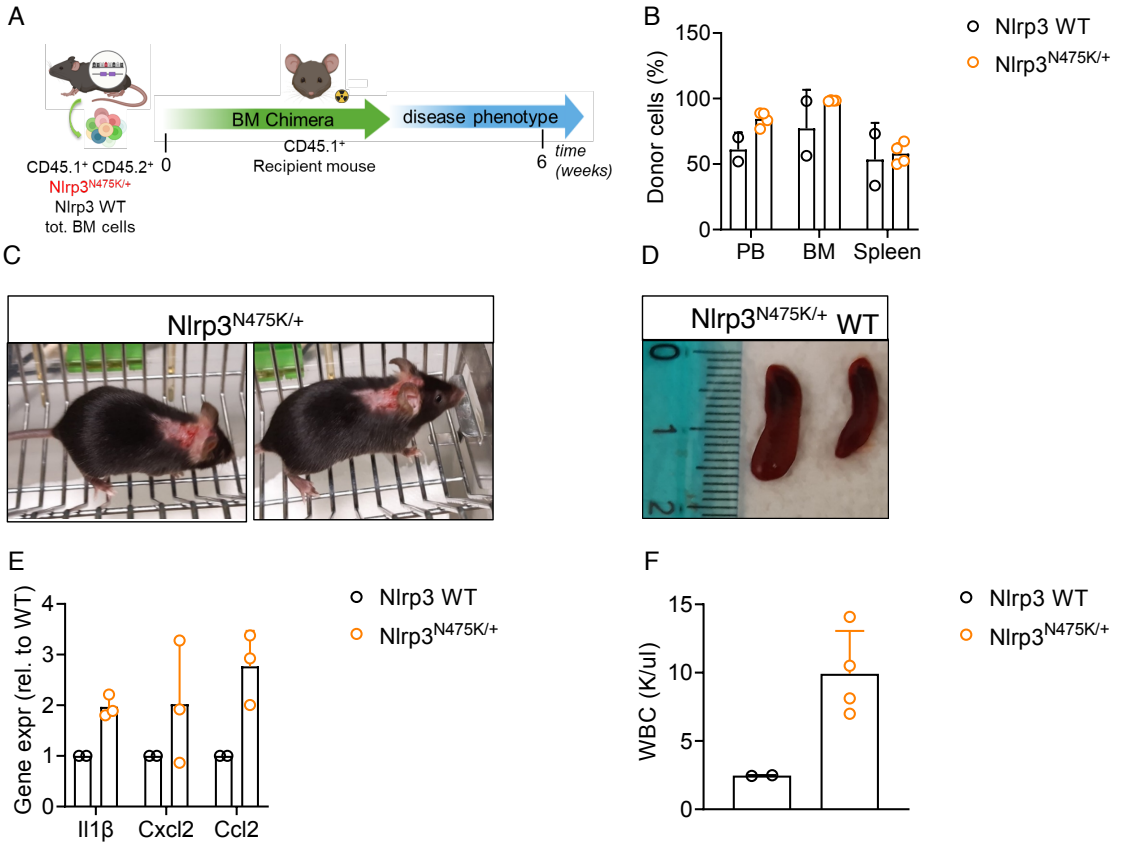


Figure 25. Generation and characterization of Nlrp3$^{N475K/+}$ chimaeras.
(A) Illustration of the experimental design. Total BM cells isolated from Nlrp3$^{N475K/+}$ (CD45.2^{+} CD45.1^{+}) mice were transplanted in lethally irradiated C57BL/6 (CD45.1^{+}) recipient mice. (B) CD45.2^{+} CD45.1^{+} donor cells were measured in the peripheral blood, bone marrow and spleen at 6 weeks post-transplantation by flow cytometry. (C, D) Photos showing dermatitis (C) and splenomegaly (D) in Nlrp3$^{N475K/+}$ mice. (E) Expression of the proinflammatory cytokines IL-1β, Cxcl2 and Ccl2 in splenocytes of Nlrp3$^{N475K/+}$ chimaeras evaluated by real-time RT-PCR and presented as relative fold expression compared with Nlrp3$^{+/+}$ splenocytes using the $2^{-\Delta\Delta Ct}$ method. (F) White blood cell count measured by haemocytometer in the peripheral blood of Nlrp3$^{N475K/+}$ and Nlrp3$^{+/+}$ chimaeras. Dots represent individual mice, the mean +/- StD of Nlrp3$^{+/+}$ (n=2) and Nlrp3$^{N475K/+}$ (n=3/4) mice are shown.

3.2.4 IL-1RA expression ameliorates CAPS associated inflammatory symptoms in the inducible Nlrp3$^{A350V/+}$CreT mouse model

Considering the complications that arose from employing a spontaneous mouse model of CAPS, we redirected our investigations to Nlrp3$^{A350V/+}$CreT mice, an inducible model for IL-1-mediated inflammation that well recapitulates a mild phenotype of the CAPS disease spectrum (described in paragraph 1.5.2)(Brydges *et al*, 2009). These mice carry a human NLRP3 coding sequence carrying the NLRP3^{A350V} mutation inserted in one of the two mouse NLRP3 alleles. NLRP3^{A350V} mutant mice were crossed with mice carrying a Cre recombinase under the control of the tamoxifen-inducible promoter (named CreT^{+} mice). Upon tamoxifen administration *in vivo*, the NLRP3^{A350V} mutant protein is produced, and the CAPS-like phenotype manifests with weight loss, neutrophil infiltrates in organs and high levels of proinflammatory cytokines in the serum. For simplicity in the following sessions, we used CreT^{+} and CreT^{-} to identify Nlrp3$^{A350V/+}$CreT and Nlrp3$^{A350V/+}$ mice, respectively.

To assess the efficacy of IL-1RA GT in preventing the onset and progression of CAPS disease, BM cells isolated from CD45.2^{+} CreT^{+} mice were transduced with the LV.IL-1RA and transplanted into lethally irradiated CD45.1^{+} C57BL/6 recipient mice (Figure 26A). A group of CD45.1^{+} C57BL/6 mice transplanted with untransduced (MOCK) CreT^{+} BM cells was used as control. Vector-positive cells were identified in the peripheral blood, BM, and spleen of CreT^{+} chimaeras with a VCN ranging between 3 and 5 in all the organs analysed (Figure 26B). Full donor chimerism was obtained in all mice already at 5 weeks post-transplant (Figure 26C). The most abundant cell populations in the blood were granulocytes, monocytes, B and T cells in the IL-1RA and MOCK groups (Figure 26D). Moreover, IL-1RA level was on average 70 ng/ml in the plasma of CreT^{+} chimaeras (Figure 26E).

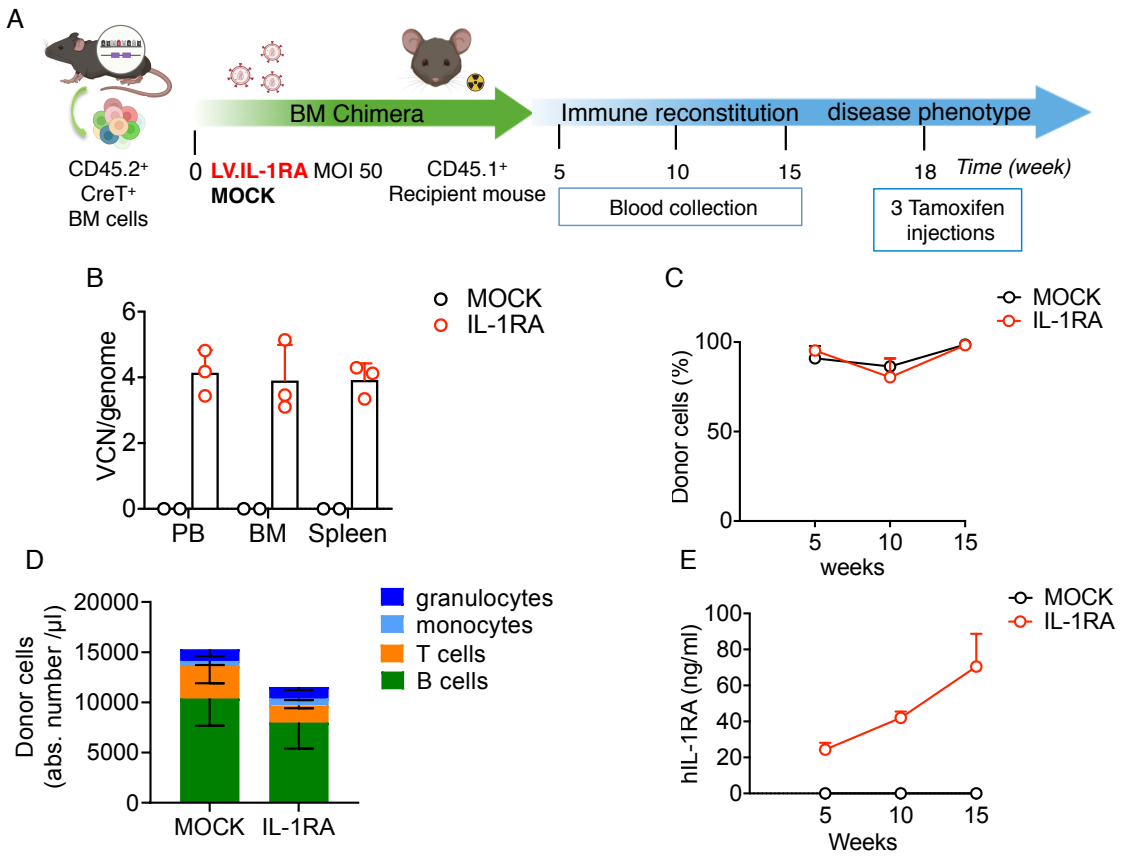


Figure 26. Generation of Nlrp3$^{A350V/+}$CreT chimaeras expressing IL-1RA by immune cells.
(A) Illustration of the experimental design. Total BM cells were isolated from CreT^{+} CD45.2^{+} mice, transduced with the LV.IL-1RA at the indicated MOI and transplanted into lethally irradiated C57BL/6 (CD45.1^{+}) mice. Mice receiving untransduced cells (MOCK) were used as control. At 18 weeks post-transplant, chimeric mice were treated i.p. with tamoxifen for three days to activate the cre-lox system leading to the CAPS-like phenotype. (B) The mean VCN/genome was calculated on total blood leukocytes, BM, and spleen by ddPCR. (C) Time-course analysis of donor cells (CD45.1^{+}) chimerism in the peripheral blood at 5, 10, and 15 weeks post-transplantation measured by flow cytometry. (D) The absolute number of donor-derived immune cell types (granulocytes, monocytes, B and T cells) in the peripheral blood of MOCK and IL-1RA chimaeras at 15 weeks post-transplant. (E) Longitudinal analysis of IL-1RA production in the plasma at 5, 10, 15 weeks post-transplant assessed by ELISA. Mean +/- StD of CreT^{+} MOCK (n=2) and CreT^{+} IL-1RA (n=3) mice. Dots represent individual mice.

Eighteen weeks after transplant, CAPS-like phenotype was induced by treating IL-1RA CreT^{+} and MOCK chimaeras with three i.p. injections of tamoxifen (Figure 26A). MOCK mice developed dermatitis in the muzzle, whereas LV-derived IL-1RA ameliorated skin inflammation in CreT^{+} chimaeras (Figure 27A). Moreover, IL-6 level was reduced in the plasma of IL-1RA chimaeras compared to MOCK mice (Figure 27B). Nevertheless, mice of both groups similarly lost weight following tamoxifen

treatment (Figure 27C) and developed morphological features of inflammation moderate to marked in spleen, liver and BM (Table 3).

These preliminary findings indicate that vector-derived IL-1RA production can partially ameliorate CAPS inflammation in Nlrp3$^{A350V/+}$CreT mice.

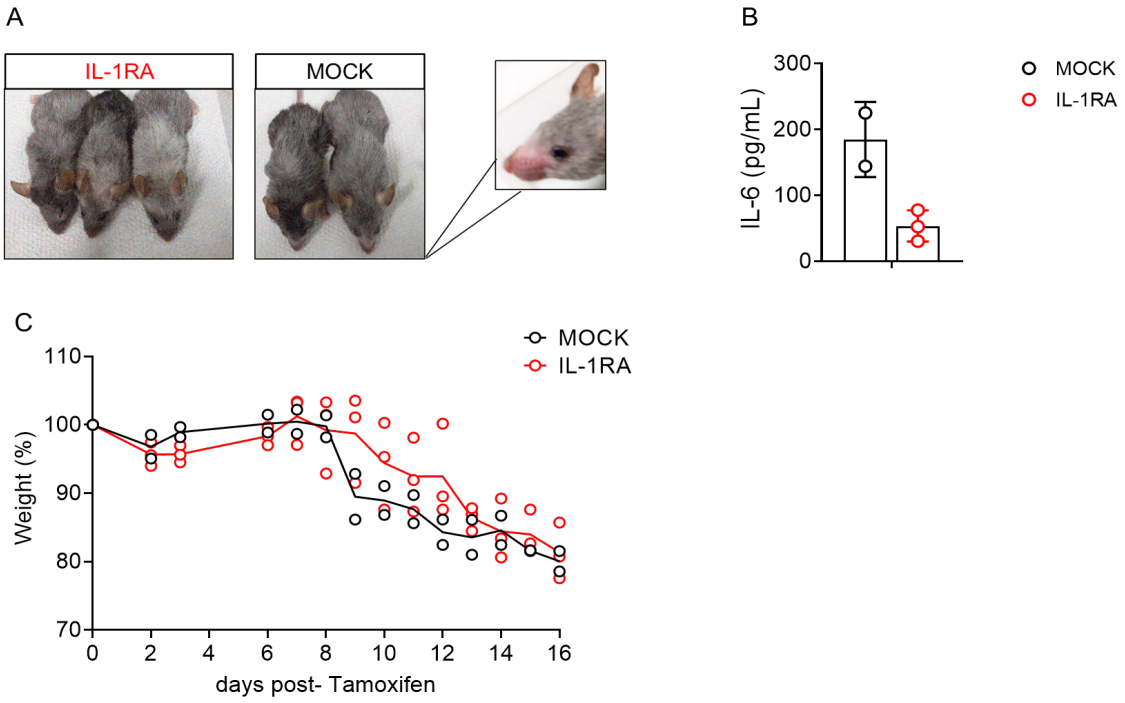


Figure 27. IL-1RA GT ameliorated dermatitis and reduced serum IL-6 levels in Nlrp3$^{A350V/+}$CreT mice

(A) Photos show dermatitis in tamoxifen-treated MOCK mice. (B) IL-6 production in plasma of tamoxifen-treated MOCK and IL-1RA CreT^{+} mice. Mean +/- StD of two technical replicates obtained from CreT^{+} MOCK (n=2) mice and CreT^{+} IL-1RA (n=3) mice. Dots represent individual mice. (C) Time-course analysis of weight loss after tamoxifen treatment evaluated as the percentage of initial weight. Lines represent means in each group and dots define individual mice.

Table 3. Pathology report showing morphological features of inflammation in the spleen, liver and bone marrow of MOCK and IL-1RA CreT^{+} mice following tamoxifen treatment.
Analyses performed by Dr Francesca Sanvito (SR-Tiget). Grade 1: minimal; Grade 2: mild; Grade 3: moderate; Grade 4: marked.

Organs	***Mice groups***				
Liver	***MOCK***		***IL-1RA***		
Inflammatory cells infiltrations, lympho-granulocytic, diffuse	2	3	2	4	1
Hepatocyte degenerative changes (cytoplasmic microvacuolation/eosinophilia/ rerefaction)	2	2	2	2	2
Extramedullary haematopoiesis, myeloid	1	1	1	2	1
Spleen					
Increased cellularity: EMH (granulopoiesis)	3	4	4	4	3
Bone marrow femur					
Increased cellularity, granulocytic	4	4	4	4	4
Bone marrow sternum					
Increased cellularity, granulocytic	4	4	4	4	4

3.2.5 Syngeneic transplant of IL-1RA-producing Nlrp3$^{A350V/+}$CreT HSPCs prevents CAPS disease progression

In the Nlrp3$^{A350V/+}$ CreT chimaeras we used C57BL/6 as recipient mice, thus the non-immune cells, which were responsible of tissues 'and organs' inflammation, were of wild-type origin. Therefore we aimed to evaluate whether IL-1RA GT can prevent the CAPS-like phenotype by acting on both immune and non-immune cells, such as intestinal epithelial cells since they are also responsible for the hyperinflammatory phenotype. We performed a HSPCs transplant where both the donor and recipients were CreT^{+}. Thus, HSPCs isolated from CreT^{+} mice were transduced with the LV.IL-1RA and transplanted into lethally irradiated CreT^{+} littermates (Figure 28A). A group of CreT^{+} chimaeras transplanted with untransduced (MOCK) CreT^{+} HSPCs was also prepared. Control groups were CreT^{-} mice transplanted with CreT^{-} cells, a cohort of untransplanted (UT) CreT^{-} and a cohort of untransplanted (UT) CreT^{+} mice, (Table 4).

We evaluated the haematopoietic reconstitution after 7 weeks post-transplant, and we did not observe any differences in the peripheral blood composition evaluated as B, T and myeloid cells among all groups (Figure 28B). We also measured human IL-1RA levels in the plasma of GT IL-1RA mice ranging between 100 and 150 ng/mL (Figure 28C).

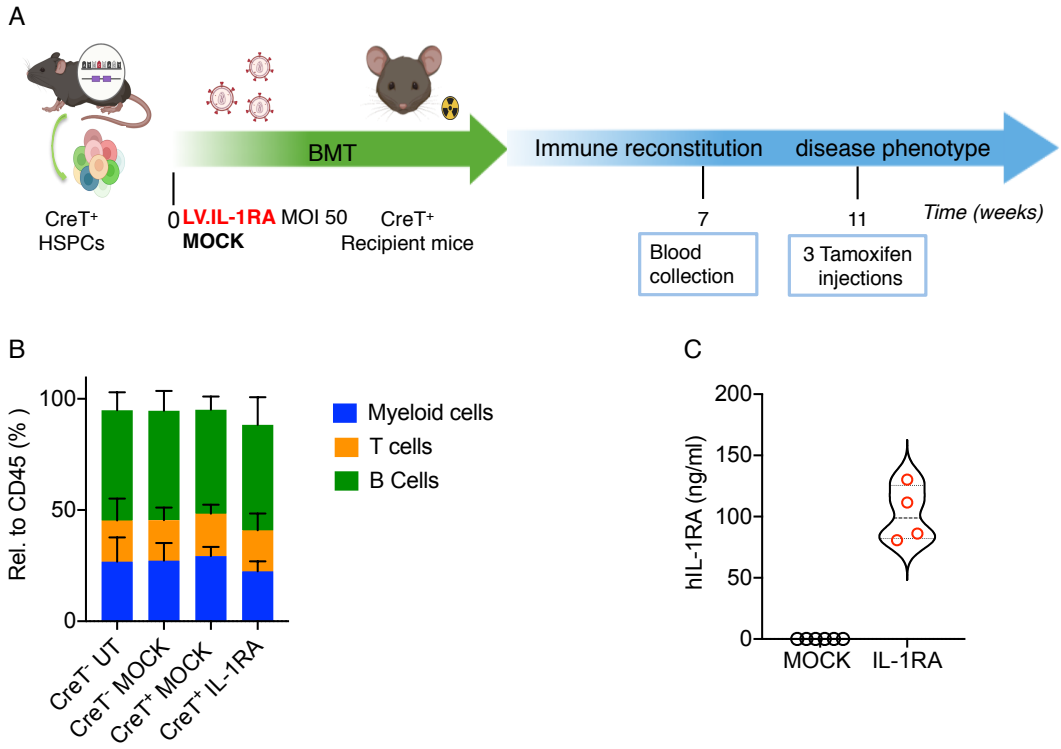


Figure 28. Immune reconstitution and IL-1RA expression in Nlrp3$^{A350V/+}$CreT mice
(A) Illustration of the experimental design. HSPCs isolated from CreT^{+} mice were transduced with the LV.IL-1RA an MOI 50 (CreT^{+} IL-1RA) or left untransduced (CreT^{+} MOCK) and transplanted into lethally irradiated CreT^{+} mice. CreT^{-} mice untreated (CreT^{-} UT) or transplanted with untransduced HSPCs (CreT^{-} MOCK) and CreT^{+} untreated (CreT^{+} UT) were also included as control groups. At 11 weeks post-transplant, chimaeric mice were treated i.p. with tamoxifen for three days to activate the Cre-lox system and elicit the CAPS-like phenotype. (B) The relative frequency of myeloid, B and T cells on the total CD45^{+} cells was evaluated by flow cytometry 7 weeks post-transplant. (C) IL-1RA production in the plasma was measured at 7 weeks post-transplant. (B) Mean +/- StD and (C) median and interquartile range of CreT^{-} UT (n=6), CreT^{-} MOCK (n=6), CreT^{+} MOCK (n=5) and CreT^{+} IL-1RA (n=4) mice. Abbreviations: BMT (bone marrow transplant).

Table 4. Summary of the different mice' groups.
Abbreviations; NA, not available.

Donor	***Recipient***	***Tamoxifen treatment***
CreT^{+}IL-1RA	CreT^{+}	+
CreT^{+}MOCK	CreT^{+}	+
CreT^{+} UT	NA	-
CreT^{-} MOCK	CreT^{-}	+
CreT^{-} UT	NA	-

Eleven weeks after transplant, disease phenotype was induced by treating *i.p.* IL-1RA and MOCK CreT^{+} with three consecutive injections of tamoxifen (Figure 28A). CreT^{-} MOCK mice were also treated to investigate possible toxic effects of tamoxifen administration itself, whereas the CreT^{-} UT and the CreT^{+} UT group remained untreated. MOCK CreT^{+} mice lost an average of 12% of their initial weight, whereas IL-1RA

CreT^{+} mice gained weight (Figure 29A). Serum IL-6 was elevated in CreT^{+} MOCK mice but not in IL-1RA CreT^{+} GT mice and all the other control groups (Figure 29B). Moreover, CreT^{+} MOCK mice showed a significant increase of total leukocytes, neutrophil and monocyte but not lymphocytes counts in the peripheral blood, while IL-1RA GT showed normal levels (Figure 29C). The histopathological analyses of the skin, BM, heart, liver and intestine sections are currently ongoing and will reveal more about the effect of the immune-mediated delivery of IL-1RA in preventing tissue inflammation.

These results indicate that HSPCs-derived IL-1RA can effectively prevent disease onset and progression characterised by weight loss, blood leucocytosis and serum IL-6 production in Nlrp3$^{A350V/+}$ CreT mice, probably by suppressing immune cell infiltrates in the tissues.

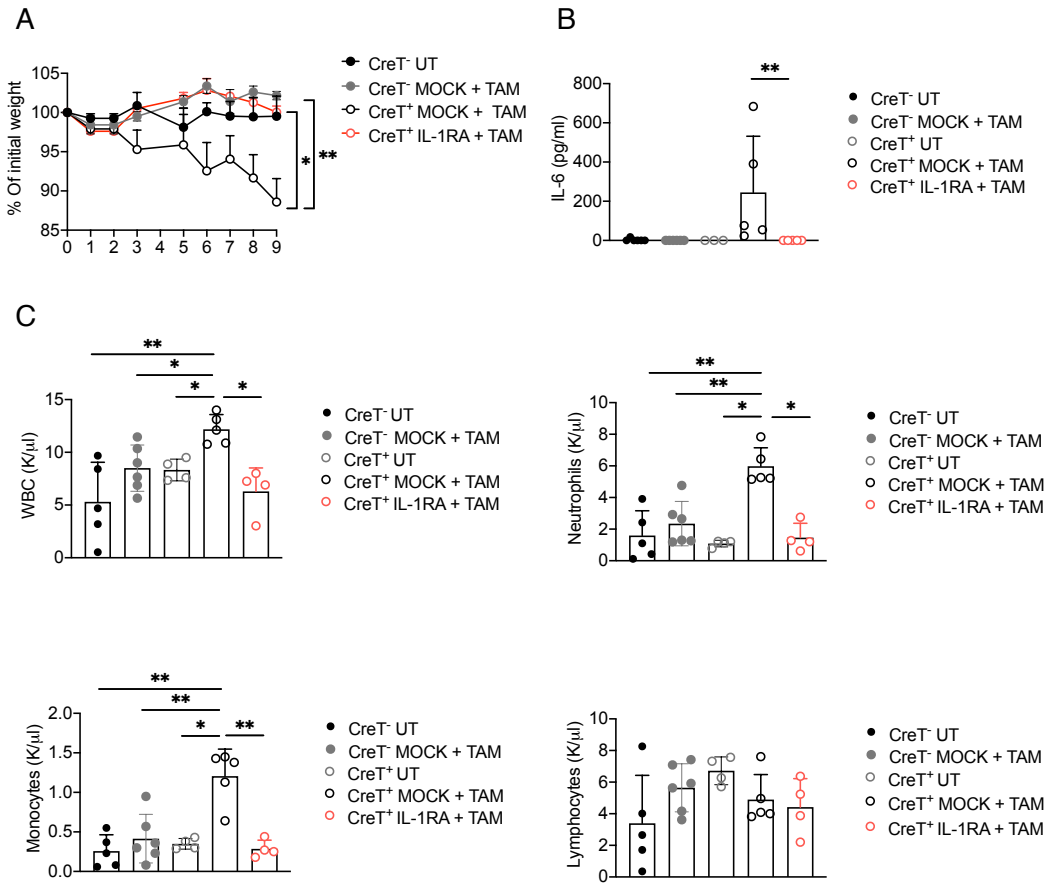


Figure 29. Transplant of IL-1RA-expressing CreT^{+} cells in Nlrp3$^{A350V/+}$CreT^{+} prevents weight loss, blood leucocytosis and plasma IL-6 overproduction.

(A) Time-course analysis of weight loss after tamoxifen treatment evaluated as the percentage of initial weight. Each dot represents the mean weight +/- StD per group at each time point. (B) IL-6 levels in the plasma of tamoxifen-treated MOCK and IL-1RA CreT^{+} mice. (C) Absolute white blood count, neutrophil, monocyte and lymphocyte counts. (A)Each dot represents the

*mean weight +/- StD per group: CreT⁻ UT (n=6), CreT⁻ MOCK (n=6), CreT⁺ UT mice (n=4), CreT⁺ MOCK mice (n=5), CreT⁺ IL-1RA (n=4). Mann Whitney test: *, p<0.05;**, p<0.005. K/µl indicates thousands cells/µl.*

3.2.6 IL-1RA gene transfer in Nlrp3$^{A350V/+}$CreT BMDCs leads to the downregulation of proinflammatory cytokines expression

Tamoxifen-treated CreT^{+} BMDCs are hyper-responsive to LPS-stimulation as they secrete significant levels of mature IL-1β in response to LPS stimulation compared to CreT^{-} BMDCs (Brydges *et al*, 2009). To test whether IL-1RA gene transfer downregulates the production of proinflammatory cytokines, CreT^{+} BM cells were transduced with LV.IL-1RA or left untransduced and differentiated toward the DC lineage by GM-CSF treatment. After six days, BMDCs were treated with tamoxifen to activate the Nlrp3^{A350V} mutant protein and stimulated with LPS (Figure 30A). IL-1RA transduced CreT^{+} BMDCs without tamoxifen treatment were used as a control. We first confirmed that vector-derived human IL-1RA was released in the culture supernatants of LV.IL-1RA-transduced BMDCs in a range between 100 and 150 ng/ml.

limbs (Figure 30B). Following LPS stimulation, the expression of *Il1β*, *Il1α,* and *Cxcl2* genes showed a tendency toward an increase in the tamoxifen-treated CreT^{+} MOCK condition compared to unstimulated cells (Figure 30C). The same trend was observed in the condition with CreT^{+} MOCK tamoxifen compared to the one without tamoxifen treatment (Figure 30C). On the other hand, IL-18 was not upregulated by LPS stimulation and remained stable in the presence of tamoxifen (Figure 30C). This latter finding was in line with previous observations suggesting a diverse licensing mechanism of NLRP3 inflammasome activation leading to IL-18 versus IL-1β release (Schmidt & Lenz, 2012). Notably, the expression of all cytokines tested was downregulated in IL-1RA-transduced BMDCs, at basal state and after LPS stimulation regardless of the type of tamoxifen treatment. These results indicate that IL-1RA production by transduced BMDCs reduces the production of cytokines regulated in LPS/IL-1β-dependent fashion.

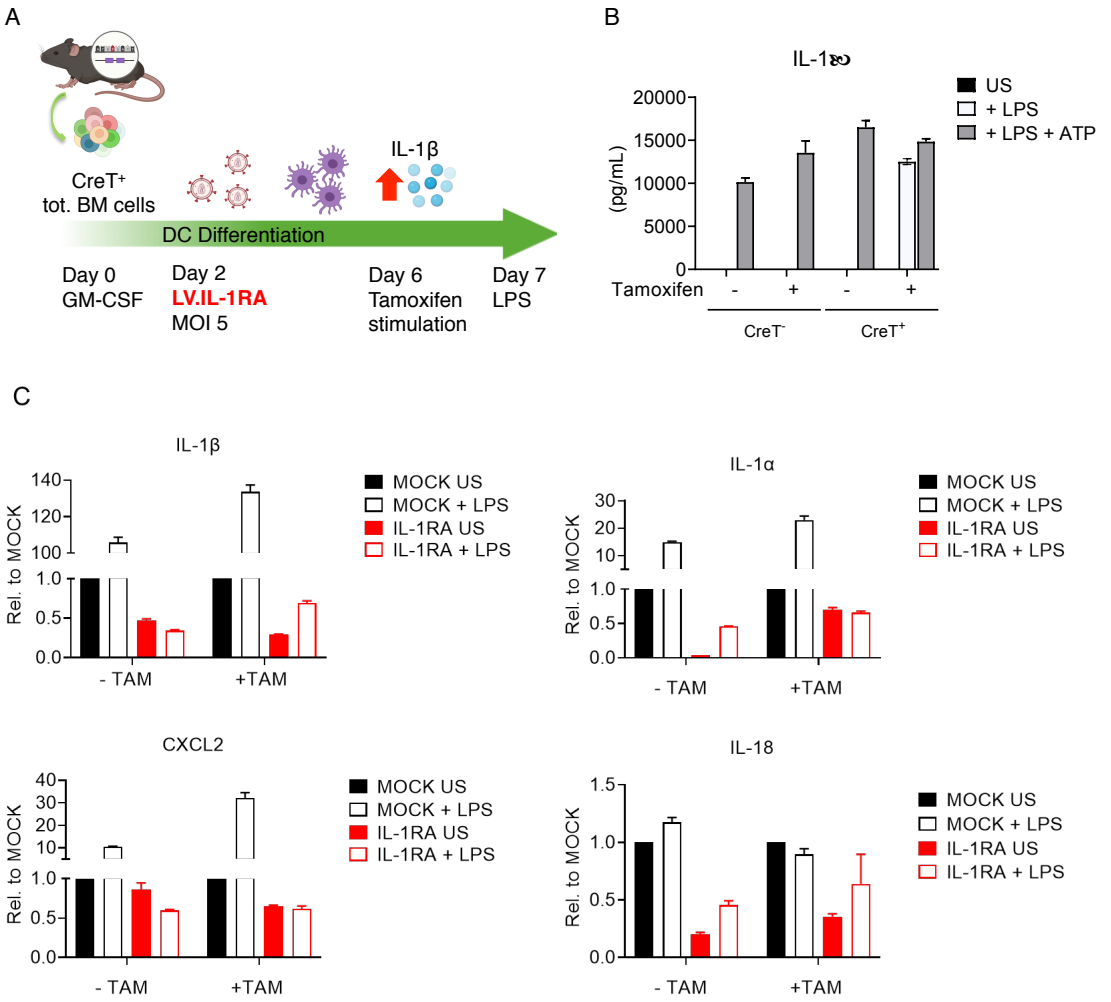


Figure 30. Effect of IL-1RA gene transfer on proinflammatory cytokines production by LPS-stimulated Nlrp3$^{A350V/+}$CreT BMDCs

(A) Illustration of the experimental design. Total BM CreT^{+} cells were transduced with the LV.IL-1RA at an MOI of 5 and differentiated in DCs in the presence of GM-CSF. After 6 days of differentiation, DCs were treated with tamoxifen (0,4µg/mL) to activate the Nlrp3$^{A350V/+}$ mutation followed by LPS stimulation. (B) IL-1β secretion was measured in culture supernatants collected from CreT^{+} and CreT^{-} BMDCs treated with or without tamoxifen by ELISA. (C) Il1β, Il1a, Cxcl2 and Il18 gene expression was measured in CreT^{+} DCs by qRT-PCR calculated by the 2$^{-\Delta\Delta CT}$ method using the MOCK US condition or the MOCK LPS condition as the calibrator. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; BMDCs, bone marrow-derived dendritic cells. Mean +/- StD of 3 technical replicates.

3.2.7 Evaluation of IL-1RA GT efficacy in the experimental autoimmune encephalomyelitis mouse model for multiple sclerosis

IL-1 cytokine is a key factor in driving neuroinflammation in multiple sclerosis (MS). The EAE model, the most used experimental model mimicking MS in mice, is an autoimmune disease associated with T-cell and monocyte/macrophages infiltration in CNS and local inflammation. EAE is generally induced through immunisation with a mouse myelin oligodendrocyte glycoprotein peptide (MOG35-55) emulsified with CFA containing killed *Mycobacterium tuberculosis* to elicit peptide-specific effector T-helper cells. The resulting clinical phenotype follows a standard clinical course characterised by an ascending paralysis beginning in the tail after 10-15 days post-immunisation followed by hind limbs paralysis reaching the fore-limbs. The worsening clinical outcome is associated with weight loss.

We established the EAE model in collaboration with Dr Luca Muzio (IRCCS San Raffaele Scientific Institute) to test the efficacy of IL-1RA-GT in the context of an autoimmune disease in which there is a critical role of IL-1-mediated inflammation. We generated BM chimaeras by transplanting IL-1RA-transduced HSPCs collected from CD45.1$^+$ C57BL/6 mice into lethally irradiated congenic CD45.2$^+$ C57BL/6 recipient mice. BM chimaeras receiving GFP-transduced HSPCs were used as control (Figure 31A). After 2 months, donor chimerism and haematopoietic reconstitution were assessed in the peripheral blood of all mice. There were no differences in the percentage of donor immune cells belonging to the myeloid and lymphoid lineages between IL-1RA and GFP mice (Figure 31B and C). IL-1RA levels in the plasma of chimeric mice ranged between 100 to 200 ng/ml (Figure 31D).

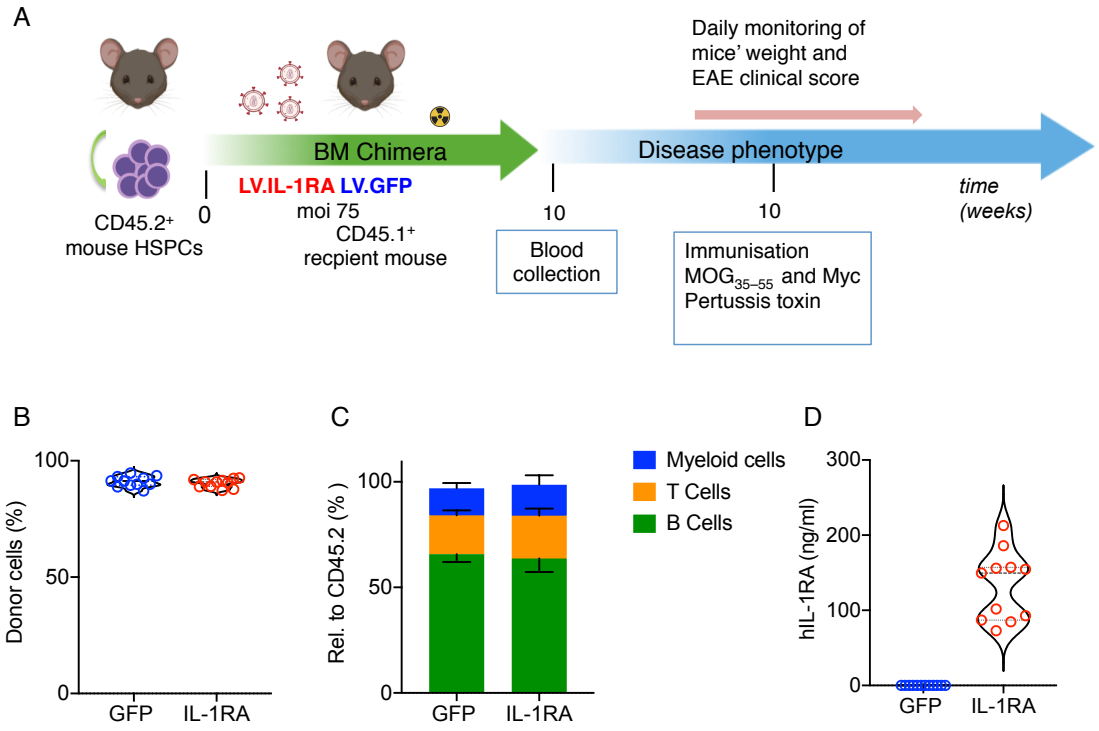


Figure 31. Immune reconstitution and IL-1RA expression in BM chimaeras before EAE induction.

(A) Illustration of the experimental design. HSPCs were isolated from CD45.1$^+$ C57BL/6 mice, transduced with the LV.IL-1RA at MOI 75 and transplanted into lethally irradiated CD45.2$^+$ C57BL/6 mice. At 10 weeks post-transplant, chimaeric mice were immunised s.c. with an injection of MOG35-55 and mycobacterium tuberculosis emulsified in CFA and i.v. with pertussis toxin. (B, C) Percentage of total CD45.2$^+$ donor cells and various immune cells (i.e., myeloid, T and B cells) was evaluated in the peripheral blood by flow cytometry 10 weeks pre-immunisation. (D) Plasma IL-1RA levels were measured in IL-1RA and GFP at 10 weeks post-transplant by ELISA. B,D). Each dot represents a single mouse, (B,D) median and interquartile range and (C) Mean +/- StD of GFP (n=11) and IL-1RA (n=11) mice. Abbreviations: Myc, Mycobacterium tuberculosis. (

At 10 weeks post-transplant, mice were treated subcutaneously with the CFA-MOG35-55 (Figure 31A). Mice also received an *i.v.* injection of pertussis toxin on the day of the immunization and after 2 days. We monitored daily mouse weight and EAE clinical score evaluated as 0=normal, 1=flaccid tail, 2=paresis of hindlimbs, 3=paralysis of hindlimbs and/or paresis of forelimbs, 4=tetraparalysis, 5=moribund or dead (Casella *et al*, 2018). In the IL-1RA group, 2 mice out of 11 did not developed EAE disease', while all the GFP mice got sick. Despite no significant differences between the cumulative scores, which indicate the EAE severity, the disease' onset and the maximum score, the mortality rate was lower in the IL-1RA group than the GFP one (Table 5). Notably, in the chronic phase of the disease, the mean clinical score was decreased in the IL-1RA group compared to the control group (Figure 32A).

Consequently, an increased mean of mice's weight was registered in the IL-1RA group compared to the GFP one (Figure 32B).

The experiment is still ongoing, and the clinical score will continue to be monitored for up to 1 more week. In the end, we will also analyse spinal cord sections to investigate whether IL-1RA could positively impact axonal damage and the demyelination process. These preliminary findings constitute proof-of-concept results on the role of IL-1RA in stabilizing IL-1 hyper-activity in the EAE mouse model.

Table 5. Summary of main parameters in the EAE progression and severity; incidence, mortality, cumulative score, onset and maximum score.

Groups	Incidence	Mortality	Cumulative score	Onset (days)	Maximum score
GFP	11/11	(6/11) 54%	37.2±9.4	16	4.2+-0.9
IL-1RA	9/11	(2/9) 22%	36.6±7.4	16	3.6+-0.8

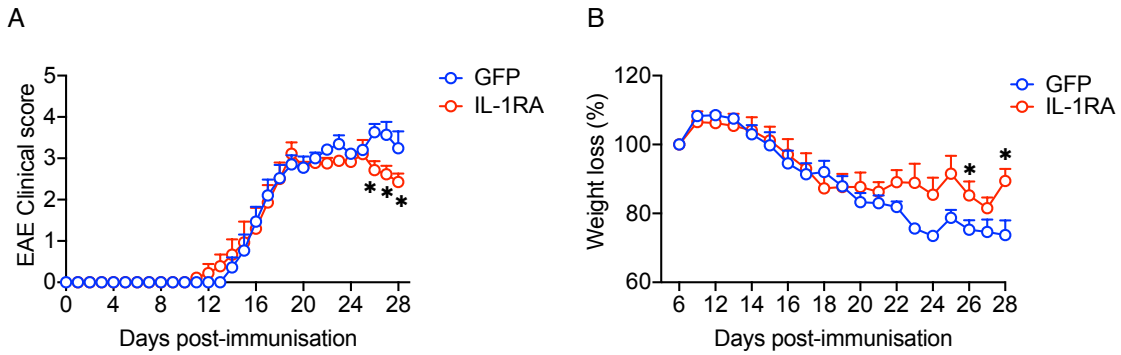


Figure 32. IL-1RA GT ameliorates mortality and disease' severity in the EAE mouse model of MS

*(A) Time course analysis of mouse EAE clinical score. (B) Gradual analysis of weight loss evaluated as the percentage of initial weight measured at day 6 post-immunisation. Each dot represents the average weight and clinical score +/- StD per each group of GFP (n=11) and IL-1RA (n=11) mice. Mann Whitney test: *, p<0.05*

4. Discussion

The NLRP3 inflammasome-IL-1 axis is a critical signalling pathway that initiates and drives an inflammatory response. However, if its activation occurs uncontrollably, it can lead to the pathogenesis of acute and chronic conditions. IL-1-mediated SAIDs comprise diseases of a clear genetic origin (CAPS, FMF, and DIRA) and multifactorial pathologies where both genetic and environmental factors contribute to the complex clinical manifestations, such as RA and Still's disease. SAID common feature is a general immune dysregulation causing recurrent episodes of non-infectious fever and irreversible organ damage due to cytokine and chemokine storms (Krainer *et al*, 2020).

There is no definitive cure for IL-1-mediated SAIDs. In recent years, the mechanisms leading to exaggerated IL-1 signalling have been revealed and contributed to developing new biological treatments for these patients. IL-1RA represents the natural antagonist form of IL-1, acting as a direct competitor for IL-1RI complex binding. Therefore, IL-1RA has been considered a potential therapeutic molecule given its anti-inflammatory properties. The mainstay therapy for IL-1-mediated SAIDs is anakinra, sold as Kineret (Sobi), the recombinant non-glycosylated form of the natural IL-1RA (Dinarello *et al*, 2012). Like other biological therapies, anakinra shows a short half-life and poor tissue distribution making disease remission challenging to achieve. Indeed, patients affected by the most severe forms of SAIDs remain refractory to IL-1 blockade therapy (Sibley *et al*, 2012; Özçakar *et al*, 2016). Additionally, daily drug injections can cause severe side effects negatively impacting patients' quality of life. Therefore, patients with SAIDs urgently need a durable therapy to counteract the excessive IL-1 production.

Most IL-1-mediated SAIDs are caused by mono- or bi-allelic gain of function mutations in genes encoding inflammasome sensor proteins, such as NLRP3, causing IL-1/IL-18 over-production. An ideal therapeutic approach should correct single mutations in the NLRP3 gene re-establishing a normal inflammasome activity in immune cells eventually derived from HSPCs. A genome editing approach might be considered in these patients, but non-immune cells would continue expressing the mutated NLRP3 protein. Also, the extent of correction to achieve disease remission remains to be determined as editing efficiency in HSPCs is currently less than 50% (Azhagiri *et al*, 2021).

Ex-vivo HSPC-based GT has become a valuable therapeutic approach for PIDs, hemoglobinopathies, metabolic diseases, and more (Tucci *et al*, 2021; Bueren *et al*, 2020). The GT arena is rapidly expanding to alternative clinical applications. GT has also been exploited to deliver soluble immunomodulatory molecules, like cytokines, growth and coagulation factors (Naldini, 2019). One of the advantages of GT mediated by HSPCs is the extraordinary ability of these cells to engraft and differentiate in immune cells, which migrate in all tissues and stably produce therapeutic proteins. We hypothesised that LV-mediated targeting of IL-1RA expression in patients' HSPCs might empower immune cells with a potent anti-inflammatory activity able to neutralise systemic and tissue inflammation via suppression of the IL-1 pathway. Therefore, this PhD project aimed to investigate the feasibility and efficacy of a GT approach based on HSPCs producing IL-1RA for treating IL-1-induced SAIDs.

We showed that mouse and human HSPCs transduced with LV.IL-1RA expressed and secreted human IL-1RA in a vector dose-dependent manner. LV.IL-1RA transduction and IL-1RA ectopic expression were well-tolerated as IL-1-expressing HSPCs proliferated and generated CFU-GM and BFU-E colonies similarly to HSPCs transduced with the control LV.GFP vector even at high VCN. These results indicate that the transduction procedure and IL-1RA expression did not affect the clonogenic potential of LV.IL-1RA-transduced HSPCs. Moreover, once transplanted in mice, IL-1RA-expressing HSPCs engrafted and generated all immune cell types similarly to MOCK and vector-control mice in amplitude and kinetic. Robust and stable IL-1RA level was measured in the plasma of IL-1RA GT mice. Moreover, LV-derived expression of human IL-1RA in mice did not cause any sign of tissue and organs toxicity. Altogether, these results demonstrated that IL-1RA is efficiently produced by immune cells derived by IL-1RA-expressing HSPCs, which retain their stem cell features *in vivo*. These findings are the first step in the preclinical development of an LV-mediated GT approach directing IL-1RA expression in HSPCs.

In the second part of the thesis, we tested *in vivo* the efficacy of IL-1RA GT in reducing the exaggerated IL-1-mediated inflammatory response in well-established mouse models of inflammasome hyperactivation. The first model is the peritonitis induced by MSU crystals, commonly used to study NLRP3/IL-1 pathophysiology and test anti-inflammatory drugs, including anakinra (Goh *et al*, 2014). Notably, we

demonstrated that LV-derived IL-1RA could reduce neutrophil recruitment induced by MSU crystals to an extent comparable to mice treated with anakinra. This reduction in the peritoneal cavity could be associated with IL-1RA overexpression both in peritoneal macrophages and in infiltrating neutrophils, resulting in the prevention of IL-1-mediated proinflammatory response to inflamed tissue.

The humanised NSG mouse model was also used to test the ability of IL-1RA-producing human $CD34^+$ cells to suppress MSU-induced inflammation. A trend toward decreased neutrophils' recruitment was identified in MSU-treated mice reconstituted with $CD34^+$ cells expressing IL-1RA. These results demonstrate that IL-1RA produced by HSPC-derived cells could suppress neutrophilic inflammation *in vivo* in the acute phase. Moreover, our findings set the stage for further use of humanised mice as the benchmark model to study the efficacy of IL-1RA GT in the context of human inflammation. Indeed, patients suffering from urate crystal-induced arthritis could benefit from an HSPC-based IL-1RA GT preventing severe comorbidities (cardiovascular disease, renal impairment, and metabolic syndrome) as severe cases are often refractory to anakinra treatment (Chen *et al*, 2010).

Mouse models harbouring gain-of-function mutations identified in patients have been established (Hoffman, 2020). An inverse inflammation status has been noticed in mouse CAPS compared to the human clinical continuum. For instance, a recently developed KI model harbouring the $Nlrp3^{N475K}$ mutation identified in NOMID, the most severe form of CAPS, showed the least inflammatory symptoms compared to other models (Bertoni *et al*, 2020). We used the tamoxifen-inducible $Nlrp3^{A350V/+}$CreT mouse model of MWS developing a severe inflammatory phenotype, previously utilised to examine other approaches (Brydges *et al*, 2009) . IL-1RA-transduced $Nlrp3^{A350V/+}$CreT BM cells were transplanted in C57BL/6 mice, and, after tamoxifen administration, disease onset and severity were evaluated compared to mice receiving untransduced MOCK BM cells. MOCK mice developed severe dermatitis, while IL-1RA mice did not and showed a trend of low IL-6 production in plasma. Mice of both groups lost weight and showed similar organ inflammation. Although the amelioration of systemic inflammation was evident, we hypothesised that IL-1RA production did not reach a therapeutic value. We planned to repeat the same experiment with more mice per group to confirm these results.

In view of an *ex vivo* GT approach, transduced HSPCs are re-infused into the patients, in which non-haematopoietic cells, like intestinal epithelial cells, continue expressing NLRP3 mutated proteins. We reasoned that the best preclinical experimental set-up that mimics the clinical situation would be to transplant IL-1RA-transduced HSPCs in mice that develop the disease. Therefore, IL-1RA-transduced or untransduced MOCK Nlrp3$^{A350V/+}$CreT BM cells were transplanted in Nlrp3$^{A350V/+}$CreT mice. MOCK mice developed significant weight loss, leucocytosis, and systemic inflammation characterised by high IL-6 levels after disease induction by tamoxifen treatment. In contrast, IL-1RA mice were protected from IL-1-mediated inflammation as much as mice that do not develop the disease (tamoxifen-treated CreT^{-} mice transplanted with untransduced cells, untransplanted CreT^{-} or CreT^{+} not treated with tamoxifen). Notably, IL-1RA-mediated correction of leucocytosis was more evident in the myeloid than lymphoid cell compartment, confirming that the inflammatory conditions associated with the CAPS mutation Nlrp3 ^{A350V} are mediated mainly by the cells of the innate immune compartment. Histopathological analyses of various organ sections are ongoing and will inform the therapeutic effect of IL-1RA GT in preventing tissue inflammation. Overall, these results indicate that IL-1RA GT can effectively prevent IL-1-mediated inflammation in mice with CAPS-like symptoms.

Despite the central role of the NLRP3-IL-1 axis in mediating the CAPS syndromes, there are currently no published experimental results demonstrating the therapeutic effect of anti-IL-1 agents in Nlrp3^{A350V+}CreT mice. The murine form of rilonacept can partly improve the survival rate of Nlrp3$^{A350V/+/}$CreL mice expressing the mutant protein constitutively but do not ameliorate skin rush or contain weight loss, suggesting the existence of other IL-1-independent pathways responsible for disease manifestations. Therefore, the contribution of other caspase-1-dependent mechanisms cannot be excluded. A prominent regulatory role of TNFα in inflammasome function and mouse CAPS pathology has also been proposed (McGeough *et al*, 2017).

Our data demonstrate that immune cell-derived IL-1RA production is sufficient to prevent acute peritonitis induced by MSU crystals, and correct systemic inflammation and leucocytosis in a mouse model of CAPS. We are currently evaluating whether our IL-1RA GT approach effectively corrected tissue inflammation and organ damage in Nlrp3$^{A350V/+}$CreT chimaeras. Future studies will compare disease onset and outcome

between GT-treated mice and mice treated long term with anakinra, the benchmark medication for CAPS.

IL-1 is a key factor contributing to MS and other neuroinflammatory diseases. MS, in particular, is an autoimmune disease mainly caused by T cells overreacting against myelin essential protein and other proteins expressed in the CNS (Høglund, 2014). In this context, IL-1 initiates the pathological process directing Th1/Th17 differentiation and activation of various immune cell subsets. Using a similar approach to ours, Parker et al. reported that *ex-vivo* IL-1RA GT effectively ameliorated IL-1-mediated neuroinflammation by reducing working memory deficit and glial activation in a mouse model of MPSIIIA, a rare inherited metabolic disorder that affects the brain (Parker *et al*, 2020). There are, however, no published data on the efficacy of IL-1RA GT in autoimmune chronic inflammatory diseases affecting CNS and other organs. We tested whether our IL-1RA GT approach could reduce disease development and progression in the EAE mouse model of MS. We prepared BM chimaeras reconstituted with IL-1RA-expressing HSPCs, and EAE was induced. The mortality rate was lower in IL-1RA mice than control mice receiving GFP-transduced HSPCs (22% *vs* 54%, respectively). EAE severity declined in IL-1RA-expressing mice, which also gained some weight, compared to control mice at the chronic stage of the disease. These preliminary findings suggest that IL-1RA might contribute to controlling IL-1-induced neuroinflammation during the chronic phase, possibly by repressing myeloid cell infiltration in the CNS rather than acting on the initial T-cell-mediated immune response. We are currently monitoring the mice, and histopathological analyses on spinal cord sections will reveal whether IL-1RA GT can contain axonal loss and demyelination.

Previous work showed that injection intra-cisterna of a non-replicative HSV-1-derived vector expressing IL-1RA ameliorated EAE progression by effectively delaying disease onset and reducing severity (Furlan *et al*, 2007). These results indicate that local IL-1RA administration might be more efficient than *ex vivo* GT in promptly preventing the leading role of the adaptive immune system. However, more studies are needed to fully elucidate the role of IL-1 in the EAE/MS pathology. In this regards, despite Anakinra has shown to be promising in preclinical studies, it has not been approved yet for MS. A Key reason resides in difficulties in assessing the pleiotropic role of IL-1 in this pathology; it is clear that IL-1 is detrimental in this disease, nevertheless potential

paradoxical effects of anti-IL-1 therapy cannot be excluded as this cytokine has demonstrated to be fundamental for repairing processes such as remyelination (Musella *et al*, 2020). We are aware of these considerations; therefore, we did not aim to produce preclinical evidence for future application of IL-1RA GT for MS, but we leveraged the EAE mouse model to have proof-of-concept results on IL-1RA efficacy in suppressing IL-1-mediating inflammation.

Several additional aspects need to be addressed in future studies. The optimal amount of IL-1RA and the proportion of IL-1RA-producing cells necessary to repress IL-1-mediated inflammation in patients with CAPS need to be determined. There is scattered information on the antinflammatory effect of IL-1R blockade in patients' cells. Monocytes isolated from CAPS patients exhibit a hyperactivated state and produce substantial amounts of mature IL-1β in response to LPS stimulation (Gattorno *et al*, 2007). In contrast, patients' treatment with anakinra reduces monocyte-derived IL-1β production compared to untreated patients (Gattorno *et al*, 2007). It has been estimated that a 100-fold excess of plasma IL-1RA is required to effectively neutralise IL-1 production during an inflammatory response in healthy donors (Granowitz *et al*, 1991). These observations suggest that a high IL-1RA production is necessary to reach the expected therapeutic benefit (Gattorno *et al*, 2007). Based on our results, a range between 30 and 150 ng/ml of plasma IL-1RA is sufficient to prevent IL-1-mediated acute inflammatory response in the MSU-peritonitis and the genetic Nlrp3$^{A350V/+}$CreT mouse model. *In vitro*, LV-derived IL-1RA secreted by DCs of Nlrp3$^{A350V/+}$CreT mice reduced the expression of genes encoding proinflammatory cytokines such as *IL-1α*, *IL-1β*, *CXCL2,* and *IL-18*, at basal level and following LPS stimulation. We will also assess the antinflammatory effect of constitutive IL-1RA production in monocytes/macrophages of patients with CAPS. We plan to characterise the phenotype and differentiation potential of CD34^{+} cells of patients, as limited information is currently available. Moreover, the feasibility of our IL-1RA approach will be examined using patients' CD34^{+} cells *in vitro* and *in vivo*. It is paramount to establish a humanised CAPS model to investigate the therapeutic effect of LV-mediated IL-1RA GT. The only available model has been developed by replacing the mouse NLRP3 alleles with one copy of the human NLRP3 gene carrying a CAPS-associated mutation. However, this model develops arthritis-like features such as bone erosion and osteoporosis, but not

autoinflammation (Snouwaert *et al*, 2016). Although the NSG model was previously used to study T-cell-mediated pathologies like RA, the suboptimal reconstitution of the myeloid-cell compartment in these mice limits their use in disease studies primarily mediated by innate immune cells (Schinnerling *et al*, 2019). In future, we may consider exploiting a new transgenic NSG model expressing human IL-3, GM-CSF, and SCF that support monocyte and granulocyte differentiation (Coughlan *et al*, 2016).

We envisaged some limitations of our current IL-1RA GT approach for a future clinical application. First, it would be mandatory to define the cohort of patients that could benefit from an *ex vivo* IL-1RA GT approach. Patients affected by NOMID, Still's disease, and the most severe form of FMF might benefit from IL-1RA GT therapy. Another critical aspect to consider is that constitutive IL-1RA production might impact the ability of patients to respond to infections appropriately, as IL-1β is a critical factor in host defence. Therefore, physiological regulation of IL-1RA production would be more desirable, and, indeed, we plan to develop an improved LV design in which IL-1RA expression is driven by its endogenous promoter. We will test whether IL-1RA chimaeras respond normally to infections.

Finally, the best conditioning regimen to use in patients with autoinflammation needs to be determined. Myeloablative conditioning might be necessary to obtain optimal and stable engraftment of IL-1RA-producing cells since innate immune cells should not be endowed with a selective advantage *in vivo*. Moreover, the persistent hyper-inflammatory state in patients might represent a challenge. However, an FMF patient successfully treated with allogeneic HSCT achieved a positive outcome (Milledge *et al*, 2002). Reduced conditioning regimen intensity followed by HSCT has also been used to cure CGD, a primary immunodeficiency disorder characterised by chronic autoinflammation and severe infections (Güngör *et al*, 2014).

In conclusion, we conducted preclinical studies on the feasibility and the efficacy of an HSPC-based IL-1RA GT approach tested in three mouse models of IL-1-mediated inflammation. We demonstrated that LV-mediated IL-1RA gene transfer in mouse and human HSPCs is safe and well-tolerated *in vivo*, allowing efficient HSPC engraftment and haematopoietic reconstitution. Antinflammatory activity of LV-derived IL-1RA prevented the excessive IL-1-mediated inflammation in two mouse models of SAIDs and an experimental model of MS.

5. Materials and methods

5.1 Cell Cultures

5.1.1 HEK293T cells

HEK293T is a human embryonic cell line derived from kidney tissue. They are suitable cells for transfection and transformation procedures, harbouring a large T antigen (LTag) from the simian vacuolating virus 40 (SV40), which inactivates p53 and the retinoblastoma suppressor protein. HEK293T cells are adherent cells cultured in Iscove's Modified Dulbecco's Medium (IMDM, #12440053, Fisher Scientific) with 10% Fetal Bovine Serum (FBS) (#ECS0182D, Euroclone), 1% Penicillin/Streptomycin (Pen-Strep #ECB3001, Euroclone), and 1% L-Glutamine (#ECM0495, Euroclone) in polystyrene flasks and plates (Corning, New York, US) under normoxic conditions (37°C, 5% CO2). Medium containing 1% trypsin was used to detach the cells.

5.1.2 Mouse BM cells

Total BM cells were obtained by perfusing the femora and tibiae of C57BL/6 mice with phosphate-buffered saline (PBS) (#D8537, Merck) using a 1 ml syringe with a 0.5x16 mm needle. Cells were filtered through a 40 μm nylon filter and washed with phosphate buffered saline (PBS). Cells were then collected by centrifugation for 7 min at 1,500 revolutions per minute (rpm) at room temperature and resuspended in a complete medium.

5.1.3 Mouse Lin-negative HSPCs

Lin-negative mouse HSPCs were isolated from total BM cells using the Lineage Cell Depletion Kit (#130-090-858, Miltenyi Biotec) combined with the LS/MS columns (MACS, Miltenyi Biotec). Cells were labelled with anti-mouse Lineage Cocktail Pacific Blue (#133310, BioLegend), and purity was assessed by flow cytometry.

5.1.4 Human $CD34^+$ HSPCs

Human HSPCs used for *in vitro* and *in vivo* experiments were purchased from Lonza and consisted of mobilized peripheral blood (mPB) $CD34^+$ cells isolated from G-CSF-

mobilized apheresis of healthy volunteers.

5.2 Production of LV.IL-1RA

5.2.1 Generation of the IL-1RA transfer vector

To obtain the PGK.IL-1RA plasmid, the codon-optimised version of the cDNA for the human secreted form of IL-1RA was inserted into the pCCLsin.PPT.hPGK.eGFP.Wpre plasmid by GenScript Biotech. The replacement of the enhanced GFP (E-GFP) cDNA fragment was performed through a restriction digestion with the BamHI/SalI restriction enzymes, and the codon-optimised form of sIL-1RA was inserted into the plasmid backbone through a ligation. The pCCLsin.PPT.hPGK.eGFP.Wpre plasmid encoding the e-GFP protein was used as control plasmid for the experiments.

5.2.2 Production of lab-grade vector preparation

The full methods to produce third-generation lentiviral vectors and measure viral titer were previously described by Follenzi and colleagues (Follenzi *et al*, 2000). Specifically, to produce VSV-G-pseudotyped third-generation monodirectional vectors a calcium phosphate-based transfection was performed in HEK293T cells. Ultracentrifugation was then followed in order to keep vectors concentrated.

5.2.3 Production of purified vector preparation

In house-purified LVs were produced by the SR-Tiget Processing Development Laboratory (PDL) according to the protocol described by Soldi et al., (Soldi *et al*, 2020). Briefly, three main steps were performed in order to get LV stocks rid of contaminating DNA and proteins: 1) an initial collection of the vector from a filtered LV-containing supernatant which allows removal of significant impurities 2) middle purification of an eluted vector that leads to concentrated-LV preparations, and 3) a

final phase of purification which aims to remove contaminants and impurities, producing a biologically active and safe product.

5.2.4 Measurement of viral titre and infectivity

HEK293T cells were plated at $0.075x10^6$ cells/mL cells per well in a complete IMDM medium in a 6-well plate. After 24 hours, cells were transduced with serial dilutions (10-3, 10-4, 10-5, 10-6, 10-7) of lab-grade and purified vector preparations. Cells were cultured and expanded for 14 days. Genomic DNA (gDNA) was extracted using the QIAmp DNA Mini Kit (#56304, Qiagen), following the manufacturer's instructions. To assess the viral titre, the genomic DNA was extracted and the Droplet Digital PCR (ddPCR) SystemTM (Biorad) was performed (Wang *et al*, 2018). Vector infectivity was calculated as the ratio between HEK293T viral titre and total particles as assessed by p24 immunoassay.

5.3 Lentiviral cell transduction

5.3.1 Transduction of mouse haematopoietic cells

Total BM and Lin- cells were plated at a cell density of $1x10^6$ cells/mL in 48 well plates and pre-stimulated in a culture medium supplemented with a cytokine cocktail described in Table 6A for 2 hours +/- 15 min. LV.IL-1RA and LV.GFP was added at the MOI indicated in each experimental set-up. The volume amount of viral supernatant was calculated following the equation below:

$$\text{Volume of viral supernatant (mL)} = \frac{\text{Number of cells x MOI (TU/cell)}}{\text{Viral titre (TU/mL)}}$$

Sixteen hours after LV exposure, cells were washed and suspended in PBS for transplantation experiments or in complete medium for liquid culture (media composition in Table 6B) or Methocult medium (Stem Cell Technologies) for CFU assay. The liquid culture was maintained for 14 days to assess IL-1RA production in the culture supernatant by enzyme-linked immunosorbent assay (ELISA), and transduction efficiency expressed as mean VCN per genome by ddPCR.

Table 6. Culture media for mouse haematopoietic cells.
Abbreviations; rmSCF, recombinant mouse stem cell factor, rmTPO; recombinant mouse thrombopoietin, rhFlt3L; recombinant human Fms-like tyrosine kinase receptor 3 ligand; rhIL-3; recombinant human Interleukin-3; P/S; penicillin/streptomycin.

A. Media for mouse haematopoietic cells pre-stimulation and transduction		
Medium composition	***Concentration***	***Supplier***
Serum-free Stem Span expansion medium	-	Stem Cell Technologies
rmSCF	100 ng/ml	Peprotech
rmTPO	50 ng/ml	Peprotech
rhFlt3-L	100 ng/ml	Peprotech
rhIL-3	20 ng/ml	Peprotech
P/S	1%	Euroclone
L-glutamine	1%	Euroclone
B. Media for mouse haematopoietic cells liquid culture		
Medium composition	***Concentration***	***Supplier***
Serum-free Stem Span expansion medium	-	Stem Cell Technologies
rmSCF	100 ng/ml	Peprotech
rmTPO	50 ng/ml	Peprotech
rhFlt3-L	100 ng/ml	Peprotech
rhIL-3	20 ng/ml	Peprotech
P/S	1%	Euroclone
L-glutamine	1%	Euroclone
FBS	10%	Euroclone

5.3.2 Lentiviral transduction of human CD34$^+$ HSPCs

Human CD34$^+$ cells were plated at a cell density of 1x10^6 cells/mL in a 96-wells pre-coated with RetroNectin® (2 μg/cm^2, cat. #rFN-CH-296, TakaraBio) in CellGenix® GMP SCGM supplemented medium (#20802-0500) containing supporting cytokines reported in Table 7A. After 22 hours, cells were incubated with the transduction enhancer prostaglandin E2 (PGE2, 10 mM; Cayman Chemical) for 2 hours before overnight (O/N) transduction with LV.IL-1RA or LV.GFP at various vectors doses. The volume of viral vectors was calculated using the equation described in Section 5.3.1. After transduction, cells were collected, washed and resuspended in PBS for transplantation experiments or fresh medium for liquid culture (Table 7B) or Methocult medium (#H4434, Stem Cell Technologies) for CFU assay. The liquid culture was maintained for 14 days and IL-1RA production was assessed in cell supernatants by ELISA, and transduction efficiency and by ddPCR.

Table 7. Culture media for human CD34^{+} cells.
Abbreviations; IMDM; Iscove's Modified Dulbecco's Medium, rhSCF; recombinant human stem cell factor, rhTPO; recombinant human thrombopoietin, rhFlt3L; recombinant human Fms-like tyrosine kinase receptor 3 ligand, hIL-3; recombinant human Interleuikin-3, P/S; penicillin/streptomycin.

A. Media for human haematopoietic cells pre-stimulation and LV transduction		
Medium composition	***Concentration***	***Vendor***
CellGenix® GMP SCGM	-	Cell Genix
rhSCF	300 ng/ml	Peprotech
rhTPO	100 ng/ml	Peprotech
rhFlt3-L	100 ng/ml	Peprotech
rhIL-3	60 ng/ml	Peprotech
P/S	1%	Euroclone
L-glutamine	1%	Euroclone
B. Media for human haematopoietic cells liquid culture		
Medium composition	***Final concentration***	***Vendor***
IMDM	-	Fisher Scientific
rhSCF	300 ng/ml	Peprotech
rhTPO	100 ng/ml	Peprotech
rhFlt3-L	100 ng/ml	Peprotech
rhIL-3	60 ng/ml	Peprotech
P/S	1%	Euroclone
L-glutamine	1%	Euroclone

5.3.3. BMDCs differentiation and LV-transduction

Bone Marrow derived DCs (BMDCs) were generated starting from total bone marrow progenitor cells. They were transduced and differentiated according to the protocol described by Comi and colleagues (Comi *et al*, 2020). In brief, bone marrow cells were cultured at a concentration of $1x10^{6}$/well in 24 well culture plate in IMDM supplemented with 10% FBS and 1% Pen-Strep in the presence of 25 ng/ml of mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF, #415-ML R&D systems). On day 2, the transduction with LV.IL-1RA at MOI of 5 was performed. The day after, the transduction was stopped by adding 1ml of IMDM+mGM-CSF (25 ng/mL). On day 5, half of culture medium was replaced with 1 ml of fresh IMDM+GM-GSF medium (25ng/mL). On day 6-7, half cells were treated overnight (O.N.) with (Z)-4-Hydroxytamoxifen (#H7904, Sigma) dissolved in ethanol at a final concentration of 0.4µg/mL, in the condition without tamoxifen the cells was treated with ethanol alone as vehicle. The day after the cells were stimulated O.N with LPS (#tlrl-eblps, Invivogen) 100 ng/mL to obtain mature DCs. After LPS-stimulation, both the cells (pellet) and the supernatants were collected for downstream analysis.

5.4 CFU assay

Mouse haematopoietic cells (5,000/plate Lin-negative, 30,000/plate total BM cells) were resuspended in mouse Methocult medium (#M3434, Stem Cell Technologies) and plated in 35-mm plates in quadruplicates per condition. After 12-14 days, the number and morphology of CFU-GM colonies were scored by light microscopy. For BFU-E colonies detection, plates were incubated in a solution containing 3,3'-diaminobenzidine (DAB, #B3383, Merck), 3% acetic acid and 30% H_2O_2, which stains haemoglobin-containing cells, thus allowing BFU-E precursors distinguishable from unstained CFU-GM.

$CD34^+$ cells (1,000/plate) were resuspended in a complete human Methocult medium (#H4034, Stem Cell Technologies) and plated in 35-mm plates in duplicate per condition. Fourteen days later, haematopoietic precursors identified as CFU-GM, BFU-E and CFU-GEMM were scored for number and morphology by light microscopy.

5.5 Measurement of vector copy number and transduction efficiency

Genomic DNA was extracted from human and mouse cells expanded *in vitro* for 14 days after transduction or from mice' organs using the QIAmp DNA mini kits (cat. #56304, Qiagen), following the manufacturer's instructions. DNA was quantified and assessed for purity by Qubit Fluorometric Quantification (Invitrogen). Single CFUs were picked and lysed in QuickExtract™ DNA Extraction Solution (#QE090050, Lucigen Corporation). Vector copies per diploid genome (vector copy number, VCN) of the integrated LVs were assessed by droplet digital PCR (ddPCR), using the following probes and primers: HIV probe, 5'-FAM-ATCTCTCTCCTTCTAGCCTC-MGBNFQ-3'; HIV forward, 5'-TACTGACGCT CTCGCACC-3'; HIV reverse 5'-TCTCGACGCAGACTCG-3'; human telomerase probe, 5'-VIC-TCAGGACGTCGAGTGGACACGGTG-TAMRA-3', human telomerase forward, 5'-GGCACACGTGGTTTTCG-3'; human telomerase reverse, 5'-GGTGAAC CTCGTAAGTTTATGCAA-3'; mouse semaphorin probe, 5'-HEX-AGAGGCCTGTCC TGCAGCTCATGG-BHQ-3', mouse semaphorin forward, 5'-ACCGATTCCAGAT GATTGGC-3', mouse semaphorin reverse, 5'-TCCATATTAATGCAGTGCTTGC-3'. Amplification was performed on a QX200 Droplet Digital PCR SystemTM (Biorad). Vector-positive colonies were scored positive

when the VCN was >=0.5 copies/genome. Transduction efficiency was calculated following the equation below:

$$\text{Transduction Efficiency (\%)} = \frac{\text{Number of vector-positive colonies}}{\text{Number of total colonies}} \times 100$$

5.6 Immunoblot analysis

Total proteins extracts were prepared using the cold RIPA buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin). Protein content was quantified using the Bio-Rad Protein Assay Dye Reagent Concentrate assay (cat. #5000006, Bio-Rad). Supernatant proteins were precipitated by mixing cell-free supernatants and cold acetone at a 1:4 ratio, followed by incubation at −20°C for at least one hour. The protein mixture was then centrifuged at 15,000 rpm for 15 min at +4°C. The supernatant was discarded, and protein pellets were re-suspended in Laemmli buffer. An equal amount of proteins (30-45 µg per lane) and precipitated cell supernatants was loaded onto pre-casted 10-12% SDS-PAGE gels (BioRad). The marker ExcelBand Enhancer - three-colour high range protein marker SMOBIO (Bio-Cell) was used. Proteins were then transferred onto a PVDF membrane (#1620174, Bio-Rad) by the Trans-Blot Turbo transfer system (Bio-Rad). Membranes were blocked in freshly prepared 5% dried milk in TBS-Tween-20 (0.05%) for 90 minutes at room temperature in oscillation. Over-night incubation with the following primary antibodies was performed at 4°C on an oscillator: rabbit anti-human IL-1RA polyclonal antibody (1:500, #PA5-13428, Thermo Fisher) and anti-human β-actin (1:10,000, cat. a3854, Sigma) diluted in 5% milk. The membranes were washed with TBS-Tween-20 and incubated in milk 5% for one hour at RT in oscillation with the anti-rabbit (1:3000, cat. #P0448, Dako) secondary antibody conjugated with HRP. The enhanced chemiluminescence Immobilon, Horse-Radish Peroxidase (HRP) substrate (cat. #WBLUC0500, Merck Millipore) was used for membrane development. Images were acquired using the ChemiDoc MP Imaging System (Bio-Rad).

5.7 Enzyme-linked immunosorbent assay

Cytokine release was measured in cell supernatants and mouse plasma by ELISA using the DuoSet kit from R&D Systems, following the manufacturer's instructions. Optical densities were measured using Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific).

5.8 Quantitative real-time RT-PCR

Total RNA was extracted with TRIzol reagent (cat. #15596026, Invitrogen) using the RNAeasy Plus Micro Kit (cat. #74034, Qiagen), according to the manufacturer's instructions. After quantification using NanoDrop 2000 (Thermo Scientific), total RNA (1 μg) was reverse-transcribed with the High-Capacity cDNA Reverse Transcription Kit (cat. #43-688-14, Applied Biosystems). Primers' annealing was performed at 25°C for 10 min, followed by elongation at 37°C for 2 hours, and enzyme inactivation at 85°C for 5 min using the Quanti fast-SYBR green PCR-kit. The following mouse primer sets were used: *IL18* (forward, 5'-TCCTTGAAGTTGACGCAAGA-3', reverse; 5'-TCCAGCATC AGGACAAAGAA-3'); *IL1β,* (forward 5'-GGTCAAAGGTTTGGAAGCAG-3', reverse 5'-TGTGAAATGCCACCTTTTGA-3'); *IL1α* (forward 5'-CCAGAAGAAAAT GAGGTCGG-3', reverse 5'-AGCGCTCAAGGAGAAGACC-3'); *CXCL2* (forward 5'-CTCTCAAGGGCGGTCAAAAAGTT-3', reverse, 5'-TCAGACAGCGAGGCACATC AGGT-3'); *GAPDH* (forward 5'-TCGTCCCGTAGACAAAATGG-3', reverse 5'-TTG AGGTCAATGAAGGGGTC-3'). Copied DNA was amplified by a ViiATM7 Real-Time PCR System (ThermoFisher).

5.9 Mouse models

Mouse studies were conducted according to protocols approved by the IRCCS San Raffaele Scientific Institute and Institutional Animal Care and Use Committee, adhering to the Italian Ministry of Health guidelines for experimental animals' use and care. All efforts were made to minimize number, pain and distress during and after experimental

procedures. C57BL/6N (strain #027), C57BL/6J (stock #005304, Jax Lab) and NSG mice (stock#005557, Jax Lab) were purchased from Charles River Laboratories (Calco, Italy). The Nlrp3$^{N475K/+}$ mice were generated by our collaborator Dr Marco Gattorno (IRCCS Istituto Giannina Gaslini, Genoa, Italy). Nlrp3$^{A350V/+}$CreT mice were purchased from Jax Lab and maintained at Charles River Laboratories by breeding heterozygote Nlrp3^{350VneoR} mice (B6.129-Nlrp3<tm1Hhf>/J, stock #017969) with the B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J (stock #004453) mice purchased from the Jackson laboratory. Mice were maintained in specific pathogen-free conditions at the IRCCS San Raffaele Scientific Institute SPF Animal Facility.

5.9.1 C57BL/6N and C57BL/6-LY5.1

C57BL/6N or B6 are the most commonly used inbred mice for research purposes. C57BL/6 mice were used as donor and recipient mice in the transplantation experiments, The C57BL/6-Ly5.1 model (strain #494, B6.SJL-Ptprca Pepcb /BoyJ) is a congenic strain of the C57BL/6 harbouring the Ly5 locus encoding the tyrosine phosphatase receptor type C (Ptprc), also known as CD45.1, expressed by all haematopoietic cells. Cells are distinguished based on the expression of the two CD45 isoforms, namely CD45.2 in the C57BL/6N model and CD45.1 variant in the C57BL/6-Ly5.1 model.

5.9.2 NOD scid gamma (NSG) mice

Immunodeficient NOD.CB17-Prkdcscid/NCrCrl (NSG) mice (stock #005557, Jax Lab) were used as recipient in xenograft transplantation experiments. NSG mice harbour a scid mutation affecting the DNA repair complex protein Prkdc and a null mutation in the IL-2 receptor common gamma chain gene, which lead to B- and T-cell deficiency and NK-cell dysfunctionality thus allowing the engraftment and differentiation of human HSPCs.

5.9.3 Nlrp3$^{N475K/+}$ mice

Nlrp3$^{N475K/+}$ mice harbour the human NOMID mutation N475K in the Nlrp3 gene (Bertoni *et al*, 2020). The authors used an already described knock-in strategy (Meng *et*

al, 2009; Brydges *et al*, 2009). Briefly, they engineered the N475K mutation associated with the CAPS phenotype into the mouse NLRP3 gene. Heterozygous Nlrp3$^{N475K/+}$ offspring were bred with CreZ mice (zona pel-lucida 3) to obtain Nlrp3$^{N475K/+}$CreZ mice (herein reported as Nlrp3$^{N475K/+}$ mice). Cells that physiologically express NLRP3 constitutively (e.g., macrophages, DCs, neutrophils, microglia and intestinal epithelial cells) re-express the mutant Nlrp3$^{N475K/+}$ protein. These mice represented the only CAPS mouse model able to develop organs' amyloidosis, moreover they are characterized by systemic inflammation manifested as skin rush, splenomegaly, blood leucocytosis, increased serum levels of IL-1β, IL-1α and IL-18, and high mortality rate. Dr Marco Gattorno (IRCCS Istituto Giannina Gaslini, Genoa, Italy) provided total BM cells of these mice for the generation and characterization of Nlrp3$^{N475K/+}$ mouse chimaeras described in section 3.2.3

5.9.4 Nlrp3$^{A350V/+}$CreT mice

The knock-in Nlrp3$^{A350V/+}$ mouse strain (B6.129-Nlrp3<tm1Hhf>/J, stock #017969, Jax Lab) harbouring the alanine 352 to valine (A350V) mutation, conserved in mouse and human and strongly associated with human MWS, was generated by Brydges and colleagues (Brydges *et al*, 2009) . To mutate this residue, a targeting construct was generated containing a 4–7 kb region upstream and downstream of a targeted position in intron 2 cloned around the flox neomycin antibiotic resistance cassette. The A350V point mutation was engineered by site-directed mutagenesis. The plasmid was electroporated into 129 SvJ stem cells, and colonies positive for the A350V mutation were used to create chimeric mice (Figure 33). Sequence analysis of cDNA from Nlrp3$^{A350VneoR/+}$ mice showed the absence of expression of the mutated allele due to the transcriptional silencing given by the reverse orientation of the neomycin resistance cassette in respect to the NLRP3 gene. Then, Nlrp3$^{A350VneoR/+}$ mice were bred with tamoxifen-inducible B6.Cg-Tg(CAG-cre/Esr1*)5Amc/ strain (stock #004453, Jax Lab) harbouring a Cre recombinase fused to the estrogen-responsive protein to generate Nlrp3$^{A350V/+}$CreT mice harbouring one A350V Nlrp3 mutant allele. The A350V Nlrp3 mutant protein expression is induced upon i.v. injections of Tamoxifen (Figure 33). The disease manifests with rapid weight loss, neutrophil infiltrates in organs and high levels

of proinflammatory cytokines, such as IL-6 in the serum. These mice were used for transplantation experiments in section 3.2.4 and 3.2.5 and for *in vitro* experiments with BMDCs in section 3.2.6. For simplicity, in the results section the Nlrp3A$^{350V/+}$CreT mice have been defined as CreT^{+} while the Nlrp3A$^{350V/+}$ as CreT^{-}.

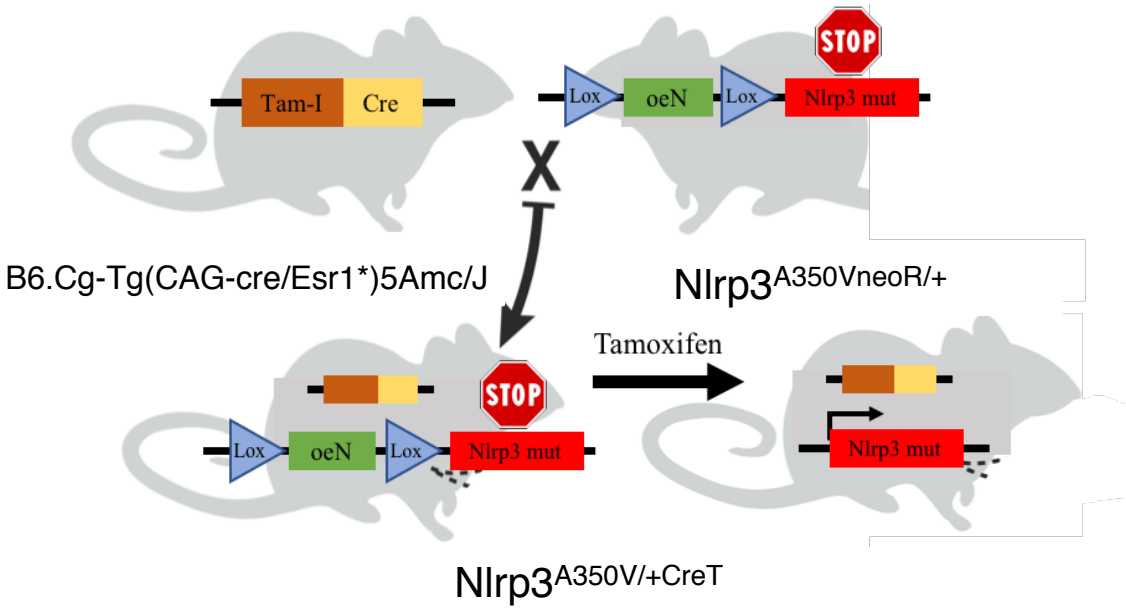


Figure 33. Breeding strategy used to generate the conditional Nlrp3$^{A350V/+}$CreT mouse strain. *Heterozygous Nlrp3$^{A350VneoR/+}$ mice were bred to B6.Cg-Tg(CAG-cre/Esr1*)5Amc/j mice carrying Cre recombinase under the control of a tamoxifen-inducible promoter. The resulting mouse strain harbours one NLRP3 mutated allele, which is not expressed due to the inverted floxed neomycin cassette and the Cre recombinase cassette. Abbreviations: mut, mutation; Tam-I, Tamoxifen-inducible.*

5.9.5 Nlrp3A$^{350V/+}$CreT mice genotyping

We established the following three colonies:

1) Nlrp3^{350VneoR} x Nlrp3^{350VneoR} to obtain the heterozygous Nlrp3$^{A350V/+}$ mice;
2) CreT^{+} (B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J) x C57BL/6J to generate mice harbouring the CreT cassette;
3) Nlrp3$^{A350V/+}$ x B6.Cg-Tg(CAG-cre/Esr1*)5Amc/ to obtain the tamoxifen-inducible Nlrp3A$^{350V/+}$CreT mouse model.

DNA was isolated from blood and tail biopsies using QIAmp DNA mini kits (QIAmp). Nlrp3$^{A350V/+}$ mouse genotyping was assessed by PCR following the protocol available on Jackson website at the following link (https://www.jax.org/Protocol?stock Number=017969&protocolID=26359). To amplify the *Cre* recombinase in the B6.Cg-

Tg(CAG-cre/Esr1*)5Amc/ and Nlrp3A$^{350V/+}$CreT mice, we set up a new genotyping PCR protocol using the following primers*:* Cre forward 5'-GCTAACCATGTTCAT GCCTTC-3' and Cre transgene reverse 5'-AGGCAAATTTTGGTGTACGG-3'. The size of the expected amplicon is 180 bp (base pairs). As positive control, we amplified the wild-type mouse Nlrp3 sequence using the following primers: Nlrp3 wild-type forward 5'CACCCTGCATTTTGTTGTTG-3', NLRP3 reverse 5'-CGTGTAGCGACTGTTGA GGT-3'. The size of the amplicon is 248 bp. All mice from the B6.Cg-Tg(CAG-cre/Esr1*)5Amc/ and the Nlrp3A$^{350V/+}$CreT colonies have an wild-type NLRP3 allele generating an amplicon of 248 pb, only half of mice inherited the CreT cassette (Figure 31). The PCR protocol used was summarized in Table 8.

Table 8. PCR Protocol for mouse genotyping.
Available on Jackson web site (https://www.jax.org/Protocol?stockNumber=017969&protocolID=26359).

Step	***Temp (°C)***	***Note***
1	94.0	
2	94.0	
3	65.0	Temperature decrease of -0.5 °C per cycle
4	68.0	
5		Repeat steps 2-4 for 10 cycles (Touchdown)
6	94.0	
7	60.0	
8	72.0	
9		Repeat steps 6-8 for 28 cycles
10	72.0	
11	10.0	Hold

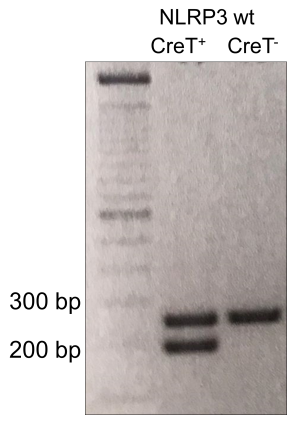


Figure 34. B6.Cg-Tg(CAG-cre/Esr1*)5Amc/ and Nlrp3$^{A350V/+}$CreT mouse genotyping.
The PCR-amplified product of 180 and 248 bp represent the CreT transgene (CreT^{+}) and the NLRP3 wt gene, respectively.

5.9.6 Tamoxifen treatment in vivo

Tamoxifen, free base (#215673883, MP Biomedicals) diluted in a solution of 90% sunflower seed oil (#S5007, Sigma-Aldrich) and 10% ethanol was injected intraperitoneally in Nlrp3$^{A350V/+}$CreT and Nlrp3$^{A350V/+}$ mice at a dose of 50mg/kg for three consecutive days. Mice were monitored for weight loss and sign of distress during the entire treatment until the euthanasia.

5.10 Transplantation experiments

In transplantation experiments, donor cells were isolated from mice aged from 6 to 10 weeks, while recipient mice were 8 to 12 weeks old.

5.10.1 Mouse-to-mouse BM chimaeras

We performed three different mouse-to-mouse BM chimaera experiments:

- In section (3.1.4) 500.000 C57BL/6-CD45.1 HSPCs were transplanted into a lethally irradiated C57BL/6 CD45.2 recipient mouse.
- In section (3.2.3) 2x10^{6} Nlrp3$^{N475K/+}$ and Nlrp3$^{+/+}$ BM CD45.1/CD45.2 cells were transferred in a lethally irradiated C57BL/6-CD45.2 recipient mice.
- In section (3.2.4) 1.5x10^{6} Nlrp3$^{A350V/+}$CreT BM cells were infused in a lethally irradiated C57BL/6-CD45.1 recipient mice.
- In section (3.2.7) 500.000 C57BL/6-CD45.2 HSPCs were infused in a lethally irradiated C57BL/6-CD45.1 recipient mice.

In all these experiments, the cells were resuspended in 200 µl of PBS and transferred via intravenous (IV) injection in lethally irradiated (9 Gy split into two doses) recipient mice.

5.10.2 Syngeneic HSPCs transplant in Nlrp3$^{A350V/+}$CreT mice

In section 3.2.5 a dose of syngeneic 300.000 CreT^{+} or CreT^{-} HSPCs were transplanted into individual lethally irradiated CreT^{+} or CreT^{-} recipient mouse. The cells were resuspended in 200 µl of PBS and transferred via intravenous (IV) injection. The letal dose of irradiation used was 9 Gy split into two doses.

5.10.3 Xenotransplantation of human HSPCs in NSG mice

For transplantation experiments with human haematopoietic cells, in NSG mice (section 3.2.2), 500,000 CD34^{+} cells derived from mPB were resuspended in 200 µl of PBS and injected via the tail vein of recipient NSG mice sub-lethally irradiated (0.15 G, one dose).

5.10.4 Haematological sampling

Blood (maximum volume of 200 µl/mouse) was obtained by lateral tail vein bleeding. Peripheral blood (PB) samples were collected into capillary blood collection tubes pre-treated with EDTA to prevent blood coagulation. A hemogram analysis was performed with 50 µl of total peripheral blood using the Hemocytometer ProCyte Dx (IDEXX). PB was then centrifuged at 13,000 rpm for 10', and the plasma was collected to measure cytokine level. Plasma volume was replaced with PBS and total blood leucocytes were subjected to flow cytometry (FACS) and DNA extraction. BM cells were obtained by perfusing the femora and the tibiae with PBS using a 1-ml syringe. Part of cells was used for FACS analysis for haematopoietic lineages and donor engraftment, whereas the remaining sample was lysed and spun down for DNA extraction and VCN analysis. Spleens were surgically collected and maintained in PBS supplemented with 2% FBS and smashed on a 40 µm nylon filter. Following centrifugation, cells were collected and washed with PBS, and a fraction was used for FACS analysis and the remaining sample for DNA and RNA extraction.

5.10.5 Histopathological analysis

A tibia and the spleen were surgically extracted, weighed and fixed in 10% buffered formalin. For histological examination, samples were embedded in paraffin wax,

sectioned, mounted on glass slides, and stained with haematoxylin and eosin. Decalcification will be performed for the tibia. Dr Francesca Sanvito from SR-Tiget GLP Test Facility performed the analysis of the specimens and provided pictures.

5.11 MSU-induced peritonitis model

Two milligrams of preformed MSU crystals in 200 µl of PBS were injected intraperitoneally in chimeric mice. A group of mice received intraperitoneally a pre-treatment of Anakinra (Kineret, SOBI) at the dose of 50 mg/kg 15' before MSU crystal injection. After 6 hours, mice were humanely euthanised and the peritoneal lavage was conducted with 5 ml of cold PBS supplemented with 2% of FBS. Peritoneal fluid was filtered through a 40 µm nylon strainer, and cells were collected by centrifugation at 1,500 rpm for 5' at RT. Cells were resuspended in PBS, counted and labelled with anti-mouse CD45-FITC (cat. #552950, BD Bioscience), anti-mouse CD11b-APC (cat. #171011283 BioLegend) and anti-mouse Ly6G-PE (cat. #551461 BioLegend) antibodies. Neutrophils were identified as CD11b+Ly6G+ cells within the CD45+ cell population. Absolute numbers were determined by multiplying the percentages of neutrophils by the numbers of cells in the peritoneal lavage.

5.12 EAE mouse model for MS

The experiments with the EAE model were performed in collaboration with Dr Muzio (IRCCS San Raffaele Scientific Institute). EAE was induced by immunization with MOG35−55 (200 µg/mouse; Espikem, Italy) followed by two injections of 500 ng pertussis toxin the day of immunization and 48 h later. Weight was recorded daily and clinical score assessed using the following scale according to Rossi et al.,(Rossi *et al*, 2018): 0=healthy, 1=flaccid tail, 2=paresis of hindlimbs, 3=paralysis of hindlimbs and/or paresis of forelimbs, 4=tetraparalysis, 5=moribund or dead. The EAE cumulative score is indicative of EAE severity. It is the average value of the sum of each mouse' clinical score in a defined time. The maximum score is the highest clinical score reached in a single mouse group.

5.13 Flow cytometry analyses

5.13.1 Flow cytometry on mouse cells in chimeric mice

Mouse PB, splenocytes and BM samples were lysed with ammonium-chloride-potassium (ACK) Lysing Buffer (cat. #07850, STEMCELL Technologies) to remove the red blood cells. Cells were incubated with FcR blocking reagent for 10' at RT (1:100, cat. #553141, BD Biosciences) and labelled with 100 μl of the antibody mix for 30' at RT. The list of antibodies is reported in Table 9. After surface marking, cells were incubated with propidium iodide (cat. #421301, BioLegend) to stain dead cells. Absolute cell quantification was performed by adding Flowcount beads (cat. #424902, BD Bioscience) to PB samples before the staining procedure. The samples were acquired by a BD Symphony A5 (BD Bioscience) and BD Canto HTS (BD Bioscience) cytofluorimeters after calibration with Rainbow beads (cat. #RCP-30-5A, Spherotech), and raw data were collected through DIVA software (BD Biosciences). Data were subsequently analysed by FlowJo software Version 10.5.3 (BD Biosciences). Absolute numbers of the various BM haematopoietic populations were determined by multiplying the percentages for the absolute numbers of cells.

Table 9. List of fluorescent antibodies for mouse haematopoietic cells phenotyping

Antibody	*Fluorochrome*	*Supplier*	*Cat. Number*
anti-mouse CD45.2	PE	BD biosciences	560695
anti-mouse CD45.1	BUV395	BD biosciences	565212
anti-mouse CD19	APC-R700	BD biosciences	565473
anti-mouse CD3	BV605	BioLegend	100237
anti-mouse CD4	BUV496	BD biosciences	612952
anti-mouse CD8a	Pe-Cy5	BD biosciences	553034
anti-mouse CD11b	BUV563	BD biosciences	741242
anti-mouse Ly6C	BUV785	BioLegend	128041
anti-mouse Ly6G	BUV737	BD biosciences	741813
anti-mouse CD11C	BV650	BD biosciences	564079
anti-mouse NK1.1	BB700	BD biosciences	566502
anti-mouse Ter119	BV711	BD biosciences	740686
anti-mouse c-Kit	APC	BioLegend	155107
anti-mouse CD150	BV421	BD biosciences	566298
anti-mouse CD48	APC-Cy7	BioLegend	103431
anti-mouse Sca-1	PE-Cy7	BD biosciences	558162

Table 10. Mouse haematopoietic populations.
The gating strategies used to identify distinct populations are showed. The surface markers in bold are specific for its associated populations. Performed in collaboration with Luca Basso-Ricci (SR-Tiget).

Haematopoietic output	***Markers***
Monocytes	CD45.1/2+ CD11b+ Ly6G-**Ly6C+**
Granulocytes	CD45.1/2+ CD11b+ Ly6C+**Ly6G+**
Dendritic Cells	CD45.1/2+ CD11b+ Ly6C-Ly6G-**CD11c+**
CD3 T cells	CD45.1/2+ CD11b- **CD3+**
CD4 T cells	CD45.1/2+ CD11b- **CD3+ CD4+**
CD8 T cells	CD45.1/2+ CD11b- **CD3+ CD8+**
B cells	CD45.1/2+ CD11b- CD3- **CD19+**
NK cells	CD45.1/2+CD11b+Ly6C-Ly6G-CD11c- or CD11b-CD3-CD19- and **NK1.1+**
Erythroid cells	CD45.1/2+CD11b+Ly6C-Ly6G-CD11c- or CD11b-CD3-CD19- and **Ter119+**
HSC	CD45.1/2+ LIN- **Sca-1+ c-Kit+ CD48- CD150+**
GMLP	CD45.1/2+ LIN- **Sca-1+ c-Kit+ CD48+ CD150-**
MPP	CD45.1/2+ LIN- **Sca-1+ c-Kit+ CD150- CD48-**
GMP	CD45.1/2+ LIN- **Sca-1- c-Kit+ CD48+ CD150-**
MEP	CD45.1/2+ LIN- **Sca-1- c-Kit+ CD150+ CD48+**
ERY	CD45.1/2+ LIN- **Sca-1- c-Kit+ CD150- CD48-**

5.13.2 Flow cytometry analyses on human cells in NSG mice

For the measurement of human haematopoietic output in NSG mice we used a multi-parametric flow-cytometry assay (Whole Blood Dissection) recently developed in the laboratory (Basso□Ricci *et al*, 2017) (Table 11). The various cell subsets identified are showed in Table 12.

For figure 24 the myeloid compartment included all the CD33+ subsets (iPMN, PMN, Monocyte, DC and Myeloblast).

Table 11. List of fluorescent antibodies for WBD phenotyping.
(Basso□Ricci et al, 2017)

Antibody	*Fluorochrome*	*Supplier*	*Cat. Number*
Mouse anti-human CD3	BV605	BioLegend	317322
Mouse anti-human CD56	PC5	BioLegend	362516
Mouse anti-human CD14	BV510	BioLegend	301842
Mouse anti-human CD33	BB515	BD Biosciences	564588
Mouse anti-human CD41/CD61	PC7	BioLegend	359812
Mouse anti-human CD66b	BB515	BD Biosciences	564679
Mouse anti-human CD7	BB700	BD Biosciences	566488
Mouse anti-human CD45	BUV395	BD Biosciences	563792
Mouse anti-human CD38	BUV737	BD Biosciences	612824
Mouse anti-human CD90	APC	BD Biosciences	559869
Mouse anti-human CD184 (CXCR4)	PE	BioLegend	306506
Mouse anti-human CD11c	BV650	BD Biosciences	563404
Mouse anti-human CD10	BV786	BD Biosciences	564960
Mouse anti-human CD34	BV421	BioLegend	343610
Mouse anti-human CD45RA	APCH7	BioLegend	304128
Mouse anti-human CD71	BV711	BD Biosciences	563767
Mouse anti-human CD19	APCR700	BD Biosciences	659121

Table 12. Human haematopoietic populations identified by WBD analysis.
The gating strategies used to identify distinct populations are showed. The surface markers in bold are specific for its associated populations. Adapted from (Basso Ricci et al, 2017).

Haematopoietic output	***Markers***
iPMN	CD45+ CD33+ CD66b+ SSChigh **CD10- and/or CD11c–**
PMN	CD45+ CD33+ CD66b+ **SSChigh CD10+ CD11c+**
Monocyte	CD45+ CD33+ **CD14+**
DC	CD45+ CD33+ CD14- **CD11c+**
Myeloblast	CD45+ CD33+ CD14– CD11c– **CD34–**
T cells	CD45+ CD33– CD66b– **CD3+ CD56–**
NKt Cell	CD45+ CD33– CD66b– **CD3+ CD56+**
NK Cell	CD45+ CD33– CD66b– CD3- CD19- **CD56+**
B cell	CD45+ CD33– CD66b– CD3– **CD19+ CD10– CD34–**
Pre-B cell	CD45+ CD33– CD66b– CD3– **CD19+ CD10+ CD34–**
Pro-B cell	CD45+ CD33– CD66b– CD3– **CD19+ CD10+ CD34+**
Pro-lymphocyte	CD45+ CD33+ CD66b– CD3– CD19– CD56– CD34– CD71– CD41/61– **CD7+ or CD10+**
Pro-erythroblast	CD45+ CD33+ CD66b– CD3– CD19– CD56– CD34– **CD71+**
Erythroblast	**CD45- CD71+**
HSC	CD45+ CD14– CD11c– CD3– CD19– CD56– **CD34+ CD38– CD90+ CD45RA-**
MPP	CD45+ CD14– CD11c– CD3– CD19– CD56– **CD34+ CD38– CD90- CD45RA-**
MLP	CD45+ CD14– CD11c– CD3– CD19– CD56– **CD34+ CD38– CD90+ CD45RA+**
ETP	CD45+ CD14– CD11c– CD3– CD19– CD56– **CD34+ CD38+ CD7+**
PreBNK	CD45+ CD14– CD11c– CD3– CD19– CD56– **CD34+ CD38+ CD7- CD10+**
GMP	CD45+ CD14– CD11c– CD3– CD19– CD56– **CD34+ CD38+ CD7- CD10- CD45RA+**
CMP	CD45+ CD14– CD11c– CD3– CD19– CD56– **CD34+ CD38+ CD7- CD10- CD45RA- CD71- CD41/61-**
MEP	CD45+ CD14– CD11c– CD3– CD19– CD56– **CD34+ CD38+ CD7- CD10- CD45RA- CD71+ CD41/61+**
EP	CD45+ CD14– CD11c– CD3– CD19– CD56– **CD34+ CD38+ CD7- CD10- CD45RA- CD71+ CD41/61-**
MKP	CD45+ CD14– CD11c– CD3– CD19– CD56– **CD34+ CD38+ CD7- CD10- CD45RA- CD71- CD41/61+**

5.14 Statistical analysis

Mann-Whitney unpaired test (two-tailed) and paired t-test were used for statistical analysis. Linear regression was used to demonstrate the direct correlation between two parameters. Results were analysed using GraphPad Prism 9.0 and presented as median and interquartile range or mean values ± standard deviation. Differences were considered significant at *, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$.

References

Abbate A, Toldo S, Marchetti C, Kron J, Van Tassell BW & Dinarello CA (2020) Interleukin-1 and the Inflammasome as Therapeutic Targets in Cardiovascular Disease. *Circ Res*: 1260–1280

Aday AW & Ridker PM (2018) Antiinflammatory Therapy in Clinical Care: The CANTOS Trial and Beyond. *Front Cardiovasc Med* 5: 1–6

Afonina IS, Müller C, Martin SJ & Beyaert R (2015) Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme. *Immunity* 42: 991–1004

Afonina IS, Tynan GA, Logue SE, Cullen SP, Bots M, Lüthi AU, Reeves EP, McElvaney NG, Medema JP, Lavelle EC, *et al* (2011) Granzyme B-dependent proteolysis acts as a switch to enhance the proinflammatory activity of IL-1α. *Mol Cell* 44: 265–278

Agostini L, Martinon F, Burns K, Mcdermott MF, Hawkins PN & Rg Tschopp J (2004) NALP3 Forms an IL-1-Processing Inflammasome with Increased Activity in Muckle-Wells Autoinflammatory Disorder containing protein called ASC binds and activates pro-caspase-1 (Martinon et al ASC contains a C-terminal CARD motif as well as an N-terminal CAR. *Immunity* 20: 319–325

Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, Baricordi C, Dionisio F, Calabria A, Giannelli S, Castiello MC, *et al* (2013) Lentiviral hematopoietic stem cell gene therapy in patients with wiskott-aldrich syndrome. *Science (80-)*

Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A, Morecki S, Andolfi G, Tabucchi A & Carlucci F (2002) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science (80-)* 296: 2410–2413

Aiuti A, Vai S, Mortellaro A, Casorati G, Ficara F, Andolfi G, Ferrari G, Tabucchi A, Carlucci F & Ochs HD (2002) Immune reconstitution in ADA-SCID after PBL gene therapy and discontinuation of enzyme replacement. *Nat Med* 8: 423–425

Akira S, Takeda K & Kaisho T (2001) Ni0801_675. 2

Akkina RK, Walton RM, Chen ML, Li QX, Planelles V & Chen IS (1996) High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope

glycoprotein G. *J Virol* 70: 2581–2585

Aksentijevich I, Centola M, Deng Z, Sood R, Balow J, Wood G, Zaks N, Mansfield E, Chen X, Eisenberg S, *et al* (1997) Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. *Cell* 90: 797–807

Aksentijevich I, Masters SL, Ferguson PJ, Dancey P, Frenkel J, van Royen-Kerkhoff A, Laxer R, Tedgård U, Cowen EW, Pham T-H, *et al* (2009) An Autoinflammatory Disease with Deficiency of the Interleukin-1–Receptor Antagonist. *N Engl J Med* 360: 2426–2437

Arend WP (1993) Interleukin-1 receptor antagonist. *Adv Immunol* 54: 167–227

Arend WP & Gabay C (2000) Physiologic role of interleukin-1 receptor antagonist. *Arthritis Res Ther* 2: 245

Arend WP, Joslin FG & Massoni RJ (1985) Effects of immune complexes on production by human monocytes of interleukin 1 or an interleukin 1 inhibitor. *J Immunol* 134: 3868–75

Arend WP, Malyak M, Guthridge CJ & Gabay C (1998) INTERLEUKIN-1 RECEPTOR ANTAGONIST: Role in Biology. *Annu Rev Immunol* 16: 27–55

Arranz L, Arriero M del M & Villatoro A (2017) Interleukin-1β as emerging therapeutic target in hematological malignancies and potentially in their complications. *Blood Rev* 31: 306–317

Ashcroft FM & Ashcroft FM (2005) ATP-sensitive potassium channelopathies : focus on insulin secretion Find the latest version : Review series ATP-sensitive potassium channelopathies : focus on insulin secretion. *J Clin Invest* 115: 2047–2058

Auron PE, Webb AC, Rosenwasser LJ, Mucci SF, Rich A, Wolff SM & Dinarello CA (1984) Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proc Natl Acad Sci U S A* 81: 7907–7911

Azhagiri MKK, Babu P, Venkatesan V & Thangavel S (2021) Homology-directed gene-editing approaches for hematopoietic stem and progenitor cell gene therapy. *Stem Cell Res Ther* 12: 1–12

Bach F, Albertini R, Joo P, Anderson J & Bortin M (1968) Bone-marrow transplantation in a patient with the Wiskott-Aldrich syndrome. *Lancet* 292: 1364–

1366

Bardhan M & Kaushik R (2022) Physiology, Complement Cascade

Basso□Ricci L, Scala S, Milani R, Migliavacca M, Rovelli A, Bernardo ME, Ciceri F, Aiuti A & Biasco L (2017) Multiparametric Whole Blood Dissection: a one□shot comprehensive picture of the human hematopoietic system. *Cytom Part A* 91: 952–965

Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, *et al* (2009) Cutting Edge: NF-κB Activating Pattern Recognition and Cytokine Receptors License NLRP3 Inflammasome Activation by Regulating NLRP3 Expression. *J Immunol* 183: 787–791

Bellingan GJ, Caldwell H, Howie SE, Dransfield I & Haslett C (1996) In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes. *J Immunol* 157: 2577–85

Bernardo ME & Aiuti A (2016) The Role of Conditioning in Hematopoietic Stem-Cell Gene Therapy. *Hum Gene Ther* 27: 741–748

Bertoni A, Carta S, Baldovini C, Penco F, Balza E, Borghini S, Di Duca M, Ognio E, Signori A, Nozza P, *et al* (2020) A novel knock-in mouse model of cryopyrin-associated periodic syndromes with development of amyloidosis: Therapeutic efficacy of proton pump inhibitors. *J Allergy Clin Immunol* 145: 368-378.e13

Biffi A, Montini E, Lorioli L, Cesani M, Fumagalli F, Plati T, Baldoli C, Martino S, Calabria A & Canale S (2013a) Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science (80-)* 341

Biffi A, Montini E, Lorioli L, Cesani M, Fumagalli F, Plati T, Baldoli C, Martino S, Calabria A, Canale S, *et al* (2013b) Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science (80-)* 341

Blasius AL & Beutler B (2010) Intracellular Toll-like Receptors. *Immunity* 32: 305–315

Boggs SS, Patrene KD, Mueller GM, Evans CH, Doughty LA & Robbins PD (1995) Prolonged systemic expression of human IL-1 receptor antagonist (hIL-1ra) in mice reconstituted with hematopoietic cells transduced with a retrovirus carrying the hIL-1ra cDNA. *Gene Ther* 2: 632–638

Boraschi D, Italiani P, Weil S & Martin MU (2018) The family of the interleukin-1 receptors. *Immunol Rev* 281: 197–232

Briskos C, Wait R, Begum S, O'Neil LAJ & Saklatvala J (2007) Mass spectrometric analysis of the endogenous type I interlukin-1 (IL-1) receptor signaling complex formed after IL-1 binding identifies IL-1RAcP, MyD88, and IRAK-4 as the stable components. *Mol Cell Proteomics* 6: 1551–1559

Brydges S, Mueller J & McGeough M (2009) Animal Models of Inflammasomopathies Reveal Roles for Innate but not Adaptive Immunity. *Immunity* 30: 875–887

Bueren JA, Quintana-Bustamante O, Almarza E, Navarro S, Río P, Segovia JC & Guenechea G (2020) Advances in the gene therapy of monogenic blood cell diseases. *Clin Genet* 97: 89–102

Burger D, Chicheportiche R, Giri JG & Dayer JM (1995) The inhibitory activity of human interleukin-1 receptor antagonist is enhanced by type II interleukin-1 soluble receptor and hindered by type I interleukin-1 soluble receptor. *J Clin Invest* 96: 38–41

Canna SW, De Jesus AA, Gouni S, Brooks SR, Marrero B, Liu Y, Dimattia MA, Zaal KJM, Sanchez GAM, Kim H, *et al* (2014) An activating NLRC4 inflammasome mutation causes autoinflammation with recurrent macrophage activation syndrome. *Nat Genet* 46: 1140–1146

Carrillo MA, Zhen A & Kitchen SG (2018) The Use of the Humanized Mouse Model in Gene Therapy and Immunotherapy for HIV and Cancer. *Front Immunol* 9: 746

Carta S, Penco F, Lavieri R, Martini A, Dinarello CA, Gattorno M & Rubartelli A (2015) Cell stress increases ATP release in NLRP3 inflammasome-mediated autoinflammatory diseases, resulting in cytokine imbalance. *Proc Natl Acad Sci U S A* 112: 2835–2840

Casella G, Colombo F, Finardi A, Descamps H, Ill-Raga G, Spinelli A, Podini P, Bastoni M, Martino G, Muzio L, *et al* (2018) Extracellular Vesicles Containing IL-4 Modulate Neuroinflammation in a Mouse Model of Multiple Sclerosis. *Mol Ther* 26: 2107–2118

Charrier S, Ferrand M, Zerbato M, Précigout G, Viornery A, Bucher-Laurent S, Benkhelifa-Ziyyat S, Merten OW, Perea J & Galy A (2011) Quantification of lentiviral vector copy numbers in individual hematopoietic colony-forming cells

shows vector dose-dependent effects on the frequency and level of transduction. *Gene Ther* 18: 479–487

Chavarría-Smith J & Vance RE (2013) Direct Proteolytic Cleavage of NLRP1B Is Necessary and Sufficient for Inflammasome Activation by Anthrax Lethal Factor. *PLoS Pathog* 9: 1–4

Chen GY & Nuñez G (2010) Sterile inflammation: Sensing and reacting to damage. *Nat Rev Immunol* 10: 826–837

Chen J & Chen ZJ (2018) PtdIns4P on dispersed trans-Golgi network mediates NLRP3 inflammasome activation. *Nature* 564: 71–76

Chen K, Fields T, Mancuso CA, Bass AR & Vasanth L (2010) Anakinra's Efficacy is Variable in Refractory Gout: Report of Ten Cases. *Semin Arthritis Rheum* 40: 210–214

Chen KW, Boucher D & Broz P (2019) Divide to conquer: NLRP3 is activated on dispersed trans-Golgi network. *Cell Res* 29: 181–182

Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, Wang X & Zhao L (2018) Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget* 9: 7204–7218

Chen QL, Yin HR, He QY & Wang Y (2021) Targeting the NLRP3 inflammasome as new therapeutic avenue for inflammatory bowel disease. *Biomed Pharmacother* 138: 111442

Cicalese MP, Ferrua F, Castagnaro L, Pajno R, Barzaghi F, Giannelli S, Dionisio F, Brigida I, Bonopane M & Casiraghi M (2016) Update on the safety and efficacy of retroviral gene therapy for immunodeficiency due to adenosine deaminase deficiency. *Blood, J Am Soc Hematol* 128: 45–54

Coeshott C, Ohnemus C, Pilyavskaya A, Ross S, Wieczorek M, Kroona H, Leimer AH & Cheronis J (1999) Converting enzyme-independent release of tumor necrosis factor α and IL-1β from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc Natl Acad Sci U S A* 96: 6261–6266

Coil DA & Miller AD (2005) Phosphatidylserine treatment relieves the block to retrovirus infection of cells expressing glycosylated virus receptors. *Retrovirology* 2: 1–7

Colomer-Lluch M, Ruiz A, Moris A & Prado JG (2018) Restriction factors: from intrinsic viral restriction to shaping cellular immunity against HIV-1. *Front Immunol* 9: 2876

Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M, Giri JG, Dower SK, Sims JE & Mantovani A (1993) Interleukin-1 Type 11 Receptor: 261

Comi M, Amodio G, Passeri L, Fortunato M, Santoni de Sio FR, Andolfi G, Kajaste-Rudnitski A, Russo F, Cesana L & Gregori S (2020) Generation of Powerful Human Tolerogenic Dendritic Cells by Lentiviral-Mediated IL-10 Gene Transfer. *Front Immunol* 11: 5–7

Constantinescu CS, Farooqi N, O'Brien K & Gran B (2011) Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol* 164: 1079–1106

Cosmi L, De Palma R, Santarlasci V, Maggi L, Capone M, Frosali F, Rodolico G, Querci V, Abbate G, Angeli R, *et al* (2008) Human interleukin 17-producing cells originate from a CD161 +CD4+ T cell precursor. *J Exp Med* 205: 1903–1916

Coughlan AM, Harmon C, Whelan S, O'Brien EC, O'Reilly VP, Crotty P, Kelly P, Ryan M, Hickey FB, O'Farrelly C, *et al* (2016) Myeloid Engraftment in Humanized Mice: Impact of Granulocyte-Colony Stimulating Factor Treatment and Transgenic Mouse Strain. *Stem Cells Dev* 25: 530–541

Dalbeth N, Lauterio TJ & Wolfe HR (2014) Mechanism of action of colchicine in the treatment of gout. *Clin Ther* 36: 1465–1479

Dasgeb B, Kornreich D, McGuinn K, Okon L, Brownell I & Sackett DL (2018) Colchicine: an ancient drug with novel applications. *Br J Dermatol* 178: 350–356

Dayer JM (2004) The process of identifying and understanding cytokines: From basic studies to treating rheumatic diseases. *Best Pract Res Clin Rheumatol* 18: 31–45

Demirkaya E, Erer B, Ozen S & Ben-Chetrit E (2016) Efficacy and safety of treatments in Familial Mediterranean fever: a systematic review. *Rheumatol Int* 36: 325–331

Deyle DR & Russell DW (2009) Adeno-associated virus vector integration. *Curr Opin Mol Ther* 11: 442–447

Dhimolea E (2010) Canakinumab. 3–13

Dinarell CA, Rosenwasser LJ & Wolff SM (2021) Demonstration of a circulating suppressor factor of thymocyte proliferation during endotoxin fever in humans .

Dinarello CA (2009) Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 27: 519–50

Dinarello CA & Bernheim HA (1981) Ability of human leukocytic pyrogen to stimulate brain prostaglandin synthesis in vitro. *J Neurochem* 37: 702–708

Dinarello CA, Simon A & Van Der Meer JWM (2012) Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov* 11: 633–652

Drayton DL, Liao S, Mounzer RH & Ruddle NH (2006) Lymphoid organ development: From ontogeny to neogenesis. *Nat Immunol* 7: 344–353

Dujmovic I, Mangano K, Pekmezovic T, Quattrocchi C, Mesaros S, Stojsavljevic N, Nicoletti F & Drulovic J (2009) The analysis of IL-1 beta and its naturally occurring inhibitors in multiple sclerosis: The elevation of IL-1 receptor antagonist and IL-1 receptor type II after steroid therapy. *J Neuroimmunol* 207: 101–106

Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D & Naldini L (1998) A third-generation lentivirus vector with a conditional packaging system. *J Virol* 72: 8463–8471

Dunne A & Neill LAJO (2003) The Interleukin-1 Receptor / Toll-Like Receptor Superfamily. 1–18

Eisenberg SP, Brewer MT, Verderber E, Heimdal P, Brandhuber BJ & Thompson RC (1991) Interleukin 1 receptor antagonist is a member of the interleukin 1 gene family: Evolution of a cytokine control mechanism. *Proc Natl Acad Sci U S A* 88: 5232–5236

Eisenberg SP, Evans RJ, Arend WP, Verderber E, Brewer MT, Hannum CH & Thompson RC (1990) Primary structure and functional expression from complementary DNA of a human interleukin-1 receptor antagonist. *Nature* 343: 341–346

Evans C (2018) Editorial: Arthritis Gene Therapy Using Interleukin-1 Receptor Antagonist. *Arthritis Rheumatol (Hoboken, NJ)* 70: 1699–1701 doi:10.1002/art.40675

Evans CH, Robbins PD, Ghivizzani SC, Wasko MC, Tomaino MM, Kang R, Muzzonigro TA, Vogt M, Elder EM, Whiteside TL, *et al* (2005) Gene transfer to human joints: progress toward a gene therapy of arthritis. *Proc Natl Acad Sci U S*

A 102: 8698–8703

Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY & Henson PM (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, PGE2, and PAF. *J Clin Invest* 101: 890–898

Faustin B, Lartigue L, Bruey JM, Luciano F, Sergienko E, Bailly-Maitre B, Volkmann N, Hanein D, Rouiller I & Reed JC (2007) Reconstituted NALP1 Inflammasome Reveals Two-Step Mechanism of Caspase-1 Activation. *Mol Cell* 25: 713–724

Favier LA & Schulert GS (2016) Mevalonate kinase deficiency: Current perspectives. *Appl Clin Genet* 9: 101–110

Feder HM & Salazar JC (2010) A clinical review of 105 patients with PFAPA (a periodic fever syndrome). *Acta Paediatr Int J Paediatr* 99: 178–184

Ferrari G, Thrasher AJ & Aiuti A (2021) Gene therapy using haematopoietic stem and progenitor cells. *Nat Rev Genet* 22: 216–234

Follenzi A, Ailles LE, Bakovic S, Geuna M & Naldini L (2000) Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* 25: 217–222

Fukumoto T, Matsukawa A, Ohkawara S, Takagi K & Yoshinaga M (1996) Administration of neutralizing antibody against rabbit IL-1 receptor antagonist exacerbates lipopolysaccharide-induced arthritis in rabbits. *Inflamm Res* 45: 479–485

Fumagalli F, Calbi V, Natali Sora MG, Sessa M, Baldoli C, Rancoita PM V, Ciotti F, Sarzana M, Fraschini M, Zambon AA, *et al* (2022) Lentiviral haematopoietic stem-cell gene therapy for early-onset metachromatic leukodystrophy: long-term results from a non-randomised, open-label, phase 1/2 trial and expanded access. *Lancet* 399: 372–383

Furlan R, Bergami A, Brambilla E, Butti E, De Simoni MG, Campagnoli M, Marconi P, Comi G & Martino G (2007) HSV-1-mediated IL-1 receptor antagonist gene therapy ameliorates MOG35-55-induced experimental autoimmune encephalomyelitis in C57BL/6 mice. *Gene Ther* 14: 93–98

Furlan R, Filippi M, Bergami A, Rocca MA, Martinelli V, Poliani PL, Grimaldi LME, Desina G, Comi G & Martino G (1999) Peripheral levels of caspase-1 mRNA

correlate with disease activity in patients with multiple sclerosis; A preliminary study. *J Neurol Neurosurg Psychiatry* 67: 785–788

Garat C & Arend WP (2003) Intracellular IL-1Ra type 1 inhibits IL-1-induced IL-6 and IL-8 production in Caco-2 intestinal epithelial cells through inhibition of p38 mitogen-activated protein kinase and NF-kappaB pathways. *Cytokine* 23: 31–40

Gatti R, Meuwissen H, Allen H, Hong R & Good R (1968) Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet* 292: 1366–1369

Gattorno M, Tassi S, Carta S, Delfino L, Ferlito F, Pelagatti MA, D'Osualdo A, Buoncompagni A, Alpigiani MG, Alessio M, *et al* (2007) Pattern of interleukin-1β secretion in response to lipopolysaccharide and ATP before and after interleukin-1 blockade in patients with CIAS1 mutations. *Arthritis Rheum* 56: 3138–3148

Gay NJ & Keith FJ (1991) Drosophila Toll and IL-1 receptor. *Nature* 351: 355–6

Gentner B, Tucci F, Galimberti S, Fumagalli F, De Pellegrin M, Silvani P, Camesasca C, Pontesilli S, Darin S, Ciotti F, *et al* (2021) Hematopoietic Stem- and Progenitor-Cell Gene Therapy for Hurler Syndrome. *N Engl J Med* 385: 1929–1940

Gertz MA (2010) Current status of stem cell mobilization. *Br J Haematol* 150: 647–662

Gicquel T, Robert S, Loyer P, Victoni T, Bodin A, Ribault C, Gleonnec F, Couillin I, Boichot E & Lagente V (2015) ILr1β production is dependent on the activation of purinergic receptors and NLRP3 pathway in human macrophages. *FASEB J* 29: 4162–4173

Goh AXH, Bertin-Maghit S, Yeo SP, Ho AWS, Derks H, Mortellaro A & Wang CI (2014) A novel human anti-interleukin-1β neutralizing monoclonal antibody showing in vivo efficacy. *MAbs* 6: 764–772

Goossens PH, Schouten GJ, 'T Hart BA, Bout A, Brok HPM, Kluin PM, Breedveld FC, Valerio D & Huizinga TWJ (1999) Feasibility of adenovirus-mediated nonsurgical synovectomy in collagen-induced arthritis-affected rhesus monkeys. *Hum Gene Ther* 10: 1139–1149

Govindarajan V, De Rivero Vaccari JP & Keane RW (2020) Role of inflammasomes in multiple sclerosis and their potential as therapeutic targets. *J Neuroinflammation* 17: 1–15

Granowitz E V, Santos AA, Poutsiaka DD, Cannon JG, Wilmore DW, Wolff SM &

Dinarello CA (1991) Production of interleukin-1-receptor antagonist during experimental endotoxaemia. *Lancet (London, England)* 338: 1423–1424

Grez M, Reichenbach J, Schwäble J, Seger R, Dinauer MC & Thrasher AJ (2011) Gene therapy of chronic granulomatous disease: The engraftment dilemma. *Mol Ther* 19: 28–35

Gritsenko A, Yu S, Martin-Sanchez F, Diaz-Del-Olmo I, Nichols E-M, Davis DM, Brough D & Lopez-Castejon G (2020) Priming Is Dispensable for NLRP3 Inflammasome Activation in Human Monocytes In Vitro. *Front Immunol* 11: 565924

Gudkov A V & Komarova EA (2016) Inflammation. 1–23

Güngör T, Teira P, Slatter M, Stussi G, Stepensky P, Moshous D, Vermont C, Ahmad I, Shaw PJ, Da Cunha JMT, *et al* (2014) Reduced-intensity conditioning and HLA-matched haemopoietic stem-cell transplantation in patients with chronic granulomatous disease: A prospective multicentre study. *Lancet* 383: 436–448

Guo H, Callaway JB & Ting JPY (2015) Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat Med* 21: 677–687

Haar N Ter, Lachmann H, Özen S, Woo P, Uziel Y, Modesto C, Koné-Paut I, Cantarini L, Insalaco A, Neven B, *et al* (2013) Treatment of autoinflammatory diseases: Results from the Eurofever Registry and a literature review. *Ann Rheum Dis* 72: 678–685

Ter Haar NM, Eijkelboom C, Cantarini L, Papa R, Brogan PA, Kone-Paut I, Modesto C, Hofer M, Iagaru N, Fingerhutová S, *et al* (2019) Clinical characteristics and genetic analyses of 187 patients with undefined autoinflammatory diseases. *Ann Rheum Dis* 78: 1405–1411

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, *et al* (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302: 415–419

Haddad A & Wilson AM (2021) Biochemistry, Complement. In Treasure Island (FL)

Hagar JA, Powell DA, Aachoui Y, Ernst RK & Miao EA (2013) Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science* 341: 1250–1253

Hashkes PJ & Laxer RM (2019) Textbook of Autoinflammation

Haskill S, Martin G, Van Le L, Morris J, Peace A, Bigler CF, Jaffe GJ, Hammerberg C, Sporn SA, Fong S, *et al* (1991) cDNA cloning of an intracellular form of the human interleukin 1 receptor antagonist associated with epithelium. *Proc Natl Acad Sci U S A* 88: 3681–3685

Hauser SL, Doolittle TH, Lincoln R, Brown RH & Dinarello CA (1990) Cytokine accumulations in CSF of multiple sclerosis patients: frequent detection of interleukin-1 and tumor necrosis factor but not interleukin-6. *Neurology* 40: 1735–1739

He WT, Wan H, Hu L, Chen P, Wang X, Huang Z, Yang ZH, Zhong CQ & Han J (2015) Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. *Cell Res* 25: 1285–1298

He Y, Franchi L & Núñez G (2013) TLR agonists stimulate Nlrp3-dependent IL-1β production independently of the purinergic P2X7 receptor in dendritic cells and in vivo. *J Immunol* 190: 334–339

Headland SE & Norling L V. (2015) The resolution of inflammation: Principles and challenges. *Semin Immunol* 27: 149–160

Heffner GC, Bonner M, Christiansen L, Pierciey FJ, Campbell D, Smurnyy Y, Zhang W, Hamel A, Shaw S & Lewis G (2018) Prostaglandin E2 increases lentiviral vector transduction efficiency of adult human hematopoietic stem and progenitor cells. *Mol Ther* 26: 320–328

Heidary M, Rakhshi N, Pahlevan Kakhki M, Behmanesh M, Sanati MH, Sanadgol N, Kamaladini H & Nikravesh A (2014) The analysis of correlation between IL-1B gene expression and genotyping in multiple sclerosis patients. *J Neurol Sci* 343: 41–45

Hirsch E, Irikura VM, Paul SM & Hirsh D (1996) Functions of interleukin 1 receptor antagonist in gene knockout and overproducing mice. *Proc Natl Acad Sci* 93: 11008–11013

Hoffman HM (2009) Therapy of autoinflammatory syndromes. *J Allergy Clin Immunol* 124: 1129–1138

Hoffman HM (2020) Autoinflammatory disease: New mouse models and therapies. *J Allergy Clin Immunol* 145: 116–118

Hoffman HM, Mueller JL, Broide DH, Wanderer AA & Kolodner RD (2001) Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet*

Høglund RA (2014) Multiple sclerosis and the role of immune cells. *World J Exp Med* 4: 27

Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E & Fitzgerald KA (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458: 514–518

Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA & Latz E (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9: 847–856

Hou B, Zhang Y, Liang P, He Y, Peng B, Liu W, Han S, Yin J & He X (2020) Inhibition of the NLRP3-inflammasome prevents cognitive deficits in experimental autoimmune encephalomyelitis mice via the alteration of astrocyte phenotype. *Cell Death Dis* 11

Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M, Kempski H, Brugman MH, Pike-Overzet K, Chatters SJ, de Ridder D, *et al* (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest* 118: 3143–3150

Hull KM, Drewe E, Aksentijevich I, Singh HK, Wong K, McDermott EM, Dean J, Powell RJ & Kastner DL (2002) The TNF receptor-associated periodic syndrome (TRAPS): Emerging concepts of an autoinflammatory disorder. *Medicine (Baltimore)* 81: 349–368

Hung GL, Galea-Lauri J, Mueller GM, Georgescu HI, Larkin LA, Suchanek MK, Tindal MH, Robbins PD & Evans CH (1994) Suppression of intra-articular responses to interleukin-1 by transfer of the interleukin-1 receptor antagonist gene to synovium. *Gene Ther* 1: 64–69

Iqbal AJ, Fisher EA & Greaves DR (2017) Inflammation-a critical appreciation of the role of myeloid cells. *Myeloid Cells Heal Dis A Synth* 4: 325–342

Ito S, Hara Y & Kubota T (2014) CARD8 is a negative regulator for NLRP3 inflammasome, but mutant NLRP3 in cryopyrin-associated periodic syndromes escapes the restriction. *Arthritis Res Ther* 16: 1–11

Jarcho S (1971) Augustus Volney Waller on blood vessels and inflammation. II. *Am J Cardiol* 28: 712–714

De Jesus AA, Canna SW, Liu Y & Goldbach-Mansky R (2015) Molecular mechanisms in genetically defined autoinflammatory diseases: Disorders of amplified danger signaling

Juliana C, Fernandes-Alnemri T, Kang S, Farias A, Qin F & Alnemri ES (2012) Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. *J Biol Chem* 287: 36617–36622

Kafri T, Blömer U, Peterson DA, Gage FH & Verma IM (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat Genet* 17: 314–317

Kaskow BJ & Baecher-Allan C (2018) Effector t cells in multiple sclerosis. *Cold Spring Harb Perspect Med* 8: 1–14

Kawai T & Akira S (2010) The role of pattern-recognition receptors in innate immunity: Update on toll-like receptors. *Nat Immunol* 11: 373–384

Kayagaki N, Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-Takamura S, Miyake K, Zhang J, Lee WP, Muszyński A, *et al* (2013) Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* 341: 1246–1249

Kelley N, Jeltema D, Duan Y & He Y (2019) The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *Int J Mol Sci* 20: 1–24

Klimatcheva E, Rosenblatt JD & Planelles V (1999) Lentiviral vectors and gene therapy. *Front Biosci* 4: 481–496

Koboziev I, Jones-Hall Y, Valentine JF, Reinoso Webb C, Furr KL & Grisham MB (2015) Use of humanized mice to study the pathogenesis of autoimmune and inflammatory diseases

Kofoed EM, Vance RE & Biology C (2012) Dictates Inflammasome Specificity. 477: 592–595

Krainer J, Siebenhandl S & Weinhäusel A (2020) Systemic autoinflammatory diseases. *J Autoimmun* 109

Kyriazopoulou E, Huet T, Cavalli G, Gori A, Kyprianou M, Pickkers P, Eugen-Olsen J, Clerici M, Veas F, Chatellier G, *et al* (2021) Effect of anakinra on mortality in

patients with COVID-19: a systematic review and patient-level meta-analysis. *Lancet Rheumatol* 3: e690–e697

Laudisi F, Spreafico R, Evrard M, Hughes TR, Mandriani B, Kandasamy M, Morgan BP, Sivasankar B & Mortellaro A (2013) Cutting Edge: The NLRP3 Inflammasome Links Complement-Mediated Inflammation and IL-1β Release. *J Immunol* 191: 1006–1010

Levy BD, Clish CB, Schmidt B, Gronert K & Serhan CN (2001) Lipid mediator class switching during acute inflammation: Signals in resolution. *Nat Immunol* 2: 612–619

Lin C-C & Edelson BT (2017) New Insights into the Role of IL-1β in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis. *J Immunol* 198: 4553–4560

Liu L, Xue Y, Zhu Y, Xuan D, Yang X, Liang M, Wang J, Zhu X, Zhang J & Zou H (2016) Interleukin 37 limits monosodium urate crystal-induced innate immune responses in human and murine models of gout. *Arthritis Res Ther* 18: 1–14

Liu X & Quan N (2018) Microglia and CNS interleukin-1: Beyond immunological concepts. *Front Neurol* 9: 1–11

Lopez-castejon G, Luheshi NM, Compan V, High S, Whitehead RC, Flitsch S, Kirov A, Prudovsky I, Swanton E & Brough D (2013) Deubiquitinases Regulate the Activity of Caspase-1 and Interleukin-1 Secretion via Assembly of the. 288: 2721–2733

Lyseng-Williamson KA (2018) Anakinra in Still's disease: a profile of its use. *Drugs Ther Perspect* 34: 543–553

Ma Y, Thornton S, Boivin GP, Hirsh D, Hirsch R & Hirsch E (1998) Altered susceptibility to collagen□induced arthritis in transgenic mice with aberrant expression of interleukin□1 receptor antagonist. *Arthritis Rheum Off J Am Coll Rheumatol* 41: 1798–1805

Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA & Donath MY (2002) Glucose-induced β cell production of IL-1β contributes to glucotoxicity in human pancreatic islets. *J Clin Invest* 110: 851–860

Majno G (1975) Communautd Scientifique,. 1975

Malim MH, Hauber J, Le SY, Maizel J V & Cullen BR (1989) The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of

unspliced viral mRNA. *Nature* 338: 254–257

Malkin HM (1984) Julius Cohnheim (1839-1884). His life and contributions to pathology. *Ann Clin Lab Sci* 14: 335–342

Malyak M, Guthridge JM, Hance KR, Dower SK, Freed JH & Arend WP (1998) Characterization of a low molecular weight isoform of IL-1 receptor antagonist. *J Immunol* 161: 1997–2003

Man SM & Kanneganti TD (2016) Converging roles of caspases in inflammasome activation, cell death and innate immunity. *Nat Rev Immunol* 16: 7–21

Mantovani A, Dinarello CA, Molgora M & Garlanda C (2019) Interleukin-1 and Related Cytokines in the Regulation of Inflammation and Immunity. *Immunity* 50: 778–795

Manz MG & Boettcher S (2014) Emergency granulopoiesis. *Nat Rev Immunol* 14: 302–314

Marktel S, Scaramuzza S, Cicalese MP, Giglio F, Galimberti S, Lidonnici MR, Calbi V, Assanelli A, Bernardo ME & Rossi C (2019) Intrabone hematopoietic stem cell gene therapy for adult and pediatric patients affected by transfusion-dependent ß-thalassemia. *Nat Med* 25: 234–241

Martin P, Palmer G, Rodriguez E, Palomo J, Lemeille S, Goldstein J & Gabay C (2020) Intracellular IL-1 Receptor Antagonist Isoform 1 Released from Keratinocytes upon Cell Death Acts as an Inhibitor for the Alarmin IL-1α. *J Immunol* 204: 967–979

Martinon F, Burns K & Tschopp J (2002) The Inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL-β. *Mol Cell* 10: 417–426

Martinon F, Mayor A & Tschopp J (2009) The inflammasomes: Guardians of the body. *Annu Rev Immunol* 27: 229–265

Martinon F, Pétrilli V, Mayor A, Tardivel A & Tschopp J (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440: 237–241

Masters SL, Dunne A, Subramanian SL, Hull RL, Tannahill GM, Sharp FA, Becker C, Franchi L, Yoshihara E, Chen Z, *et al* (2010) Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1β 2 in type 2 diabetes. *Nat Immunol* 11: 897–904

McClements ME, Butt A, Piotter E, Peddle CF & Maclaren RE (2021) An analysis of the Kozak consensus in retinal genes and its relevance to gene therapy. *Mol Vis* 27: 233–242

McDermott MF, Aksentijevich I, Galon J, McDermott EM, William Ogunkolade B, Centola M, Mansfield E, Gadina M, Karenko L, Pettersson T, *et al* (1999) Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. *Cell* 97: 133–144

McGeough MD, Wree A, Inzaugarat ME, Haimovich A, Johnson CD, Peña CA, Goldbach-Mansky R, Broderick L, Feldstein AE & Hoffman HM (2017) TNF regulates transcription of NLRP3 inflammasome components and inflammatory molecules in cryopyrinopathies. *J Clin Invest* 127: 4488–4497

McKee CM & Coll RC (2020) NLRP3 inflammasome priming: A riddle wrapped in a mystery inside an enigma. *J Leukoc Biol* 108: 937–952

Medzhitov R (2010) Inflammation 2010: New Adventures of an Old Flame. *Cell* 140: 771–776

Medzhitov R & Janeway CA (1997) Innate immunity: The virtues of a nonclonal system of recognition. *Cell* 91: 295–298

Meng G, Zhang F, Fuss I, Kitani A & Strober W (2009) A Mutation in the Nlrp3 Gene Causing Inflammasome Hyperactivation Potentiates Th17 Cell-Dominant Immune Responses. *Immunity* 30: 860–874

Merlin S & Follenzi A (2019) Transcriptional Targeting and MicroRNA Regulation of Lentiviral Vectors. *Mol Ther Methods Clin Dev* 12: 223–232

Mertens M & Singh JA (2009) Anakinra for rheumatoid arthritis: A systematic review. *J Rheumatol* 36: 1118–1125

Milledge J, Shaw PJ, Mansour A, Williamson S, Bennetts B, Roscioli T, Curtin J & Christodoulou J (2002) Allogeneic bone marrow transplantation: Cure for familial Mediterranean fever. *Blood*

Miller AD, Miller DG, Garcia JV & Lynch CM (1993) [40] Use of retroviral vectors for gene transfer and expression. *Methods Enzymol* 217: 581–599

Milone MC & O'Doherty U (2018) Clinical use of lentiviral vectors. *Leukemia* 32: 1529–1541

Modlich U, Navarro S, Zychlinski D, Maetzig T, Knoess S, Brugman MH, Schambach A, Charrier S, Galy A, Thrasher AJ, *et al* (2009) Insertional transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors. *Mol Ther* 17: 1919–1928

Mullard A (2019) NLRP3 inhibitors stoke anti-inflammatory ambitions. *Nat Rev Drug Discov* 18: 405–407

Murakami T, Ockinger J, Yu J, Byles V, McColl A, Hofer AM & Horng T (2012) Critical role for calcium mobilization in activation of the NLRP3 inflammasome. *Proc Natl Acad Sci U S A* 109: 11282–11287

Musella A, Fresegna D, Rizzo FR, Gentile A, De Vito F, Caioli S, Guadalupi L, Bruno A, Dolcetti E, Buttari F, *et al* (2020) 'Prototypical' proinflammatory cytokine (IL-1) in multiple sclerosis: role in pathogenesis and therapeutic targeting. *Expert Opin Ther Targets* 24: 37–46

Muzio M, Polentarutti N, Sironi M, Poli G, De Gioia L, Introna M, Mantovani A & Colotta F (1995) Cloning and characterization of a new isoform of the interleukin 1 receptor antagonist. *J Exp Med* 182: 623–628

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

Naldini L (2015) Gene therapy returns to centre stage. *Nature* 526: 351–360

Naldini L (2019) Genetic engineering of hematopoiesis: current stage of clinical translation and future perspectives. *EMBO Mol Med* 11: 1–12

Nathan C (2006) Neutrophils and immunity: Challenges and opportunities. *Nat Rev Immunol* 6: 173–182

Nathan C & Ding A (2010) Nonresolving Inflammation. *Cell* 140: 871–882

Nedjai B, Hitman GA, Yousaf N, Chernajovsky Y, Stjernberg-Salmela S, Pettersson T, Ranki A, Hawkins PN, Arkwright PD, McDermott MF, *et al* (2008) Abnormal tumor necrosis factor receptor I cell surface expression and NF-κB activation in tumor necrosis factor receptor-associated periodic syndrome. *Arthritis Rheum* 58: 273–283

Nixon AJ, Grol MW, Lang HM, Ruan MZC, Stone A, Begum L, Chen Y, Dawson B, Gannon F, Plutizki S, *et al* (2018) Disease-Modifying Osteoarthritis Treatment With Interleukin-1 Receptor Antagonist Gene Therapy in Small and Large Animal

Models. *Arthritis Rheumatol (Hoboken, NJ)* 70: 1757–1768

Oelkers G (2012) Kineret® (anakinra) for injection, for subcutaneous use. 1

Özçakar ZB, Özdel S, Yılmaz S, Kurt-Şükür ED, Ekim M & Yalçınkaya F (2016) Anti-IL-1 treatment in familial Mediterranean fever and related amyloidosis. *Clin Rheumatol* 35: 441–446

Pal A, Neo K, Rajamani L, Ferrer FJ, Lane DP, Verma CS & Mortellaro A (2019) Inhibition of NLRP3 inflammasome activation by cell-permeable stapled peptides. *Sci Rep* 9: 4913

Palmer G, Talabot-Ayer D, Szalay-Quinodoz I, Maret M, Arend WP & Gabay C (2003) Mice transgenic for intracellular interleukin-1 receptor antagonist type 1 are protected from collagen-induced arthritis. *Eur J Immunol* 33: 434–440

Di Paolo NC & Shayakhmetov DM (2016) Interleukin 1α and the inflammatory process. *Nat Immunol* 17: 906–913

Park YH, Wood G, Kastner DL & Chae JJ (2016) Pyrin inflammasome activation and RhoA signaling in the autoinflammatory diseases FMF and HIDS. *Nat Immunol* 17: 914–921

Parker H, Ellison SM, Holley RJ, O'Leary C, Liao A, Asadi J, Glover E, Ghosh A, Jones S, Wilkinson FL, *et al* (2020) Haematopoietic stem cell gene therapy with IL-1Ra rescues cognitive loss in mucopolysaccharidosis IIIA. *EMBO Mol Med* 12: e11185

Parkin J & Cohen B (2001) An overview of the immune system. *Lancet (London, England)* 357: 1777–1789

Parolin C, Dorfman T, Palú G, Göttlinger H & Sodroski J (1994) Analysis in human immunodeficiency virus type 1 vectors of cis-acting sequences that affect gene transfer into human lymphocytes. *J Virol* 68: 3888–3895

Perregaux D & Gabel CA (1994) Interleukin-1β maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J Biol Chem* 269: 15195–15203

Petrillo C, Thorne LG, Unali G, Schiroli G, Giordano AMS, Piras F, Cuccovillo I, Petit SJ, Ahsan F & Noursadeghi M (2018) Cyclosporine H overcomes innate immune restrictions to improve lentiviral transduction and gene editing in human hematopoietic stem cells. *Cell Stem Cell* 23: 820–832

Pietras EM, Mirantes-Barbeito C, Fong S, Loeffler D, Kovtonyuk L V., Zhang S, Lakshminarasimhan R, Chin CP, Techner JM, Will B, *et al* (2016) Chronic interleukin-1 exposure drives haematopoietic stem cells towards precocious myeloid differentiation at the expense of self-renewal. *Nat Cell Biol* 18: 607–618

Reis BCS, Cunha DP, Bueno APS, Carvalho FAA, Dutra J, Mello F V., Ribeiro MCM, Milito CB, da Costa ES & Vasconcelos Z (2021) Chronic granulomatous disease and myelodysplastic syndrome in a patient with a novel mutation in cybb. *Genes (Basel)* 12

Ren GM, Li J, Zhang XC, Wang Y, Xiao Y, Zhang XY, Liu X, Zhang W, Ma WB, Zhang J, *et al* (2021) Pharmacological targeting of NLRP3 deubiquitination for treatment of NLRP3-associated inflammatory diseases. *Sci Immunol* 6

Reue K (2009) The lipin family: Mutations and metabolism. *Curr Opin Lipidol* 20: 165–170

Rhen T & Cidlowski JA (2005) Antiinflammatory Action of Glucocorticoids — New Mechanisms for Old Drugs. *N Engl J Med* 353: 1711–1723

Río P, Navarro S, Wang W, Sánchez-Domínguez R, Pujol RM, Segovia JC, Bogliolo M, Merino E, Wu N & Salgado R (2019) Successful engraftment of gene-corrected hematopoietic stem cells in non-conditioned patients with Fanconi anemia. *Nat Med* 25: 1396–1401

Riteau N, Baron L, Villeret B, Guillou N, Savigny F, Ryffel B, Rassendren F, Le Bert M, Gombault A & Couillin I (2012) ATP release and purinergic signaling: a common pathway for particle-mediated inflammasome activation. *Cell Death Dis* 3: e403–e403

Robbins GR, Wen H & Ting JP-Y (2014) Inflammasomes and metabolic disorders: old genes in modern diseases. *Mol Cell* 54: 297–308

Romberg N, Al Moussawi K, Nelson-Williams C, Stiegler AL, Loring E, Choi M, Overton J, Meffre E, Khokha MK, Huttner AJ, *et al* (2014) Mutation of NLRC4 causes a syndrome of enterocolitis and autoinflammation. *Nat Genet* 46: 1135–1139

Rossi C, Cusimano M, Zambito M, Finardi A, Capotondo A, Garcia-Manteiga JM, Comi G, Furlan R, Martino G & Muzio L (2018) Interleukin 4 modulates microglia homeostasis and attenuates the early slowly progressive phase of amyotrophic

lateral sclerosis article. *Cell Death Dis* 9

Rossi S, Studer V, Motta C, Germani G, Macchiarulo G, Buttari F, Mancino R, Castelli M, De Chiara V, Weiss S, *et al* (2014) Cerebrospinal fluid detection of interleukin-1β in phase of remission predicts disease progression in multiple sclerosis. *J Neuroinflammation* 11: 1–8

Rowczenio D, Shinar Y, Ceccherini I, Sheils K, Van Gijn M, Patton SJ & Touitou I (2019) Current practices for the genetic diagnosis of autoinflammatory diseases: results of a European Molecular Genetics Quality Network Survey. *Eur J Hum Genet* 27: 1502–1508

Sakuma T, Barry MA & Ikeda Y (2012) Lentiviral vectors: basic to translational. *Biochem J* 443: 603–618

Salmon P, Kindler V, Ducrey O, Chapuis B, Zubler RH & Trono D (2000) High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. *Blood* 96: 3392–3398

Santilli G, Almarza E, Brendel C, Choi U, Beilin C, Blundell MP, Haria S, Parsley KL, Kinnon C, Malech HL, *et al* (2011) Biochemical correction of X-CGD by a novel chimeric promoter regulating high levels of transgene expression in myeloid cells. *Mol Ther* 19: 122–132

Sauer A V, Di Lorenzo B, Carriglio N & Aiuti A (2014) Progress in gene therapy for primary immunodeficiencies using lentiviral vectors. *Curr Opin Allergy Clin Immunol* 14: 527–534

Savill J (1997) Apoptosis in resolution of inflammation. *J Leukoc Biol* 61: 375–380

Schett G, Dayer JM & Manger B (2016) Interleukin-1 function and role in rheumatic disease. *Nat Rev Rheumatol* 12: 14–24

Schinnerling K, Rosas C, Soto L, Thomas R & Aguillón JC (2019) Humanized mouse models of rheumatoid arthritis for studies on immunopathogenesis and preclinical testing of cell-based therapies. *Front Immunol* 10: 1–24

Schmidt RL & Lenz LL (2012) Distinct Licensing of IL-18 and IL-1β Secretion in Response to NLRP3 Inflammasome Activation. *PLoS One* 7: 1–9

Schreuder H, Tardif C, Trump-Kallmeyer S, Soffientini A, Sarubbi E, Akeson A, Bowlin T, Yanofsky S & Barrett RW (1997) A new cytokine-receptor binding mode revealed by the crystal structure of the IL-1 receptor with an antagonist.

Nature 386: 194–200

Schroder K, Muruve DA & Tschopp J (2009) Innate Immunity: Cytoplasmic DNA Sensing by the AIM2 Inflammasome. *Curr Biol* 19: 262–265

Schroder K & Tschopp J (2010) The Inflammasomes. *Cell* 140: 821–832

Seckinger P, Lowenthal JW, Williamson K, Dayer JM & MacDonald HR (1987) A urine inhibitor of interleukin 1 activity that blocks ligand binding. *J Immunol* 139: 1546–1549

Shang K, Wei Y, Su Q, Yu B, Tao Y, He Y, Wang Y, Shi G & Duan L (2019) IL-33 ameliorates the development of MSU-induced inflammation through expanding MDSCs-like cells. *Front Endocrinol (Lausanne)* 10: 1–9

Shi J, Xia Y, Wang H, Yi Z, Zhang R & Zhang X (2022) Piperlongumine Is an NLRP3 Inhibitor With Anti-inflammatory Activity. *Front Pharmacol* 12: 1–10

Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, Zhuang Y, Cai T, Wang F & Shao F (2015) Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* 526: 660–665

Sibley CH, Plass N, Snow J, Wiggs EA, Brewer CC, King KA, Zalewski C, Kim HJ, Bishop R, Hill S, *et al* (2012) Sustained response and prevention of damage progression in patients with neonatal-onset multisystem inflammatory disease treated with anakinra: A cohort study to determine three- and five-year outcomes. *Arthritis Rheum* 64: 2375–2386

Smith BL, Rajendiran TM, Kuffa P & Martı G (2013) K + Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter.

Snouwaert JN, Nguyen MT, Repenning PW, Dye R, Livingston EW, Kovarova M, Moy SS, Brigman BE, Bateman TA, Ting JPY, *et al* (2016) An NLRP3 Mutation Causes Arthropathy and Osteoporosis in Humanized Mice. *Cell Rep* 17: 3077–3088

So AK & Martinon F (2017) Inflammation in gout: mechanisms and therapeutic targets. *Nat Rev Rheumatol* 13: 639–647

Soldi M, Sergi Sergi L, Unali G, Kerzel T, Cuccovillo I, Capasso P, Annoni A, Biffi M, Rancoita PMV, Cantore A, *et al* (2020) Laboratory-Scale Lentiviral Vector Production and Purification for Enhanced Ex Vivo and In Vivo Genetic

Engineering. *Mol Ther - Methods Clin Dev* 19: 411–425

Song N, Liu Z, Xue W, Bai Z, Wang Q, Dai J, Liu X, Huang Y & Cai H (2017) NLRP3 Phosphorylation Is an Essential Priming Event for Inflammasome Activation Article NLRP3 Phosphorylation Is an Essential Priming Event for Inflammasome Activation. *Mol Cell* 68: 185-197.e6

Sönmezgöz E, Özer S, Gül A, Yılmaz R, Kasap T, Takcı Ş, Gümüşer R & Demir O (2019) Clinical and Demographic Evaluation According to MEFV Genes in Patients with Familial Mediterranean Fever. *Biochem Genet* 57: 289–300

Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, Koyasu S, Locksley RM, McKenzie ANJ, Mebius RE, *et al* (2013) Innate lymphoid cells-a proposal for uniform nomenclature. *Nat Rev Immunol* 13: 145–149

Stoffels M, Szperl A, Simon A, Netea MG, Plantinga TS, Van Deuren M, Kamphuis S, Lachmann HJ, Cuppen E, Kloosterman WP, *et al* (2014) MEFV mutations affecting pyrin amino acid 577 cause autosomal dominant autoinflammatory disease. *Ann Rheum Dis* 73: 455–461

Stramer BM, Mori R & Martin P (2007) The inflammation-fibrosis link? A Jekyll and Hyde role for blood cells during wound repair. *J Invest Dermatol* 127: 1009–1017

Styczyński J, Tridello G, Koster L, Iacobelli S, van Biezen A, van der Werf S, Mikulska M, Gil L, Cordonnier C & Ljungman P (2020) Death after hematopoietic stem cell transplantation: changes over calendar year time, infections and associated factors. *Bone Marrow Transplant* 55: 126–136

Sutton C, Brereton C, Keogh B, Mills KHG & Lavelle EC (2006) A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med* 203: 1685–1691

Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC & Mills KHG (2009) Interleukin-1 and IL-23 Induce Innate IL-17 Production from γδ T Cells, Amplifying Th17 Responses and Autoimmunity. *Immunity* 31: 331–341

Svenson M, Hansen MB, Heegaard P, Abell K & Bendtzen K (1993) Specific binding of interleukin 1 (IL-1) beta and IL-1 receptor antagonist (IL-1ra) to human serum. High-affinity binding of IL-1ra to soluble IL-1 receptor type I. *Cytokine* 5: 427–435

Swanson K V., Deng M & Ting JPY (2019) The NLRP3 inflammasome: molecular

activation and regulation to therapeutics. *Nat Rev Immunol* 19: 477–489

Takacs L, Kovacs EJ, Smith MR, Young HA & Durum SK (1988) Detection of IL-1 alpha and IL-1 beta gene expression by in situ hybridization. Tissue localization of IL-1 mRNA in the normal C57BL/6 mouse. *J Immunol* 141: 3081–3095

Takeuchi O & Akira S (2010) Pattern Recognition Receptors and Inflammation. *Cell* 140: 805–820

Tassi S, Carta S, Delfino L, Caorsi R, Martini A, Gattorno M & Rubartelli A (2010) Altered redox state of monocytes from cryopyrin-associated periodic syndromes causes accelerated IL-1β secretion. *Proc Natl Acad Sci U S A* 107: 9789–9794

Tauber AI (2003) Metchnikoff and the phagocytosis theory. *Nat Rev Mol Cell Biol* 4: 897–901

Taylor SL, Renshaw BR, Garka KE, Smith DE & Sims JE (2002) Genomic organization of the interleukin-1 locus. *Genomics* 79: 726–733

Thomas C, Bazan JF & Garcia KC (2012) Structure of the activating IL-1 receptor signaling complex. *Nat Struct Mol Biol* 19: 455–457

Thompson AA, Walters MC, Kwiatkowski J, Rasko JEJ, Ribeil J-A, Hongeng S, Magrin E, Schiller GJ, Payen E & Semeraro M (2018) Gene therapy in patients with transfusion-dependent β-thalassemia. *N Engl J Med* 378: 1479–1493

Thornley I, Nayar R, Freedman MH, Stephens D, Crump M, Messner HA & Sutherland DR (2001) Differences in cell cycle kinetics of candidate engrafting cells in human bone marrow and mobilized peripheral blood. *Exp Hematol* 29: 525–533

Triantafilou K, Hughes TR, Triantafilou M & Morgan BP (2013) The complement membrane attack complex triggers intracellular Ca2+ fluxes leading to NLRP3 inflammasome activation. *J Cell Sci* 126: 2903–2913

Tucci F, Scaramuzza S, Aiuti A & Mortellaro A (2021) Update on Clinical Ex Vivo Hematopoietic Stem Cell Gene Therapy for Inherited Monogenic Diseases. *Mol Ther* 29: 489–504

Viganò E, Diamond CE, Spreafico R, Balachander A, Sobota RM & Mortellaro A (2015) Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes. *Nat Commun* 6: 1–13

Waldner H (2009) The role of innate immune responses in autoimmune disease development. *Autoimmun Rev* 8: 400–404

Wang Y, Cooper R, Bergelson S & Feschenko M (2018) Quantification of residual BHK DNA by a novel droplet digital PCR technology. *J Pharm Biomed Anal* 159: 477–482

Weber A, Wasiliew P & Kracht M (2010) Interleukin-1 (IL-1) Pathway. 3: 1–7

Weber K & Schilling JD (2014) Lysosomes integrate metabolic-inflammatory cross-talk in primary macrophage inflammasome activation. *J Biol Chem* 289: 9158–9171

Wehling P, Reinecke J, Baltzer AWA, Granrath M, Schulitz KP, Schultz C, Krauspe R, Whiteside TW, Elder E, Ghivizzani SC, *et al* (2009) Clinical responses to gene therapy in joints of two subjects with rheumatoid arthritis. *Hum Gene Ther* 20: 97–101

Wei P, Garber ME, Fang SM, Fischer WH & Jones KA (1998) A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* 92: 451–462

Werman A, Werman-Venkert R, White R, Lee JK, Werman B, Krelin Y, Voronov E, Dinarello CA & Apte RN (2004) The precursor form of IL-1α is an intracrine proinflammatory activator of transcription. *Proc Natl Acad Sci U S A* 101: 2434–2439

Williamson LM, Hull D, Mehta R, Reeves WG, Robinson BH & Toghill PJ (1982) Familial Hibernian fever. *Q J Med* 51: 469–480

Xu H, Yang J, Gao W, Li L, Li P, Zhang L, Gong YN, Peng X, Xi JJ, Chen S, *et al* (2014) Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome. *Nature* 513: 237–241

Xue Y, Enosi Tuipulotu D, Tan WH, Kay C & Man SM (2019) Emerging Activators and Regulators of Inflammasomes and Pyroptosis. *Trends Immunol* 40: 1035–1052

Yilmaz E, Ozen S, Balci B, Duzova A, Topaloglu R, Besbas N, Saatci U, Bakkaloglu A & Ozguc M (2001) Mutation frequency of Familial Mediterranean Fever and evidence for a high carrier rate in the Turkish population. *Eur J Hum Genet* 9: 553–555

Zambetti LP, Laudisi F, Licandro G, Ricciardi-Castagnoli P & Mortellaro A (2012) The rhapsody of NLRPs: master players of inflammation...and a lot more. *Immunol Res* 53: 78–90

Zheng C-X, Wang S-M, Bai Y-H, Luo T-T, Wang J-Q, Dai C-Q, Guo B-L, Luo S-C,

Wang D-H, Yang Y-L, *et al* (2018) Lentiviral Vectors and Adeno-Associated Virus Vectors: Useful Tools for Gene Transfer in Pain Research. *Anat Rec (Hoboken)* 301: 825–836

Zhou R, Tardivel A, Thorens B, Choi I & Tschopp J (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 11: 136–140

Zhou R, Yazdi AS, Menu P & Tschopp J (2011) A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469: 221–225

Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, Monticelli S, Lanzavecchia A & Sallusto F (2012) Pathogen-induced human T H17 cells produce IFN-γ or IL-10 and are regulated by IL-1β. *Nature* 484: 514–518

Zonari E, Desantis G, Petrillo C, Boccalatte FE, Lidonnici MR, Kajaste-Rudnitski A, Aiuti A, Ferrari G, Naldini L & Gentner B (2017) Efficient ex vivo engineering and expansion of highly purified human hematopoietic stem and progenitor cell populations for gene therapy. *Stem cell reports* 8: 977–990

Zufferey R, Donello JE, Trono D & Hope TJ (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* 73: 2886–2892

Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L & Trono D (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol* 72: 9873–9880