IL-1R8 acts as an immune checkpoint in CD8\(^+\) T cells

Thesis submitted by

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1. Abstract

IL-1R8 is a member of the Interleukin-1 receptor (ILR) family, acting as a negative regulatory receptor that inhibits inflammation by dampening ILR and Toll-like receptor (TLR) signaling pathways. IL-1R8 deficiency was shown to enhance IL-18-promoted NK cell effector functions restraining tumor metastasis and viral dissemination. IL-1R8 is thus considered a new immunocheckpoint of innate lymphoid cells.

CD8+ T cells represent a potent and target-specific component of anti-tumor immunity, which can be targeted to overcome tumor-associated immune dysfunction in the context of cancer immunotherapy. The aim of this project was to dissect the role played by IL-1R8 in Cytotoxic T lymphocytes (CTLs). Expression profile analysis indicated that IL-1R8 was transcriptionally and epigenetically controlled along immune cell differentiation. In particular, IL-1R8 was upregulated as a consequence of T cell maturation in physiologic and pathologic contexts and its expression was associated with the acquisition of early-effector markers in healthy donors and tumor patients.

We demonstrated that IL-1R8 genetic deficiency promoted CD8+ T cell-mediated protection in immunogenic tumor models and enhanced effector functions in IL-1R8-silenced human CD8+ T cells. Surprisingly, we found that IL-1R8 regulated T cells through the integration of two independent cell-autonomous mechanisms. IL-1R8 deficiency promoted Type-1 responses and
enhanced IL-18-mediated pathway by inducing TBET. In addition, IL-1R8 regulated the IL-2/EOMES/CD25 signaling axis consequently affecting T cell maturation, proliferation and cytokines production in TBET\(^+\) EOMES\(^+\) CD8\(^+\) T cells.

The analysis of IL-1R8 transcription showed a complex mechanism of regulation, including epigenetic modifications, two transcription-starting sites and transcription of truncated isoforms.

Collectively, our data showed that IL-1R8 acts as a checkpoint for mouse and human CD8\(^+\) T cells and its expression is upregulated during the transition from naïve to mature CD8\(^+\) T cells, hence suggesting that IL-1R8 genetic targeting represents a tool to enhance the activity of CD8\(^+\) T cells, including CAR-T cells, in cancer immunotherapy.
2. Introduction

2.1 Interleukin-1 receptor 8 (IL-1R8)

2.1.1 IL-1R family members

The IL-1 receptor family is a phylogenetically conserved group of molecules acting as key players of inflammation and orchestration of the immune response [1-3]. The nomenclature has recently been revised and reported here: IL-1R1 (IL-1RI), IL-1R2 (IL-1RII), IL-1R3 (IL-1RαC), IL-1R4 (ST2), IL-1R5 (IL-18Rα), IL-1R6 (IL-1Rp2, IL-36R), IL-1R7 (IL-18Rβ), IL-1R8 (TIR8 or SIGIRR), IL-1R9 (TIGIRR-2), IL-1R10 (TIGIRR-1) (Figure 1) [1].

![IL-1R and TLRs diagram](image-url)
**Figure 1. ILR and TLR receptor families.** ILR family members (IL-1R1, IL-1R4, IL-1R5, IL-1R6, IL-1R7, IL-1R9 and IL-1R10) and TLRs are characterized by a conserved intracellular TIR domain. ILRs recognize agonist molecules (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ), receptor antagonists (IL-1Ra, IL-36Ra, and IL-38) and anti-inflammatory cytokines (IL-37), while TLRs sense DAMPs and PAMPs. The IL-1 system also includes the accessory protein IL-1R3, the decoy receptor IL-1R2 and the regulatory receptor IL-1R8. Two amino acid replacements in the TIR domain of IL-1R8 are shown in blue (Adapted from Immunol Rev. 2018 Jan;281(1):233-247. doi: 10.1111/imr.12609).

ILR proteins are composed by three domains conserved among ILR family members: 1) an extracellular immunoglobulin (Ig)-like region composed by one to three Ig domains, which determines the ligand specificity, 2) a hydrophobic aminoacidic sequence that anchors the receptor at the cellular membrane, 3) an intracellular Toll-IL-1 resistance (TIR) domain, shared with TLRs [4], essential in recruiting the signaling machinery. Because of the presence of the shared TIR domain which activates a conserved signaling pathway, ILRs and TLRs are structurally and functionally integrated in a common superfamily [5]. Recognition of IL-1 family members (IL-1α and IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ) and Pathogen/Damage Associated Molecular Patterns (PAMPs and DAMPs) leads to activation of a common signaling pathway [2, 5].

Upon the ligand binding, ILRs or TLRs dimerize and assemble their TIR domains in a molecular scaffold that recruits cytoplasmic TIR-containing
adaptor proteins such as MyD88, MAL, TRIF, and TRAM. Then, the “signalosome“ complexes activate downstream protein kinases (e.g. IL-1R associated kinases (IRAKs), and tumor necrosis factor receptor-associated factor 6 (TRAF6)), and in turn Transcription Factors (TFs) linked to cellular activation, such as nuclear factor-κB (NFκB), activator protein-1 (AP-1), c-Jun N-terminal kinase (JNK), p38 mitogen-associated protein kinase, extracellular signal-regulated kinases (ERKs), mitogen-activated protein kinases (MAPKs), and members of the interferon (IFN)-regulatory factor (IRF) [1-3]. The final output is the induction and the orientation of immune responses through regulated expression of inflammatory genes [1-3].

IL-1 system plays a fundamental role in physio-pathological processes and the regulation of inflammation contributes to preserve the homeostatic equilibrium [6, 7]. Indeed, uncontrolled inflammation contributes to the etiology and pathogenesis of several pathologic conditions including cardiovascular diseases, sepsis, graft rejection, neoplastic transformation and tumor progression [3, 7-10]. The IL-1 system comprises intracellular and extracellular regulators, which coevolved with agonist molecules and participate at different levels during ILR- and TLR-mediated signaling pathways.

First, the IL-1 system is finely tuned by the regulatory receptors IL-1R2 and IL-1R8. IL-1R2 dampens IL-1-mediated signaling, acts as dominant negative
receptor and as decoy IL-1 scavenger molecule both in full length and its soluble form [11, 12]. Moreover, an intracellular form of IL-1R2 prevents the enzymatic cleavage of pro-IL-1α [13]. In parallel, the negative regulatory receptor IL-1R8, described in detail in the next chapters, interferes with Myddosome assembly [14]. In addition, IL-1R8 acts as co-receptor of IL-1R5/IL-18Rα that recognizes the anti-inflammatory molecules IL-37 [15-18]. Then, the receptor antagonist IL-1Rα, IL-36Rα and IL-38 inhibit IL-1R1- and IL-36R-mediated pathways by competing with the agonist cytokines for the engagement of their receptors [19, 20]. Finally, non-coding RNA are involved in tuning the IL-1 system, in particular miRNAs (e.g. miR-155, miR-21, miR-146a, miR-132, miR-9 and miR-147) that post-transcriptionally regulate signaling proteins or TFs associated with ILR pathways [21-26].

However, cellular mechanisms that involve TIR containing adaptor proteins are not fully elucidated. Recently, the mammalian adaptor protein SARM1 (for long time considered a pro-inflammatory adaptor protein) was described as a negative regulator of TRIF-mediated pathways [27, 28]. In addition, the bacterial TIR containing protein NADases, as well as SARM1, were shown to oligomerize thought a self-assembly process to generate a NAD⁺-cleaving enzyme. The capacity of TIR domains in hydrolyzing the NAD⁺ to ADP-ribose/cyclic ADP-ribose was indicated as a fundamental step in cell death
initiation, opening a new scenario about unconventional roles displayed by ILR and TIR containing adaptor proteins [27, 28].

2.1.2 IL-1R8: expression, protein structure and downstream pathways

*IL-1R8*, also indicated as *SIGIRR* or *TIR8*, is a well conserved gene located in chromosomes 11 and 7 in human and mouse, respectively [29]. The human orthologue is characterized by a long 5’ Untranslated Region (5’ UTR) and three main isoforms, which code a 410 aminoacid long common protein. IL-1R8 is ubiquitously expressed in mammalian cells, in particular in epithelia of the liver, kidney, lung and digestive tract [30, 31]. IL-1R8 is also expressed in lymphoid organs and leukocytes [30-32].

IL-1R8 possesses a high identity level across species (for instance, 82% comparing human and mouse) and among ILRs family members [31]. However, the IL-1R8 extracellular region contains a single extracellular Ig domain and the intracellular domain has a longer aminoacidic tail. Furthermore, the TIR domain is characterized by two amino acid substitutions (Cys222 and Leu305) replacing the polar residues Ser447 and Tyr536, which are phosphorylated in ILRs during TIR-mediated signaling [33].
These peculiar structural features possibly explain the IL-1R8 regulatory activity on several ILRs (IL-1R1, IL-1R5/IL-18Rα and IL-1R4/ST2) and TLRs (TLR1/2, TLR3, TLR7, and TLR9) [12, 33-38]. In fact, two main mechanism of action have been proposed for the regulatory role of IL-1R8. First, the IL-1R8 extracellular domain may interfere with the dimerization of receptors such as IL-1R3/IL-1RαC with IL-1R1 or ILR4/ST2 [34]. Then, the TIR domain was demonstrated to inhibit the Myddosome and TRAM complex formation preventing the activation of downstream kinases IRAK and TRAF6 [14, 38]. In addition, IL-1R8 was also shown to inhibit JNK and mTOR activation in intestinal epithelial cells and in immune cells such as NK and Th17 T cells (Figure 2) [39].

As mentioned in the overview about ILRs, IL-1R8 is the co-receptor with IL-1R5/IL-18Rα for IL-37 [15, 16, 18]. IL-37 acts an anti-inflammatory cytokine that restrains TLR- and ILR-mediated pathways in epithelial cells, myeloid cell lines (THP-1) and primary immune cells, such as peripheral blood mononuclear cells (PBMCs) and bone marrow derived macrophages (BMDMs) [7, 15, 40]. Taking advantage of IL-37-transgenic mice and IL-37 recombinant protein, it was shown that IL-1R8 deficiency abolished the IL-37 anti-inflammatory properties in non-resolving Aspergillus infection and in OVA-induced asthma [16, 41]. Moreover, the IL-37/IL-1R8 axis was demonstrated to sustain cell metabolism and enhance oxidative
phosphorylation in mitochondria, counteracting the inflammation-induced fatigue [42, 43].

Post-translational modifications also regulate IL-1R8 protein. Indeed, IL-1R8 functions were shown to be impaired as a result of N-glycosylation defects [44].
Figure 2. Dampening activity of IL-1R8. IL-1R8 inhibits ILR- and TLR-induced signaling pathways by interfering with receptor complex formation or by competing with adaptor protein recruitment (e.g. MyD88). In addition, IL-1R8 is a coreceptor chain for the anti-inflammatory cytokine IL-37. By dampening mTOR, JNK and NFkB activation, IL-1R8 affects cell proliferation, survival and inflammation (Adapted from Immunol Rev. 2018 Jan;281(1):233-247. doi: 10.1111/imr.12609).

2.1.3 IL-1R8 in infections

The immune response is governed by a complex network of pathways, co-evolves with pathogens, and allows the survival in a not sterile environment [45]. TLR and ILR gene conservation is a direct effect of their biological relevance in governing the host defense from invertebrates to mammals [1, 5]. Since IL-1R8 was demonstrated to dampen ILR- and TLR-mediated pathways, its role in infections has been largely characterized.

For instance, IL-1R8-deficient mice were shown to be protected in a uropathogenic Escherichia coli infection model due to enhanced TNFα and chemokine production (e.g. CXCL1, CCL2, and CCL3) in tubular epithelial cells [46]. Indeed, the chemoattractant micro-environment led to enhanced recruitment of leukocytes that in turn controlled the bacteria outgrowth and spreading to kidney lobes [46]. Similar observations emerged in the Streptococcus pneumoniae infection model, where local perivascular inflammation accelerated the early phase response against pneumonia [47].
However, unresolved inflammation impairs key processes required for the resolution phase, such as the tissue repair [48, 49]. In line with multifaceted roles of inflammatory reactions, IL-1R8-deficient mice showed higher mortality rate compared to wild-type mice in *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* infection models [50-52]. The susceptibility of *Il1r8<sup>−/−</sup>* mice was linked to the severity of local tissue damage dependent on neutrophil and macrophage infiltration in the lung [50]. The deubiquitinating enzyme (DUB) USP13 was recently demonstrated to inhibit inflammation by stabilizing IL-1R8 protein [53]. USP13 deficiency increased IL-1R8 poly-ubiquitination/degradation and consequently sensitized mice to *Pseudomonas aeruginosa* infections. Notably, IL-1R8 overexpression rescued the phenotypes in USP13-deficient mice and in transfected cell lines [53]. *Il1r8<sup>−/−</sup>* mice were also susceptible to murine *Citrobacter rodentium* infection model, which mimics the immune response against human enteric pathogens such as enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella typhimurium* [54]. IL-1R8 deficiency promoted an excessive anti-microbial response and exacerbated the ILR1-MyD88-mediated pathway in intestinal epithelial cells, hence depleting resident microbiota in favor of enteric pathogen colonization [54]. The consequence of excessive inflammation was also observed in fungal infections by *Candida albicans* or *Aspergillus fumigatus*, in which IL-1R8 deficiency favored fungal burden on mucosa,
contributed to Th17 polarization and dampened Treg activity [55]. Further proofs emerged by silencing the human gene in a bladder epithelial cell line (BECs), which sustained IL-6 and IL-8 production and JNK, p38 and ERK1/2 activation. In line with the evidences provided in Il1r8−/− mice, 3 human SNPs in SIGIRR gene region (rs10902158, rs7105848, rs7111432) have been associated with clinical risk of developing pulmonary tuberculosis and tuberculous meningitis [56].

IL-1R8 receptor has been observed on platelet and megakaryocyte surface where it was shown to inhibit LPS, IL-1β and IL-18 signaling. In particular, IL-1R8 deficiency was shown to promote platelet hyper activation thus increasing platelet/neutrophil aggregation and thromboembolism [57]. In addition, the stimulation with LPS was demonstrated to induce IL-1R8 downregulation by enhancing its shedding in Microparticles (MPs). Consistently with experimental data, IL-1R8 was downregulated in platelets from septic patients and the enrichment of IL-1R8-containing MPs was observed in patient sera [57].

Fine tuning of ILR and TLR system further emerges as a crucial player of Central Nervous System (CNS) development [58-60]. IL-1R8 was demonstrated to be expressed in CNS cell types such as neurons, astrocytes and microglial cells where it restrained LPS-induced neuroinflammation [61, 62]. However, cognitive defects were reported also in absence of exogenous
stimulations emphasizing how ILRs influenced the CNS [58]. In fact, IL-1R1 constitutive activation was demonstrated to induce the epigenetic regulator methyl-CpG-binding protein (MeCP2), hence altering synaptic connectivity in hippocampus [63]. Since IL-1β was shown to influence synaptic maturation, treatment with the recombinant IL-1 receptor antagonist Anakinra/Kineret was proposed as a therapeutic strategy to treat specific neurological disorders [6].

2.1.4 IL-1R8 in sterile inflammation

Exacerbated immune responses can lead to tissue deteriorations also in acute tissue damage such as ischemia or surgery [64]. It was demonstrated that IL-1R8 affects sterile inflammation by dampening TLR mediated pathways, thus regulating the immune response evoked in response to DAMPs.

For instance, IL-1R8 inhibited myeloid cell activation in post ischemic acute renal failure reducing the release of pro-inflammatory cytokines and chemokines [65]. In parallel, IL-1R8 deficiency contributed to allorejection in a fully mismatched kidney allotransplantation model; here the absence of IL-1R8 enhanced neutrophil and macrophage migration and DC proliferation, leading to a severe alloreactive immune response [65-67].
IL-1R8 is abundantly expressed also in Intestinal Epithelial Cells (IEC) that are specialized in cross-talking with the commensal microbiota [54]. To assess the role played by IL-1R8 in colitis, the fragile equilibrium between tolerance and inflammation was broken treating mice with dextran sodium sulfate (DSS), which damages the epithelial barrier [35]. In particular, Il1r8−/− mice showed increased susceptibility to acute and chronic colitis models because of IEC over-reactivity, which in turn favored leukocyte infiltration and secretion of pro-inflammatory cytokines (IL-1β, IL-6, TNFα, IL-12p40 and IL-17) and chemokines (CXCL1, CCL2). IL-1R8-deficiency exacerbated the inflammation and it was associated with reduced survival rate and increased weight loss due to the extensive bleeding and tissue damage in the gut [35].

2.1.5 IL-1R8 in autoimmune diseases

In autoimmune disorders and allergies, the immune response is chronically unbalanced and the homeostatic disequilibrium progressively impacts on quality of life. Autoimmune disorders include all pathological conditions determined by immune responses directed against self-antigens. ILR deregulation was demonstrated to contribute to autoimmune pathogenesis.

For instance, IL-1β and IL-18 were described as key pro-inflammatory molecules involved in autoimmune disorders (e.g. inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis and psoriasis) by orchestrating
Th1/Th17 polarization [10, 68]. In agreement, IL-1R8 genetic deficiency was shown to increase the severity of experimental autoimmune encephalomyelitis (EAE) and rheumatoid arthritis in Th17-dependent manner [39, 69, 70]. Il1r8−/− mice showed an increased Th17 cell infiltration in response to IL-1β in inflamed tissues thus leading to the activation of JNK, mTOR and 4EBP1, three signaling molecules involved in immune cell responses. Moreover, the treatment with IL-1Ra reduced the aggressiveness of zymosan-induced arthritis in Il1r8−/− mice, highlighting the pathological relevance of IL-1β. Similar observations were reported by characterizing the immune cells in Aldara and rIL23-psoriasis models, in which γδ+ T cells were shown to mainly contribute to the pathogenesis [68].

In agreement with previous observations, IL-1R8 deficiency was demonstrated to fuel autoimmune disorders dependent on DAMPs. An elegant example was provided by characterizing C57BL/61pr/lpr mice that harbors lymphoproliferation spontaneous mutation Faslpr. The defect in Fas-dependent apoptosis is a sufficient genetic condition to drive a dramatic lymphoproliferative disorder and massive autoantibody production, phenomena that resemble systemic lupus erythematosus (SLE) [71]. IL-1R8-deficient C57BL/61pr/lpr mice suffered an aggravation of general conditions with severe hypergammaglobulinemia, nephritis and lung inflammation [72]. In particular, DCs and autoreactive B cells were shown to internalize immune
complexes through endocytosis, a mechanism that was demonstrated to carry nucleosomes and autoantigenic ribonucleoproteins to TLR7 and TLR9 [73, 74]. In support of experimental evidences, the IL-1R8 SNP rs7396562 was shown to correlate to SLE onset in human [75].

TLR deregulation was also associated with Alzheimer’s disease (AD), a neurodegenerative pathology determined by the accumulation of β-amyloid (Aβ) neuritic plaques [76, 77]. Indeed, Aβ immunogenic peptides were demonstrated to exacerbate neuroinflammation by engaging TLR2 and by downregulating IL-1R8. In AD, treatment with anti-TLR2 antibody (αTLR2) sustained IL-1R8 expression in microglial cells, ameliorating neurological parameters such as the long-term potential (LTP) that is an electrophysiological measurement of synaptic connectivity [78]. Moreover, IL-1R8 was reported to control neuroinflammation in consequence of the release of DAMPs such as High Mobility Group Box 1 (HMGB1) [79].

In antithesis to other ILR family members, the IL-33 receptor complex IL-1R4/ST2 is a critical regulator of Th2 humoral-mediated immunity and governs type 2 cytokine productions (e.g. IL-4, IL-5 and IL-13) [7, 34, 80-82]. According to the hygiene hypothesis, the reduced exposure to parasites imbalances the human immune system toward a Th2 profile, so predisposing highly industrialized communities to asthma and allergies [83]. IL-1R8 deficiency was shown to increase the susceptibility to Ovalbumin (OVA)-
induced allergic asthma associated with immune cell infiltration in the lung. In particular, \( IIr8^{-/-} \) mice displayed high levels of anti-OVA IgE and Th2 cytokines by enhancing IL-1R4/ST2-mediated signaling pathway [81].

2.1.7 IL-1R8 in cancer-related inflammation

Tumor development is determined by a “bio-logical” step series that drives malignant transformation [84]. Chronic inflammation is recognized as a tumor promoting phenomenon and one of the hallmarks of cancer [85]. In cancer, the immune reaction is influenced to favor the neoplastic evolution through cellular intrinsic pathways (e.g. genetic instability, pro-mitogenic signaling) or extrinsic mechanisms (e.g. inflammation-associated angiogenesis, immune cell exhaustion) [84-88].

\( IIr8^{-/-} \) mice were shown to be susceptible to a variety of chemically-induced malignancies and genetic tumor models as a consequence of exacerbated cancer-related inflammation. For instance, IL-1R8 deficiency increased the number and the size of tumor lesions in a colitis-associated cancer (CAC) model that consists in the administration of the carcinogen Azoxymethane (AOM) and of DSS which induces epithelial damage and inflammation [89]. IL-1R8 tuned the NF-kB signaling axis in colonic epithelium cells of AOM-DSS treated mice, hence enhancing cellular proliferation and survival [89]. Gut inflammation was also associated with dysbiosis, possibly due to the
altered gut permeability and defective crosstalk among commensal bacteria [90]. Moreover, IL-1R8 was shown to regulate chemokine production and consequently the migration of immune-suppressive cells such as Th2 and T regulatory (Treg) cells, as indicated by high levels of IL-10 and TGF-β in tumor lesions [35].

Taking advantages of the Apc^{min/+} genetic background, IL-1R8 deficiency was demonstrated to promote cell hyper-activation thus leading to genome instability and inefficient DNA-repair [91, 92]. Since the APC gene is a potent onco-suppressor, Apc^{min/+} mice spontaneously develop aggressive tumors when the single APC allele is lost [93]. IL-1R8 deficiency was demonstrated to accelerate the loss of heterozygosity of APC in Apc^{min/+} mice by enhancing the Akt/mTOR, pathway, a cellular signal that promoted uncontrolled colon crypt cell proliferation [93]. Intriguingly, colon carcinogenesis in humans was associated to an IL-1R8 dominant negative isoform (IL-1R8Δ8) generated as result of the exon skipping in the transcript [44]. Indeed, IL-1R8Δ8 gained the property of altering IL-1R8 function and glycosylation by trapping the full-length protein in the cytoplasm. In support of these evidences, transgenic mice that express the dominant negative isoform IL-1R8^{N85/S101S} manifested high susceptibility to CAC models [44].

IL-1R8 deficiency was also described as a tumor-promoting factor by contributing to B cell leukemogenesis and lymphomagenesis [94-98]. T-cell
leukemia/lymphoma 1 (TCL1) transgenic mice overexpress the TCL1 oncogene, phenocopying human chronic lymphocytic leukemia (CLL) progression [95]. IL-1R8 deficiency accelerated monoclonal B cell expansion and was linked to prognosis aggravation in TCL1-tg mice [95, 96]. In line with the genetically driven model, we recently demonstrated that IL-1R8 inhibited autoimmune-driven lymphomagenesis in aged C57BL/6^{lpr/lpr} mice [99]. It was shown that C57BL/6^{lpr/lpr} mice spontaneously developed Diffuse Large B Cell Lymphoma (DLBCL), a rare event promoted by ageing and autoimmune conditions. We observed that IL-1R8 genetic deficiency prompted NF-κB constitutive activation in B cells and, consequently, increased DLBCL incidence in C57BL/6^{lpr/lpr} mice. Moreover, we also reported that IL-1R8 downregulation occurred during human B cell neoplastic transformation which represented a poor prognostic marker in DLBCL patients [99].

In antithesis to colon cancer and hematological malignancies, IL-1R8 was described as a tumor-promoting molecule in MMTV-neu breast cancer model [100]. In this genetic model, IL-1R8 was shown to dampen the IL-1-downstream pathway and to reduce pro-inflammatory cytokine production in breast epithelial cells, hence favoring tumor evasion [100]. In agreement with these observations, IL-1R8 deficiency sustained NK and CD8\(^+\) T cell infiltration in primary lesions, favored M1 myeloid polarization and protected mice from tumor dissemination to the lung. Similar evidences emerged in
human clinical samples, in which IL-1R8 expression correlated with low expression of pro-inflammatory cytokines and poor immune cell activation [100].

2.1.8 IL-1R8 as checkpoint molecule in NK cells

Uncontrolled inflammation was described as a tumor-promoting factor. However, mechanisms involved in triggering inflammation are also responsible for sustaining immune responses against tumors and pathogens. This ambivalent relation between “bad” and “good” inflammation emerges in the context of IL-1R8 deficiency, which exacerbates cancer-related inflammation (as described above) but also awakes immune cell responses [101].

NK cells are an innate cytotoxic population mainly involved in controlling viral infections, hematological malignancies and hematogenous tumor dissemination [102-105]. IL-1R8 was demonstrated to be highly expressed in mature NK cells both in human and mouse [32]. IL-1R8 deficiency was shown to promote NK cell maturation as observed by assaying CD27, CD11b and KLRG1 expression. Moreover, IL-1R8-deficient NK cells expressed higher levels of Interferon-γ (IFNγ), Granzyme B (GZMB), and Fas ligand compared to IL-1R8-proficient NK cells [32]. Indeed, IL-1R8 was shown to tune IL-18-mediated signaling pathway through a cell-autonomous mechanism by
interacting with the IL-18R complex, as observed by stimulated emission depletion (STED) microscopy. Consequently, IL-1R8 was demonstrated to affect IL-18 downstream targets such as mTOR and JNK that are involved in NK cell activation [32]. In support of these evidences, IL-1R8-deficient mice were shown to be more resistant in a variety of cancers and viral experimental models such as diethylnitrosamine (DEN)-induced hepatocellular carcinoma, sarcoma-derived lung metastasis, colorectal-cancer-derived liver metastasis and murine cytomegalovirus (MCMV) infection [32]. Increased levels of anti-tumor cytokines (e.g. IFNγ) and reduced levels of pro-tumoral cytokines (e.g. IL-6, TNF, IL-1β, CCL2, CXCL1) were observed in Il1r8−/− mice. When IL-1R8-deficient mice were treated with anti-NK1.1 antibody, a strong reduction of anti-tumor and anti-viral resistance was observed, indicating that the phenotype was NK cell-dependent. In addition, NK cell over-activation was confirmed through Adoptive Cell Transfer (ACT) experiments. Finally, genetic silencing of IL-1R8 suggested that this receptor may represent a new targetable immune checkpoint in human NK cells [32]. Intriguingly, IL-37 secreting T regulatory cells (Treg) were shown to inhibit NK cells by affecting the expression of immune checkpoints such as TIM-3, PD-1. In addition, IL-37 induced the expression of IL-1R8 (that is part of the tripartite ligand-receptor complex IL-37–IL-1R8–IL-18Rα) thus suggesting a new mechanism involved in Treg-mediated suppression of NK cells [106].
Figure 3. IL-1R8 deficiency and its biological role in pathology. IL-1R8 genetic deficiency promotes inflammation and immune cell activation. IL-1R8-deficiency fosters an uncontrolled immune reaction thus favoring autoimmune disorders, tissue damages and the process of cancer-related inflammation. However, IL-1R8-deficiency also triggers NK cell anti-tumor and anti-viral activity and enhances anti-microbial response.

2.2 CD8$^+$ T cells
2.2.1 The origin of the adaptive immune system (AIS)

The AIS is a complex cellular system mainly characterized by three fundamental family molecules: 1) a pattern of clonotypic B and T cell receptors (BCR or TCR) generated through somatic rearrangement, 2) the recombination-activating genes RAG1 and RAG2, which are essential to edit the TCR loci, 3) the Major Histocompatibility Complexes I and II (MHC-I/II), unique to each individual [107-109].

The origin of adaptive immunity was initially explained as a late evolutionary step prompted by increased pathogen complexity [108]. On the contrary, phylogenetic studies demonstrated that divergences between B- and T-like lymphoid cells appeared thousands of years before the lymphoid and myeloid cell bifurcation. In fact, Gnathostome already possessed MHC-II-expressing Antigen Presenting Cells (APCs) and a well-defined splenic and thymic anatomical architecture, divided in medullary and cortical area. Primary lymphoid organs are functionally mature in Gnathostome and express the autoimmune regulator AIRE, a key gene involved in T cell negative selection [108, 110, 111]. In addition, conventional αβ+ T cell receptors and MHC-II arose in ancient vertebrates that still lacked NK receptors (NKRs), γδ+ TCR and nonclassical MHC complexes [108, 109].

So immune adaptation could be explained as the convergence of stochastic genomic phenomena, ecosystem transformations and an increased interaction
with commensal microorganisms. First, the identification of Recombination Signal Sequences (RSSs) in transposons possibly explained TCR and MHC loci frailty. In fact, it was speculated that an ancient genetic event, the invasion of RAG transposons, included RSS sequences recognizable by recombinase RAG [107, 112]. Then, whole-genome duplications in the vertebrate ancestor expanded MHC gene family in a variety of paralogous groups, hence providing new polymorphic sequences [113, 114]. Finally, it was proposed that the saturation of ecosystems reduced the offspring numbers and forced the ancient vertebrates in new complex feeding strategies, so exposing the immune system to a new microbiota [114].

2.2.2 T cell ontogenesis

In the 60’ humoral immunity and passive immune transfers were largely documented, while the existence of T lymphocytes and their biological functions were unexplored. Evidences about T helper (Th) cells emerged in 1968, when Miller and Mitchell demonstrated that the humoral response was augmented in immunized mice by co-transferring B cells and thymic cells, then indicated as antigen-reactive cells [115-117]. In the same years, it was reported that lymphoid cells were involved in graft rejection and directly killed not-self and tumor cells. Moreover, it was discovered that Ly-2 (CD8α) and Ly-3 (CD8β) depletion abrogated cellular-mediated cytotoxicity, suggesting a molecular marker of the phenotypic identification [115, 116, 118]. These three
evidences opened the dam around the T cell fields, and in few decades concepts as MHC restriction and clonal selection became familiar to immunologists.

As introduced in the incipit, the thymus was early indicated as a primary lymphoid organ. In all mammals, hematopoietic stem cells (HSCs) from the bone marrow colonize the thymus cortex, where stroma cells instruct HSC commitment to Thymocytes/Early T Cell Progenitors (ETPs) [119, 120]. Surprisingly, ETPs lack long-term self-renewal potential, so it was hypothesized that mobilization and homing from the bone marrow to thymus persist, at least in part, in adults [121]. The biological relevance of thymus along ETP ontogenesis is demonstrated by severe lymphodeficiency reported in thymectomized animals and in GEM mice harboring the nude mutation, a genetic condition that impairs cortical thymus development [122, 123]. In line with observations highlighted in animal models, the deletion in 22q11.2 loci is associated with a multi-organ disease, termed DiGeorge syndrome (OMIM # 188400), in which a dysfunctional thymus affects AIS maturation, thus sensitizing children to infections.

CD4 and CD8 T cell co-receptors are absent in first developmental steps, so thymocytes are named Double Negative (DN) T cells [119, 120]. These undergo 4 maturation stages distinguished by progressive TCR rearrangement and expression of surface markers such as CD44 (an adhesion molecule) and
CD25 (Interleukin-2 receptor α chain) [124]. Along T cell differentiation, TCR sequences further define the αβ⁺ T or γδ⁺ T cell commitment, which represent respectively the 95% and 5% of successful recombination [125]. Taking advantage of single-cell RNA sequencing, it was recently reported that TCR stimulation dictated γδ⁺ T cell development, which was shown to be favored by a strong TCR avidity [125].

In the DN3 phase, RAG proteins mediate TCR-β chain rearrangement and give raise to the pre-TCR complex constituted by the β chain, the pre-Tα chain and CD3. In turn, pre-TCR engagement unleashes a continuum of processes collectively indicated as β-selection, the first developmental step that confers clonality among T cell populations [107, 126, 127]. In summary, TCR signaling induces thymocyte proliferation, inactivates RAG proteins, prevents aberrant β chain rearrangement and regulates the simultaneous CD4 and CD8 expression, leading to Double Positive (DP) cell expansion [119, 126-129].

At this stage, DP cells migrate to the subcapsular region where they expose a conventional TCR complex constituted by α/β chains, the invariant CD3 receptor and ζ-chain accessory molecule. Here, cortical thymic epithelial cells (cTECs) cross-present autologous peptides by expressing high levels of MHC-I and MHC-II complexes [130, 131]. The TCR interaction with peptide–MHC (pMHC) ligandome triggers the antigenic-dependent survival of T cells (positive selection), while all precursors that do not receive a tonic stimulation
by autologous complexes undergo apoptosis [119, 126, 132]. In summary, the transition from DN-DP T cells is regulated through the thymic central selection, in which the somatic TCR rearrangement is required to orchestrate pro-mitogenic signaling in thymocytes [126, 133].

Since the vast majority of T cell clonotypes show autoreactivity, the AIS has also evolved a negative selection mechanism, or central tolerance, that eliminates 90% of DP cells [126]. Medullary thymic epithelial cells (mTECs) express AIRE, a gene that codifies a TF that induces the expression of tissue-restricted antigens [110, 111, 134, 135]. Consequently, autoimmune regulator proteins AIRE play a fundamental role in clonotypic selection covering ubiquitous self-antigens [110]. Therefore, AIRE loss of function mutations causes autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)(OMIM # 240300), a monogenic syndrome associated with hypothyroidism, hypogonadism, diabetes, adrenal insufficiency and enhanced susceptibility to yeast infections [111]. MHC-expressing mTECs and medullary APCs, such as macrophages and dendritic cells (DCs), cross-present the entire plethora of peripheral tissue-specific antigens expressed by AIRE [130]. The negative selection further determines the mutual exclusion of CD4 and CD8 molecules; TCR-pMHC-II interaction preferentially selects CD4+ T cells, while MHC-I favors CD8+ T cell selection [126, 128, 133]. Criteria that establish an insufficient avidity or, at the contrary, an excessive
avidity still remain elusive. TCR was shown to determine clonal selection both in consequence of low affinity interaction (“death by neglect”) and upon an excessive tonic stimulation (clonal deletion) [136].

Although the central selection has been observed in the majority of conventional T cells, same exceptions were reported in tolerogenic and immune suppressive CD4+ T regulatory cells (Tregs) that benefit from the high affinity of TCR-pMHC interaction [137]. Epithelial cells of Hassall's corpuscles were shown to polarize MHC-II-expressing DCs by secreting thymic stromal lymphopoietin (TSLP) in thymus [138]. In turn, DCs expressed high affinity pMHC-II and co-stimulatory molecules such as CD80 and CD86 thus leading to positive selection of Tregs [138]. This process contributes to “non deletional ” tolerance consisting of mechanisms that sustain the survival of the tolerogenic population [139, 140].

2.2.3 T cell homeostasis and peripheral tolerance

Naive T cells are essentially long-lived quiescent cells characterized by a nucleus full of heterochromatin and unarmed until the encountering with a
clone-specific antigen [141-143]. Elimination of autoreactive T cell clones is not restricted to the central tolerance, but it also occurs in peripheral tolerance by DCs hosted in secondary lymphoid organs (SLOs) [140, 144]. T cells that successfully overcome the negative selection still require a periodic TCR stimulation to survive [132, 141]. Naïve T cells express specific adhesion molecules such as CD62L, a selectin that favors the migration to SLOs [142, 143]. Here, it was reported that lymphoid DCs mediated the tonic stimulation through pMHCs homologous to thymic MHCs [132]. On the contrary, pMHCs not encountered in the thymus or an excessive TCR-pMHCs avidity determines Activation-Induced Cell Death (AIC) or T cell anergy [145, 146]. Anergy is a functional refractory status, unresolvable, in which T cells lose the effector function potential and became unable to produce interleukin-2 (IL-2) [146, 147], the main cytokine involved in T cell maturation and proliferation [148-150]. Naïve T cell survival is also supported by interleukin-7 (IL-7), as demonstrated by IL-7 blocking antibodies or adoptive cell transfer of naïve T cells in IL-7 deficient mice [151]. Fibroblastic reticular cells (FRCs) were shown to release IL-7 in the T zone, a lymph node compartment dedicated to T cell trafficking [152-154]. Moreover, FRCs and DCs secreted the CC-chemokine ligands 19 and 21 (respectively CCL19 and CCL21), both recognized by the chemokine receptor CCR7 expressed by naïve T cells [155, 156]. Other important sources of chemoattractant were shown to be High
Endothelial Venules (HEVs) in the lymph nodes and in Peyer patches [156, 157].

The T cell fate is also determined by APC polarization. In absence of a pro-inflammatory stimuli, resting APCs sustain T cell dormancy by lacking co-stimulatory molecules such as CD40, LICOS, CD80 and CD86. On the contrary, the TCR-pMHCs interaction combined with co-stimulation promotes T cell priming [158-160]. However, T cell maturation and expansion is not a stereotypical process and qualitative aspects of adaptive response were shown to be influenced by post-thymic maturation [161]. When primed, CD8+ T cells differentiate to a CD127- KLRG1+ terminal effector phenotype or to a CD127+ KLRG1- memory-like phenotype, a cellular subset that mainly contributes to long-term protection [162-164]. However, Recent Thymic Emigrant (RTE) CD8+ T cells, abundant in newborns, were preferentially oriented to terminal effector profile thus enhancing the short-term-protection [162].

2.2.4 T cell priming

T cell priming consists of selective clonotype expansion, which contributes to primary immune responses and establishes the immunological memory
against pre-encountered antigens [165, 166]. For decades CD4+ T cells have been mainly described as pivotal participants of adaptive humoral responses against bacteria, whereas CD8+ T, vice versa, as a prototypical adaptive population involved in controlling viral infections [167, 168]. Despite the oversimplification, this scheme summarizes the key events upstream adaptive immune orientation. Upon pathogen encounters, the innate immune system successfully controls infections through anti-bacterial and anti-viral molecules (defensins, collectins, lysozyme, type I interferons), the complement system, soluble factors (pentaxins and antibodies) and innate cells such as Neutrophils, NK cells and Macrophages [101, 168]. In consequence, a high number of antigens are collected by APCs. Peptides bound to MHC-I are generated through cytosolic pathways [169, 170], shared between immune and not immune cells, whereas peptides exposed through MHC-II are processed through APC endocytic pathways [167, 169, 171]. Since APCs possess an enormous heterogeneity, selective tissue-resident and circulating populations were shown to be highly specialized in T cell priming orchestration [165, 172]. For instance, it was demonstrated that antigen presentation in skin is a double-step process in which dermal DCs, but not the Langerhans cells, transport antigens to lymph node-resident CD8+ DCs [172]. APC activation is also regulated by immune complexes (ICs), namely antigens bound to antibodies. Indeed, it was observed that draining lymph nodes collect circulating ICs that interact with Fcγ receptors (FcγRs) on tissue resident CCR7+ DCs [173].
addition, it was shown that activated monocytes migrate to SLOs, where they differentiate to CCR2-expressing DCs. Here, CCR2+ DCs release high levels of interleukin-12p70 (IL-12), a crucial molecule involved in Th1 polarization and Type-1 response, as confirmed by dramatic reduction of IFNγ-secreting T cells in CCR2-deficient mice [165]. Taking advantage of intravital microscopy, it was described that multiple T cells form a stable cellular cluster with a single neighbor DC in proximity of intranodal HEVs [174]. Here activated T cells self-sustain oligoclonal proliferation through a positive feedback loop by releasing IL-2 and upregulating CD25 [174]. The interaction between antigen-pulsed DCs and viral antigen specific TCR naïve T cells was further dissected. By two-photon laser scanning microscopy (TPLSM) analysis, it was confirmed that emigrant T cells require the TCR-pMHC interaction to constitute a stable immunological synapse [175].

2.2.5 Co-stimulatory receptors and immune checkpoints

TCR engagement is a necessary but not sufficient condition to allow T cell functionalization. Indeed, T cells are finely tuned by stimulatory and inhibitory signals that govern cellular activity [176, 177]. Among co-
stimulatory mechanisms, the reciprocal interaction between T cell co-receptor CD28 and CD80/CD86 (named also B7.1/B7.2) expressed by polarized APCs was largely characterized [177, 178]. Since the CD28-CD80/CD86 crosstalk emerged as a fundamental step in T cell priming, anti-CD3/CD28 antibodies are adopted in all current protocols that mimic clonal expansion *in vitro*. In addition, other co-stimulatory molecules have been identified and are here schematized in two main clusters, the Immunoglobulin Superfamily (IgSF) receptors and TNF receptor superfamily (TNFRSF) [176].

<table>
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<tr>
<th>IgSF</th>
<th>T cell receptor</th>
<th>Ligand or interactor</th>
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<tbody>
<tr>
<td>CD28</td>
<td>CD80/CD86</td>
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<td>CRTAM</td>
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<tr>
<th>TNFRSF</th>
<th>T cell receptor</th>
<th>Ligand or interactor</th>
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<td>CD40</td>
<td>CD40L</td>
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<td>CD137/4-1BB</td>
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<td>CD27</td>
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As a consequence of TCR signal amplification, experienced T cells undergo to a deep molecular remodeling that includes the expression of surface markers, effector function molecules and TFs [141, 179-181]. Primed T cells downregulate CCR7 and CD62L, which are required for lymph nodes homing, and upregulate the adhesion glycoprotein CD44 that anchors T cell to Fibronectin, Hyaluronic Acid and Osteopontin [179, 180]. The distribution of these markers is largely considered a strategy to dissect T cell maturation from naïve to central memory ($T_{CM}$) and effector memory ($T_{EM}$) cells by FACS.
analysis. $T_{CM}$ are described as long-lived cells, with self-renewal potential and proliferative capacity, whereas $T_{EM}$ show strong effector functions when stimulated with cognate antigens [179, 180, 182]. In addition, a rare subset of multipotent T Stem Cell Memory ($T_{SCM}$) has been identified [183, 184]. $T_{SCM}$ consist of poorly differentiated cells characterized by the high expression of Bcl2, CD95 and the interleukin-2 receptor subunit beta CD122 [183, 185]. Similar to $T_{CM}$, $T_{SCM}$ originate from naïve $T$ cells, benefit from the stimulation with IL-7 and IL-15 but exhibit superior proliferation capacity compared to the other T cell subsets [183, 186, 187]. Following TCR stimulation, $T_{SCM}$ rapidly undergo clonal expansion thereby generating more differentiated effector and memory cells [188]. Concomitantly, $T_{SCM}$ show self-renewal potential by maintaining a undifferentiated naïve-like pool [187]. In line with these evidences, $T_{SCM}$ were demonstrated to survive for years in patients after haploidentical hematopoietic stem cell transplantation (HSCT) and CAR-$T_{SCM}$ were shown to confer long-term protection from hematological malignancies [188, 189].

The lymphoid maturation is further characterized by testing CD45RA and CD45RO isoform expression in human T cells. In particular, CD45RO represents a $T_{CM}$ and $T_{EM}$ molecular marker since it is not expressed by CD4$^+$ and CD8$^+$T naïve cells [179, 183]. At the contrary, CD45RA characterizes
 naïve T cells and a mature T cell subset named CD45RA+ TEM (TEMRA). CD8+ T cells produce high levels of effector cytokines such as IFNγ, GZMB and Tumor Necrosis Factor-α (TNF-α) in TEM and TEMRA compartments. In addition, GZMK was described in recent years as an early-effector function molecule expressed in TCM, but its contribution in anti-tumor resistance is still matter of debate [190]. It was also demonstrated that CD8+ T cells differentiate to KLRG1+ short-lived effector cells (SLECs) and CD127+ long-lived memory precursor effector cells (MPECs) [164]. In particular, IL-12 was shown to promote SLECs by over-expressing the transcription factor TBET, while IL-7 favored MPEC expansion [151, 164]. Moreover, it was suggested that IL-2 and IL-15 sustained the homeostatic survival of both cellular populations [150]. T cell maturation was also associated to EOMES, a transcription factor mainly expressed by NK and experienced CD8+ T cells [191, 192]. Taking advantage of deficient mice, it was demonstrated that EOMES was required both to T cell development and to mount an efficient adaptive response. In particular, it was reported that IL-2 governs EOMES expression, which in turn dictates IFNγ and Granzymes transcription [192-194]. Moreover, EOMES was recently indicated as a fundamental TF linked to innate memory T cells (Timm) commitment, a cellular subset that consists of antigen-independent CD8+ cells mainly involved in restraining viral infections [195, 196].
During T cell maturation, a group of co-inhibitory receptors or “immune checkpoints” is also expressed. This family of molecules was demonstrated to influence cellular activation mainly during T cell priming or following target cell engagement (e.g. tumor cells, viral infected and senescent cells) [176, 197-199]. Pioneering evidence demonstrated the inhibitory role of Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) and Programmed Cell Death 1 (PD-1) [199], discoveries recently awarded with the Nobel Prize. Extensive studies conducted in the context of cancer and viral infections highlighted new candidates such as T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), glucocorticoid-induced TNFR-related protein (GITR), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and V-domain Ig suppressor of T cell activation (VISTA) [176, 200]. Immune checkpoints were demonstrated to influence a network of cell-autonomous mechanisms that included glucose metabolism and apoptosis [199, 201].

2.2.6 IL-1 family members and CD8+ T cells

T cell polarization is a dynamic phenomenon guided by environmental cytokines. In particular, the IL-1 system includes several members that
contribute to T cell orientation [3, 202]. IL-1β and IL-18 were demonstrated to potentiate IFN-γ production by triggering Myddosome and NF-κB activation [203]. In line with evidences emerged in other cellular contexts (e.g. Th17 and Th9 cells), IL-1β was demonstrated to favor Th1 polarization in primary and secondary CD4+ T cell responses [202]. In particular, IL-1β enhanced antigen-stimulated expansion in conventional CD4+ T cells through a cell-autonomous mechanism as confirmed by adoptively transferring wt T cells to IL-1R1-deficient recipients [202]. In addition, IL-1β promoted Th1 polarization by inhibiting Treg cells and was shown to drive Th17 cells toward Th1 in presence of IL-23 [204, 205].

In parallel, it was reported that the pro-inflammatory cytokine IL-18 sustained an IL-12-mediated pathway in conventional CD8+ T cells [203]. In particular, IL-18 enhances the translation of newborn mRNA induced upon the stimulation with IL-12, a process shared with NK cells and that requires TBET expression both in CD8+ cells and in DCs [206, 207]. IL-18 was also shown to be released as a consequence of colonization with the commensal protozoan Tritrichomonas musculis, a mucosal infection that burst host epithelial inflammasome in mice [208]. IL-18 sustained the adaptive response against the mucosal infection, but it also triggered the expansion of colitogenic T cells increasing the incidence of sporadic colorectal tumors [208]. Intriguingly, the IL-1 system displays a cellular-specific impact in unconventional CD8-
expressing cells. In skin, IL-17A-producing CD8+ Tc17 cells were shown to cross-talk with non-invasive microbes under physiological condition. Because of tissue injuries, IL-1 and IL-18 were demonstrated to be released as alarmin molecules thus skewing the commensal-specific Tc17 population to Type-2 response. Here Type-2 cytokines IL-5 and IL-13 promoted tissue-repair process [209].

2.2.7 CD8+ T cells and anti-tumor resistance

Tumor promotion and progression depend on the integration of several pathways that contribute to breaking immune surveillance [210, 211]. Among immune populations described in the tumor tissue, Tumor Infiltrating Lymphocytes (TILs) are commonly associated with a favorable prognosis in a variety of human malignancies [212-215]. Anti-tumor properties of CD8+ T cells are the subject of numerous scientific studies, especially since pioneering experiments demonstrated that autologous T cells, previously stimulated in vitro, reduced the number of metastasis in patients with melanoma[216, 217].

Most cancers become symptomatic, unresponsive to drugs and invasive despite the CTL infiltration. Today we know that tumor cells escape adaptive
immune surveillance through two mechanisms here summarized: the inability of CTL to recognize tumor-antigens and the exhaustion of T cells.

TILs consist of heterogeneous populations that include a limited amount of reactive T cell clones capable of recognizing tumor antigens such as differentiation molecules, cancer testis antigens, immunogenic proteins expressed by oncogenic viruses and MHC-I neoantigens emerged as a consequence of mutational load of tumors [215, 218, 219]. In support of this hypothesis, non-reactive intra-tumor CD8+ T cells were shown to acquire a significant cytotoxic activity when transduced with tumor-specific TCRs [220]. Recently, new computational methods predicted the existence of MHC class II-restricted antigens recognized by CD4+ T cells. For instance, mITGB1 emerged as a major MHC-II-restricted neoantigen that conferred immunogenicity to sarcoma and transfected tumor cells. Here, Th1 antigen-specific CD4+T cells were demonstrated to trigger the activation of CD8+ T cell in situ [221]. Furthermore, the expression of MHC-II-restricted neoantigens on tumor cells appeared as an essential target recognized by memory cells in vaccination experimental models [221].

As mentioned, in tumors CD8+ T cells are also influenced by inhibitory signals that contribute to disrupting immune surveillance during tumor promotion and progression [210, 211]. Indeed, persistent stimulation of the TCR and the influence of immune suppressive cytokines induce a variety of mechanisms
that attenuate the activation of T cells. Collectively, these factors were shown to promote T cell “exhaustion”. Exhausted CD8+ T cells expressing high levels of immune checkpoints are unable to kill tumor cells [87, 222-224]. Similar evidences were reported in chronic viral infections, in which CTLs showed poor cytotoxic activity, dysfunctional immunological memory and reduced cell survival [87, 222].

Over the past decade, pre-clinical and clinical research has highlighted the opportunity to counteract cellular dysfunction through a class of immune checkpoint blocking antibodies, a therapy named immune checkpoint blockade [198, 225, 226]. The anti-CTLA-4 antibody Ipilimumab showed significant clinical responses in melanoma, prostate cancer, renal and urothelial carcinoma [227, 228]. Similar results were observed treating patients with anti-PD-1 pembrolizumab [229, 230]. However, human tumors have a dramatic heterogeneity that at least in part explains the sensitivity or, on the contrary, the resistance to anti-cancer drugs and immunotherapy. Tumors are stratified according to the Immunoscore, a clinical parameter that mainly depends on the frequency of immune cells (in particular CD8+ T cells) and their location in the tumor stroma [214]. “Hot” tumors are highly infiltrated and inflamed, while “cold” tumors show low immune infiltration. The introduction and analysis of new parameters, such as PD-1/PD-L1 expression, was shown to predict the clinical response to immunotherapy
Taking advantage of new RNA-sequencing technology, characterization of infiltrating immune cells was further investigated by gene “signatures” including effector function molecules, inhibitory and pro-inflammatory receptors and cytokines, pathways involved during the immune suppression [231, 232]. Unarmed TILs also possess a specific TF signature consisting of TBET downregulation and the acquisition of exhausted-related molecules such as TOX [233, 234]. In addition, the expression of EOMES in absence of TBET was associated with an unfavorable clinical outcome [235, 236].

Effectiveness of immunotherapy and abundance of antigen-specific T cells were described as interconnected processes [219, 237, 238]. Immune checkpoint blockade, as well as chemotherapy regimens, overcome the immunological ignorance by favoring the release of tumor MHC-I neoantigens [238, 239]. TCR-sequencing analysis demonstrated that the promotion of Immunogenic Cell Death (ICD) enforced the adaptive immune compartment through new waves of CTL clonal selection [240, 241]. For instance, reinvigoration of the adaptive immune response was associated with the selective expansion of clonotypes that recognize the MART1 melanoma antigen [242]. Remarkably, tumors deficient in mismatch repair showed high metastatic potential and drug resistance but, on the other hand, they were
susceptible to immunotherapy in consequence of the increased expression of neoantigens [243].

Identification of new immune checkpoints still remains a key goal of pre-clinical researches. Indeed, immune checkpoint blockade was demonstrated to affect the expression of other checkpoint molecules as a compensatory mechanism. In particular, PD-1 and PD-L1 blockade was shown to upregulate TIM-3 on CD8+ T cells and LAG3 on Treg, thus inhibiting the anti-tumor activity of TILs [244]. Therefore, the combinatorial immunotherapy approach emerges as an important field to investigate.

2.2.8 CAR-T cells

Killing of tumor cells mainly depends on the recognition of tumor-specific epitopes and tumor escape mechanisms include downregulation of MHC complexes [245, 246]. Genetic manipulation arises as a consolidate tool to confer specificity to ex vivo T cells reinfused into patients in an MHC-unrestricted fashion [247]. Among cell-based immunotherapy approaches, Chimeric antigen receptor (CAR) T cells have achieved impressive results and have recently been approved by the European Commission (EU) for the treatment of refractory malignancies.
CARs are synthetic immunoglobulin/TCR molecules designed to recognize MHC-independent ligands [247-249]. CAR consists of the extracellular domain, which recognizes a tumor-specific antigen, and of the intracellular region that transduces signals driving cellular proliferation, cytokine productions and target cell killing [247]. In detail, the extracellular domain is formed by heavy and light variable chains of antibodies while the intracellular domain derives from CD3ζ chain. In the 3rd generation of CAR-T cells, tumoricidal properties were enhanced by introducing multiple co-stimulatory endodomains into the CAR sequence. [250, 251].

CAR-T cells have promising applications in hematological malignancies, such as CD19, CD20 and CD22 expressing B-cell tumors. CD33, CD123 and Lewis Y antigen (LeY) emerged as potential targets in myeloid tumors [247, 252].

Clinical results highlighted the strengths but also the limitations of this cellular technology. First, CAR-T cells recognize cancer cells that express high levels of a specific tumor antigen, a feature commonly found in hematological malignancies but rare in most solid tumors. Clinical trials are currently investigating Mesothelin, Epidermal Growth Factor Receptor (EGFR), disialoganglioside GD2 and tyrosine-protein kinase erbB2 (HER2) as new targets [247, 253]. Moreover, screenings of tumor neoantigens are
characterizing upregulated epitopes as consequence of the anticancer therapies [254].

In addition, CAR-T cell therapy is associated to cytokine-release syndrome (CRS) and neurotoxicity depending on pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα. Treatment with Anakinra prevented these adverse events in humanized mice models [255].

Then, efficiency of CAR-T cells depends on their ability to reach the target sites. CAR-T cell migration was enhanced by the constitutive expression of chemokines receptors such as CXCR1 and CXCR2, thus promoting the frequency and the persistence of engineered cells in solid tumors [256]. Similar results were observed by overexpressing CCL19 that is a crucial molecule involved in the migration to lymph nodes [257]. Hence, CAR-T cells are affected by immune suppressive and pro-inflammatory cytokines. New strategies combine cell-based immunotherapy with immune checkpoint blockade, an approach referred to as “extrinsic” [250]. Alternatively, “intrinsic” checkpoint blockade consists in abolishing inhibitory receptor expression in autologous CAR-T cells [250, 258, 259]. Augmentation of anti-tumor properties was also achieved by transducing the cytokines involved in activation and proliferation of lymphoid cells. For instance, CAR-T cells were engineered to release IL-18 thereby supporting anti-tumor activity against large pancreatic and lung cancer [260]. In addition, CAR-T cells were shown
to benefit of autocrine secretion of IL-7, an interleukin that plays a crucial role in clonal expansion [257]. Multiple steps of CRISPR/Cas9 gene modifications were also demonstrated to confer superior antitumor properties. In particular, triple deficient PD-1/TCR/HLA class I CAR-T cells showed reduced alloreactivity combined to increased tumoricidal activity *in vivo* [261]. Recently, it was discovered that T cells upregulated IL-23 receptor as a consequence of TCR stimulation. IL-23-secreting CAR-T cells showed low PD-1 expression and strong tumoricidal activity depending on Granzyme B production in xenograft and syngeneic tumor mouse models [262].

Identification of new molecular targets to strengthen cell-based immunotherapy is currently underway.
3. Aim of the work

IL-1R8 is an anti-inflammatory molecule that tunes NK cell anti-tumor activity. Since NK and CD8+ T cells share the vast majority of immune checkpoints and are influenced by a common pattern of cytokines, the aim of our work was to elucidate the biological role played by IL-1R8 in CTLs and pave the way for its exploitation in therapeutic approaches in immunotherapy.

Our scientific approach first consisted in dissecting IL-1R8 expression in conventional T cells both in healthy donors and in oncological patients, in order to understand whether IL-1R8 was expressed and could consequently represent a candidate immune checkpoint. Moreover, we characterized IL-1R8 expression in T cell subsets to understand mechanisms that govern its expression. To clarify IL-1R8 biological function in adaptive immunity, we investigated the impact of IL-1R8 genetic deficiency on anti-tumor resistance, highlighting similarity and differences between NK and CD8+ T cells in immunogenic tumor models. We finally investigated the signaling pathways tuned by IL-1R8 in CD8+T cells and the functional consequences of IL-1R8-silencing in human CD8+ T cells.
4 Material and Methods

4.1 Animals

All male mice used were on a C57BL/6J genetic background and 8-12 weeks-old. Wild-type mice were purchased from Charles River Laboratories, Calco, Italy. IL-1R8-deficient mice were generated as described. All colonies were bred Charles in River Laboratories (Calco, Italy) and used in the SPF animal facility of Humanitas Clinical and Research Center in individually ventilated cages. Mice were randomized based on age and weight. Procedures involving animals handling and care were conformed to protocols approved by the Humanitas Clinical and Research Center (Rozzano, Milan, Italy) in compliance with national (D.L. N.116, G.U., suppl. 40, 18-2-1992 and N. 26, G.U. March 4, 2014) and international law and policies (EEC Council Directive 2010/63/EU, OJ L 276/33, 22-09-2010; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 2011). All efforts were made to minimize the number of animals used and their suffering. In most in vivo experiments, the investigators were unaware of the genotype of the experimental groups.
4.2 Human primary cells

Human peripheral mononuclear cells (PBMCs) were isolated from peripheral blood or buffy coats of healthy donors, upon approval by Humanitas Research Hospital Ethical Committee. PBMCs were obtained through a Ficoll density gradient centrifugation (GE Healthcare Biosciences). CD8\(^+\) T cells were purified by a negative selection, using a magnetic cell-sorting technique according to the protocols given by the manufacturer (MojoSort\(^\text{TM}\) Human CD8 T Cell Isolation Kit, Biolegend). Human monocytes were purified by PBMCs from Buffy Coat of healthy donors by a positive selection, using a magnetic cell-sorting technique according to the protocols given by the manufacturer (CD14 Microbeads, human, Miltenyi) or by Percoll gradient centrifugation (65% iso-osmotic; Pharmacia, Uppsala, Sweden) when specified. Residual T and B cells were removed from monocyte fraction by plastic adherence. Human CD8\(^+\) T cells were stimulated with Dynabeads\(^\text{TM}\) Human T-Activator CD3/CD28 (ratio bead/cell 1:2, Thermofisher) and were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1%Pen/Strept, IL-2 (10 ng/ml, Proleukyn). Monocytes were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% Pen/Strept and 100 ng/ml M-CSF or 100ng/ml GM-CSF (Peprotech) for 7 days.

Monocyte-derived macrophages were polarized with LPS (50ng/ml), IFN\(\gamma\) (20ng/ml, Peprotech) or TNF-\(\alpha\)(20ng/ml) for 24h. PBMCs of ALL patients
were collected from Humanitas Biobank, upon approval by Humanitas Research Hospital Ethical Committee (Authorization 1516, issued on February 26, 2016). PBMC of CLL patients and CAR-T cells were kindly provided by Prof. Andrea Biondi, Fondazione M. Tettamanti, Monza, Italy. Frozen samples were thawed and vitality was assessed by trypan blue and Aqua LIVE/Dead-405 nm staining (Invitrogen), before flow cytometry analysis. Informed consent was obtained from all subjects.

4.3 Transplantable models

Adenocarcinoma cell line MC38 and sarcoma cell line FS6 were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) 1% L-Glutammine, 1% Pen/Strept. Cells were detached with Tripsin/EDTA solution (Lonza, Basel, Switzerland), washed twice in PBS−/− and diluted in PBS−/− before injection. Mice were anesthetized and shaved on the back, 2x10^6 FS6 or 5x10^5 MN-MCA1 in 100 μl PBS were injected subcutaneously. Primary tumor growth was monitored twice weekly using a caliper. Tumor size limit at which mice were sacrificed was based on major diameter (≤ 2cm). Data are shown as tumor incidence and tumor volume according to the formula: Volume = (Dxd2)/2, where D = larger tumor diameter and d = smaller tumor diameter. Cell lines were authenticated morphologically by microscopy in vitro and by histology ex vivo.
4.4 Tumor digestion.

Transplanted tumors were dissociated manually and digested for 30 min (MC38) or 1h (FS6) at 37°C in PBS⁻⁻ + 0.1 mg/mL Type IV Collagenase (Sigma-Aldrich). Samples were mechanically dissociated and filtered on a gauze. Complete medium was added to single-cell suspensions to block collagenase activity. Samples were then centrifuged, suspended in PBS⁻⁻ and filtered with a 70 µm cell strainer (Corning).

4.5 Depletion experiments

Mice were treated intraperitoneally (ip) injecting 200 µg of specific mAbs the day before tumor cell transplantation and with 100 µg twice/week (anti-mouse CD8, InVivoPlus, Clone YTS169.4 and the relative isotype control rat IgG2b, InVivoPlus, Clone LTF2) for the entire duration of the experiment. All mAbs were purchased by BioxCell.

4.6 FACS analysis
Extracellular staining was performed using a PBS−− buffer containing FCS 2%, EDTA 2mM and NaN3 0.05% (FACS buffer). Single-cell suspensions were first incubated with Aqua LIVE/Dead-405 nm staining (Invitrogen) in PBS−−, 15 min at 4°C. Then, cell suspension was incubated with murine anti-CD16/32 (Clone 24G2) or FcR Blocking Reagent, human (Miltenyi) for 10 min at 4°C. An antibody mix in FACS buffer was then prepared and added to cell suspension for 20 min at 4°C (extracellular staining) or 1h at room temperature (intracellular staining). Formalin 1% were used to fix samples in extracellular staining, while Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used for intracellular staining. The following murine antibodies were used: CD45-BV605 or -PerCp-Cy5.5 (Clone 30-F11); CD3e-APC (Clone 145-2C11); TCRβ-BV711 (Clone H57-597); TCRγδ-BV421 (Clone GL3); NK1.1-PECF594 or -BV650 (Clone PK136); CD11b-BV786 (Clone M1/70); CD4-AF700 (Clone RM 4-5); CD8a-BV570 or -PE(Clone 53-6.7); CD44-FITC or –BV780 (Clone IM7); CD45RB-BV605 (Clone 16A); CD62L-APC (Clone MEL-14); CD25-BV786 (Clone PC61); CD27-PE-Cy7 (Clone LG.7F9); PD1-PE or –BV421 (Clone J43); CTLA4-APC (Clone UC10-4F10-11); EOMES-AF488 (Clone Dan11mag); TBET-PE (Clone O4-46); RORγT- PECF594 (Clone Q31-378); GATA3-BV421 (Clone L50-823); IFNγ-eF450 (Clone XMG1.2); GZMB-PeF610 (Clone NGZB); GZMA-APC (Clone GzA-3G8.5); Perforin-PE (Clone OM46-D). The following human antibodies were used: CD194-PECF594 (Clone 1G1); CD3-BUV496
(Clone UCHT1); CD183-PE (Clone G025H7); CD127-PE-Cy5 (Clone ebioRDR5); CD25-AF700 (Clone 2A3); CD45RO-PerCp-Cy5.5 or –BV570 (Clone UCHL1); CD8-BV786 (Clone RPA-T8); CD4-FITC (Clone M-T477); CCR7-BV711 (Clone 1501503); CD95-BV421 or –PE-Cy5 (Clone DX2); TIGIT-PerCp-Cy5.5 (Clone MBSA43); GNLY-FITC (Clone RB1); KLRG1-APC-H7 (Clone 2F1/KLRG1); GZMB - AF700 (Clone GB11); GZMK-AF647 (Clone GM6C3); CD19-BV650 (Clone HIB19); GITR-BV421 (Clone V27-580); PD1-BV480 (Clone EH12.1); CD8- BUV805 (Clone SK1); CD28-BUV737 (Clone CD28.2); CD4- BUV615 (Clone SK3); CD45RA – BUV563 (Clone H1100); CD127-BUV395 (Clone HIL-7R-M21); TBET-PE-Cy7 (Clone 4-B10); EOMES- PECF594 (Clone WD1928); CD27-PE (M-T271). Antibodies purchased from BD Bioscience, eBioscience, Miltenyi Biotec, ThermoFisher and BioLegend. Biotinylated anti-hSIGIRR (BAF990, R&D Systems) and Streptavidin-Alexa647 or -BV421 (Invitrogen™) were used to stain IL-1R8 in human cells. Results are reported as mean fluorescence intensity (MFI) or relative MFI normalized on fluorescence minus one (FMO). Cell viability was determined by Aqua LIVE/Dead-405 nm staining, negative cells were considered viable. Cells were analyzed on LSR Fortessa or FACSymphony (BD Bioscience). Data were analyzed with FlowJo software (Treestar).

4.7 PrimeFlow RNA assay
A single-cell suspension from spleen and MC38 primary lesion was obtained. Tumor infiltrating leukocytes were enriched with CD45 (TIL) MicroBeads, mouse (Miltenyi). Cells were stained for surface antigens as described, and the hybridization with Sigirr-AlexaFluor647 (VB1-3032710-PF) - or actb-AlexaFluor647-conjugated target probes was performed following manufacturer’s instructions (Thermofisher). Results are reported as mean fluorescence intensity (MFI) normalized on fluorescence minus one (FMO).

4.8 In vitro and ex vivo functional assays

Splenic CD8^+ T cells were isolated with CD8a^+ T Cell Isolation Kit, mouse (Miltenyi) according to manufacturer’s instructions. Isolated cells were cultured in IMDM medium supplemented with 10% Fetal Bovine Serum (FBS) 1% L-Glutamine, 1% Pen/Strept, Gibco™ 2-Mercaptoethanol 50 uM (Thermofisher) and stimulated with IL-2 (20ng/ml, Proleukyn), IL-12 (20ng/ml, Peprotech) and Dynabeads™ Mouse T-Activator CD3/CD28 (ratio bead/cell 1:1, Thermofisher) for 48h. Then, IFNγ and Granzyme B intracellular staining was performed upon 18 hours of re-stimulation with IL-2 (20ng/ml), IL-12 (20 ng/ml) and IL-18 (50ng/ml) when reported. BD GolgiPlug™ (containing Brefeldin) and Cell Stimulation Cocktail (eBioscience) were added 4 hours prior to intracellular staining.

TILs were isolated from a tumor single-cell suspension with CD4/CD8 (TIL) MicroBeads, mouse (Miltenyi). Isolated cells were cultured in IMDM medium
supplemented with 10% Fetal Bovine Serum (FBS) 1% L-Glutamine, 1% Pen/Strept, Gibco™ 2-Mercaptoethanol 50 uM. The intracellular staining was performed upon 18 hours of stimulation with IL-2 (20ng/ml), IL-12 (20 ng/ml) and IL-18 (50ng/ml), BD GolgiPlug™ (containing Brefeldin) were added 4 hours prior to intracellular staining.

4.9 In vitro proliferation and maturation assays

Splenic T cells were isolated with Pan T Cell Isolation Kit II, mouse (Miltenyi) according to manufacturer’s instructions. Isolated T cells were pre-incubated with Vybrant™ CFDA SE Cell Tracker Kit BV421 (Thermofisher), 1uM, 10 min at 37°C. Then, Vybrant™ CFDA SE Cell Tracker tracker was blocked with Complete Medium, 20 min at 37°C. Enriched T cells were cultured in IMDM medium supplemented with 10% Fetal Bovine Serum (FBS) 1% L-Glutamine, 1% Pen/Strept, Gibco™ 2-Mercaptoethanol 50 uM (Thermofisher) and they were stimulated with IL-2 (20ng/ml, Proleukyn), IL-12 (20ng/ml, Peprotech), IL-18 (20ng/ml) and Dynabeads™ Mouse T-Activator CD3/CD28 (ratio bead/cell 1:1, Thermofisher) for 72h when reported.

4.10 Human primary CD8⁺ T cell nucleofection
Human CD8\(^+\) T cells were enriched and cultured for 48 as previously described. Then, CD8\(^+\) T cells were vigorously resuspended and Dynabeads\textsuperscript{TM} were removed with a magnetic support (Mojosort Magnet, Biolegend). Cells were extensively washed with PBS\(-/-\). Alt-R crRNA HS.CAS9.SIGIRR.1.AA, Alt-tracrRNA-ATTO550 and Alt-R CRISPR-Cas9 Negative Control crRNA from Alt-R CRISPR-Cas9 Control Kit, Human, were reconstituted to 200 µM with IDTE pH 7.5 (1X TE Solution). 3µl of tracrRNA and 3µl of crRNA were incubated at 95°C, 5 min, and then left 5 min at room temperature. 5µl of tracrRNA:crRNA duplex were incubated with 6.8 ul of Alt-R S.p. HiFi Cas9 Nuclease V3 (100 µg, 10 µg/µL) and 8.2 of PBS\(-/-\) for 15 min at room temperature. 4ul of Alt-R Cas9 Electroporation Enhancer were added to the mix (reagents from IDT).

15x10\(^6\) Human CD8\(^+\) T cells were resuspended in 80 ul of Buffer Amaxa from Human T Cell Nucleofector Kit (Lonza), supplemented with CRSPR/Cas9 complex solution and then nucleofected with Nucleofector 2b device, program T-023 (Lonza). Cells were collected in 5 ml of complete RPMI-1640 medium, leaving in the incubator 30 min. Nucleofected CD8\(^+\) T cells were washed and resuspended in RPMI-1640 complete medium supplemented with IL-2 (10 ng/ml, Proleukyn) for 72h. CAS9 efficiency was assessed by FACS and qPCR analysis. IFN\(\gamma\) e Granzyme B intracellular staining was performed upon 18 hours of re-stimulation with IL-2 (20ng/ml, Proleukyn), IL-12 (20 ng/ml,
Peprotech) and IL-18 (50ng/ml, MBL). BD GolgiPlug™ (containing Brefeldin) and Cell Stimulation Cocktail (eBioscience) were added 4 hours prior to intracellular staining when reported.

4.11 Quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer’s instructions. RNA was purified with Direct-zol™ RNA MiniPrep Plus kit (Zymo Research). cDNA was synthesized by using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and qPCR was performed with SybrGreen PCR Master Mix (Applied Biosystems) in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and QuantStudio 7 Flex (Thermofisher). Relative gene expression was calculated with the Δ2CT method. Primers were designed according to the published sequences or designed/checked with Primer-BLAST.

Gapdh: forward 5’-GCA AAG TGG AGA TTG TTG CCA T-3’, reverse 5’-CCT TGA CTG TGC CGT TGA ATT T-3’; β-actin: forward 5’-TTC TTT GCA GCT CCT TCG TT-3’, reverse 5’-ATG GAG GGG AAT ACA GCC C-3’; hprt1: forward 5’- CTC AGA CCGCTT TTT GCC G -3’, reverse 5’- CGC CTA ATC ACG CTG GG -3’; il1r8: forward 5’- AGA GGT CCC AGA GCC AT-3’, reverse 5’- AAG CAA CTT CTC TGC CAA GG-3’; IL-1R8 (exon 6, reference primers): forward 5’- ATG TCA AGT GCC GTC TCA
ACG -3', reverse 5'- GCT GCG GCT TTA GGA TGA AGT-3'; IL-1R8 (exon 9-10): forward 5'- TGA CTC CTT CCT CCG ATT TTT G-3', reverse 5'- CGG CCT CGA AGA ATC CAG CAT-3'; β-ACTIN forward 5’-CCC AAG GCC AAC CGC GAG AAG ATC -3’, reverse 5’-GTC CCG GCC AGC CAG GTC CAG-3’

4.11 Western Blot

Human macrophages were lysate in RIPA-buffer containing 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and EDTA-free protease inhibitor cocktail (Roche). Total proteins were measured by DC Protein Assay according to manufacturer’s instructions (Bio-Rad Laboratories). Western blot analysis was performed by loading 20 µg of protein lysate on SDS-PAGE (10% acrylamide). Rabbit anti-SIGIRR (C-Terminal) (AHP1784; R&D Systems) and Goat anti-SIGIRR (N-terminal) (AF990, R&D Systems) were used. The anti-actin (sc-1615, Santacruz) was used as loading control. Chemiluminescent signal was quantified by ImageJ as a ratio of mean gray intensity values of each protein relative to actin bands, or as absolute values.

4.12 Statistical analysis

For animal studies, sample size was defined on the basis of past experience on cancer models in order to detect differences of 20% or greater between the
groups (10% significance level and 80% power). Values were expressed as mean ± SE of biological replicates. Wilcoxon matched-pairs signed-rank test was used to compare the tumor incidence, 2way ANOVA was used to compare the tumor volume. Two-tailed Student’s t test and Mann–Whitney test were used to compare unmatched groups with Gaussian and non-Gaussian distribution. Welch’s correction was applied in cases of significantly different variance. Wilcoxon matched-pairs signed-rank test was also used to compare stimulated cells from same donors. A ROUT test was applied to exclude outliers. Statistics were calculated with GraphPad Prism version 7, GraphPad software. p≤0.05 was considered significant. *p< 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.

5. Results

5.1 IL-1R8 expression in T cells

5.1.1 IL-1R8 is upregulated during lymphoid cell maturation

T cells include heterogeneous cell populations that possess distinct molecular and functional features. We first characterized IL-1R8 expression in different
CD3⁺ T cell subsets from healthy donors, namely conventional CD4⁺, CD4⁺ Treg and CD8⁺ T cells. We found that human CD3⁺ T cells express IL-1R8 (Figure 1A). IL-1R8 was shown to be upregulated during NK cell maturation. We thus investigated its expression in T cells in different maturation stages, which included naïve T cells, Stem Cell Memory TSCM [183], TCM, TEM and fully mature TEFF/TEMRA. Mature CD4⁺ T cells are functionally plastic and their skewing can be dissected through Th1 and Th2 polarization markers such as CXCR3 and CCR4. We observed that IL-1R8 was upregulated during CD4⁺ T cell maturation (Figure 1B, 1C) independently from the cellular polarization (Figure 1C). In CD8⁺ T cells, the transition from CD45RO⁻CCR7⁺ naïve T cells to antigen experienced CD45RO⁺CCR7⁺ TCM cells was associated with IL-1R8 upregulation, which emerged in early stages of T cell maturation (Figure 1D).
Figure 1. IL-1R8 is upregulated in human experienced T cells. Flow cytometry analysis of IL-1R8 protein expression in human circulating primary CD3+ T cells (A) and in regulatory CD4+ (B), conventional CD4+(C) and CD8+(D) T cell maturation and polarization subsets of healthy donors. Gating strategy (A-D): Conventional CD4+ T cells (CD3++, CD4+), CD4+ Treg cells (CD3+ CD4+, CD25+, CD127), CD8+ T cells (CD3++, CD8+), Naïve T cells (CCR7+, CD45RO−), Stem Cell Memory (SCM) T cells (CCR7+, CD45RO−, CD95+), Central Memory T cells (CCR7+, CD45RO+), Effector Memory T cells (CCR7+, CD45RO+). Effector Memory Th1 T cells (CCR7+, CD45RO+, CXCR3+), Effector Memory Th2 T cells (CCR7+, CD45RO+, CCR4+), Terminal Effector T cells (CCR7+, CD45RO−). (*p < 0.05, **p < 0.01, ***p < 0.001, Mean ± SEM is shown, unpaired Student’s t test).
IL-1R8 was shown to be regulated through transcriptional-independent processes, in different cell types. To further investigate the regulation of IL-1R8 expression, we analyzed *IL-1R8* gene expression in a variety of immune cells by RNA-sequencing. To follow the entire lymphoid ontogeny, we analyzed public available datasets generated by isolating immune cell populations from human bone marrow (Figure 2A). First, we did not observe differences comparing male and female healthy donors (data not shown). Then, *IL-1R8* gene was preferentially expressed by NK cells and by memory and effector T cells (Figure 2A).

5.1.2 Regulation of IL-1R8 expression in mouse T cells

To investigate whether IL-1R8 expression regulation is conserved between human and mouse, we analyzed its expression in lymphocytes collected from healthy mice. Since an anti-mouse IL-1R8 antibody working in FACS is not available, we evaluated IL-1R8 mRNA distribution by Prime Flow, a technique developed to couple Flow Cytometry and transcription profiling. Taking advantages of *Il1r8−/−* mice, we established the hybridization specificity of IL-1R8 probes (data not shown). In line with transcriptional profile reported in human lymphocytes, murine CD4+ and CD8+ T cells expressed high IL-1R8 levels (Figure 2B). In addition, IL-1R8 was overexpressed along the transition from CD62L+CD44− naïve T cells to experienced CD62L+CD44+ T<sub>CM</sub> cells.
(Figure 2C), indicating that regulation of IL-1R8 expression is evolutionarily-conserved in these two species. These results suggest that experiments performed to dissect the role of IL-1R8 in mouse T lymphocytes can be relevant and translated to human T cells.

Figure 2. IL-1R8 mRNA expression in immune cells. (A) RNA-seq data of human bone marrow cells (Human Cell Atlas consortium) extrapolated from the public web portal http://www.altanalyze.org/ICGS/HCA/Viewer.php. (B) Prime Flow analysis of IL-1R8 expression in splenic CD11b+ myeloid cells, NK.1.1+ NK cells, and αβ CD4+ and CD8+ T
cells divided in naïve and mature CD44+ T cells. (*p < 0.05, ***p < 0.001, ****p<0.0001, Mean ± SEM is shown, unpaired Student’s t test).

5.2 IL-1R8 as checkpoint molecule in CD8+T cells

5.2.1 IL-1R8 regulates CD8+ T cell through distinct signaling pathways.

NK cells represent the closest immune population to CD8+ T cells and the vast majority of regulatory mechanisms are shared between them [104]. Since IL-1R8 was demonstrated to affect NK cell maturation and activation, we characterized IL-1R8-deficient CD8+T cells in vitro. We firstly evaluated CD8+ T cells maturation (Figure 3A) and TF expression (data not shown) in splenocytes, but we did not observe differences comparing unstimulated wild-type and IL-1R8-deficient naïve T cells.

As mentioned in the introduction, CD8+T cell activation requires the integration of three independent signals furnished by tonic TCR stimulation, T cell co-receptors and the cytokine environment. Thus, we primed naïve CD8+ T cells with anti-CD3/CD28 beads to evoke simultaneous TCR/CD28 activation, and then supplemented the growth medium with lymphocyte activating cytokines. We stimulated cells with IL-2, a promitogenic/antiapoptotic cytokine activating EOMES, and the Type-1
polarizing cytokine IL-12 that was shown to regulate TBET [149, 263]. Since IL-1R8 was previously demonstrated to affect NK cells by dampening an IL-18-mediated pathway, we also verified the biological relevance of this cytokine in IL-1R8-deficient CD8⁺ T cells.

To clarify the impact of IL-1R8 genetic deficiency on T cells, we characterized a spectrum of phenotypes in unpolarizing and polarizing conditions, an experimental strategy to assess whether IL-1R8 played a cell-autonomous role in the early-phase of lymphoid activation.

IL-1R8-genetic deficiency increased the percentage of CD44⁺ cells after stimulation indicating amplified CD8⁺ T cell responsiveness (Figure 3A). We also observed an enhanced proliferation index in the CFSE assay (Figure 3B, 3C). Intriguingly, IL-1R8-deficiency potentiated these two interconnected processes, maturation and proliferation, in an IL-18-independent fashion.
Figure 3. IL-1R8 deficiency enhanced CD8⁺T cell maturation and proliferation. (A) CD44⁺ cell frequency of unstimulated and stimulated CD8⁺ T cells. (B-C) Proliferation index of CD8⁺ T cell upon stimulation with anti-CD3/CD28 and cytokines by CFSE assay. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, unpaired Student’s t test)

Since CD8⁺ T cell priming correlates with the acquisition of effector functions, we analyzed IFNγ and GZMB production. As previously reported [264, 265], the stimulation with IL-18 in combination with IL-2 and IL-12 sustained the effector function of Type-1 polarized wt cells. As shown in fig 4, IL-1R8
deficiency amplified this effect and significantly increased the frequency of IFNγ+ and GZMB+ cells (Figure 4A and 4B, in red), hence highlighting a shared mechanism between NK and T cells. Notably, IL-1R8 genetic deficiency prompted CD8+ T cell activation in response to IL-2, a cytokine not recognized by ILR family members (Figure 4A and 4B, in blue). We also monitored CTLA-4 and PD-1 expression by FACS analysis and observed that IL-1R8 genetic deficiency did not affect the expression of these two immune checkpoints in stimulated cells (Figure 4C), thus suggesting that T cell hyperactivation is not associated with increased cell exhaustion.
Figure 4. IL-1R8 deficiency enhanced CD8+ T cell activation. (A-B) IFNγ+ and GZMB+ frequency of CD8+ T cells and upon stimulation with cytokines. (C) PD-1+ and CTLA-4+ frequency of CD8+ T cells (*p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001, Mean ± SEM is shown, unpaired Student’s t test).

5.2.2 IL-1R8 influences TBET and EOMES induction

To further characterize IL-1R8-deficient T cells, we dissected the expression of key TFs associated with T cell orientation. It was demonstrated that TBET, GATA3 and RORγt were linked respectively with Type-1, Type-2 and Type-17 responses [266]. We observed that IL-1R8 deficiency was associated with higher TBET expression in Type-1 polarized T cells (Figure 5A, 5D). In addition, we found significant EOMES induction after stimulation with IL-2 (Figure 5B, 5D) and expansion of the EOMES+TBET+ cell subset (Figure 5C, 5D). In contrast with these data, we did not observe significant differences in GATA3 and RORγt (not shown).
Figure 5. **IL-1R8 regulates the IL-2/EOMES signaling axis and tunes TBET\(^+\)EOMES\(^+\) co-expression through a cell-autonomous mechanism.** (A-B) TBET\(^+\) and EOMES\(^+\) frequency of stimulated CD8\(^+\) T cells. (C-D) TBET\(^+\)EOMES\(^+\) cell frequency in unstimulated and stimulated CD8\(^+\)T cells. (*p < 0.05, ***,*p < 0.001, unpaired Student’s t test).

Collectively, our data suggested that IL-1R8 acts as immune checkpoint by affecting CD8\(^+\) T cells through two distinct cellular-autonomous mechanisms. First, as regulatory receptor IL-1R8 dampened the IL-18-mediated pathway. In parallel, IL-1R8 negatively regulated IL-2-mediated induction of EOMES.
5.3 IL-1R8 deficiency augments CD8+ T cell-mediated protection in immunogenic tumor models.

5.3.1 IL-1R8-deficient CD8+ T cells confer protection against immunogenic tumors

IL-1R8-deficient NK cells were previously demonstrated to control tumor growth and metastasis.

We assessed the role of IL-1R8 in the MC38 tumor sc transplantable model, an immunogenic cell line that was shown to promote adaptive immune response [267]. In this model, II1r8−/− mice were significantly protected compared to wild-type mice showing reduced tumor growth and incidence after sc MC38 injection (Figure 6A, 6B). We next extended our analysis to a different tumor type and analyzed tumor growth in the immunogenic fibrosarcoma (FS6)-transplantable model. In this model, the incidence is 100% in wt mice, whereas 36% of II1r8−/− mice rejected the tumor (Figure 6D). II1r8−/− mice also showed a reduced tumor volume, but the difference did not reach the statistical significance (Fig. 6C).
**Figure 6. Il1r8−/− mice are protected in the MC38 and FS6 tumor transplantable models.**

MC38 primary tumor volume (A) and tumor incidence (B) in wt and Il1r8−/− tumor-bearing mice. FS6 primary tumor growth (C) and tumor incidence (D) in wt and Il1r8−/− tumor-bearing mice. (*p < 0.05, ***p < 0.001, Two-way ANOVA (A, C) and Wilcoxon matched-pairs signed rank test (B, D)).

To evaluate the role played by CD8+ T cells in anti-tumor resistance, we treated tumor-injected mice with an anti-CD8 depleting antibody (Figure 7A, 7B). The efficacy of CD8+ depletion was evaluated by flow cytometry analysis of splenic immune cells (Figure 7C). CD8+ T cell depletion totally abolished the protection observed in Il1r8−/− mice (Figure 7A). In MC38 tumor transplantable model, the tumor growth was not affected by the treatment in wt mice, suggesting a poor T cell-dependent tumor control in these mice.
Interestingly, CD8⁺ T cell depletion was associated with exacerbated tumor growth in Il1r8⁻/⁻ mice compared to wt mice, suggesting that, in the absence of CD8⁺ T cells, IL-1R8-deficiency was involved in increased promotion of cancer-related inflammation. CD8⁺ T cell depletion also abolished the protection in FS6 tumor sc transplantable model (Figure 7B).

**Figure 7. IL-1R8-deficient CD8⁺T cells promote the anti-tumor resistance.** (A-B) MC38 and FS6 primary tumor volume upon CD8⁺ T cell in vivo depletion. (C) Splenic immune cell frequency upon CD8⁺ T cell in vivo depletion. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001, Two-way ANOVA (A) and unpaired Student’s t test(C)).

5.3.2 Conventional T cell frequency in wt and IL-1R8-deficient tumor-bearing mice
To investigate the effect of IL-1R8-deficiency on T cell frequency and distribution, we characterized splenic lymphocytes and TILs. We did not observe major alterations of leukocyte frequency of the spleen, but we observed a slight but significant increase of CD8$^+$ T cells (Figure 8A). Among tumor infiltrating $\alpha\beta^+$ T cells, we observed a reduction of the CD4$^+$ T cell population (Figure 8B). Also in the FS6-transplantable model we observed a slight increase of splenic CD8$^+$ T cell frequency and a significant decrease of CD4$^+$ TILs (Figure 8C, 8D).

Figure 8. Immunophenotyping of lymphoid cells collected from wt and II1r8$^{-/-}$ tumor-bearing mice. (A-B) Immune cell frequency in spleen and primary lesion of MC38 tumor-
bearing mice. (C-D) Immune cell frequency in spleen and primary lesion of FS6 tumor-bearing mice (*p<0.05, unpaired Student’s t test).

5.3.3 TIL maturation and exhaustion in tumor-bearing mice

We next analyzed T cell maturation in splenic T cells and TILs. In particular, we observed that most of the splenic lymphocytes were early experienced CD44dimCD62L+ T cells both in MC38 and FS6 tumor-bearing mice (Figure 9A, 9C). As expected, TILs showed an increased maturation compared to T cells in the spleen (Figure 9B, 9D) and were mainly constituted by TCM and TEM cells. The comparison between wt and IL-1R8-deficient mice showed only a slight but significant increase of TCM in the spleen of sarcoma-bearing mice.
Figure 9. CD8+ T cell maturation in tumor-bearing mice. CD8+T cell subset frequency in MC38 (A, B) and FS6 (C, D) tumor-bearing mice. FACS analysis of CD44 CD62L+ naïve T cells, CD44^dimCD62L+ T cells ( naïve -> T_C, D CM), CD44^+CD62L+ T_C, CD44^+CD62L^ T EM. CD44^dimCD62L+ cells were included in T EM cells (**p<0.05, unpaired Student’s t test).

We next investigated the frequency of PD-1+ CD8+ T cells, as marker of exhaustion. As shown in Figure 10A, 10B, we observed a comparable frequency of PD-1+ T cells in splenic and intra-tumor CD8+T cells in wt and Il1r8^- mice.
Figure 10. PD-1 expression on CD8⁺ T cell in tumor-bearing mice PD1⁺ CD8⁺ T cell frequency in MC38 (A) and FS6 (B) tumor transplantable models

5.3.4 IL-1R8-deficiency affects TBET and EOMES induction in CTLs

NK cells and T cells are regulated by common pattern of TFs. In particular, TBET and EOMES were demonstrated to govern key processes such as cellular activation, maturation and expression of immune checkpoints. Since IL-1R8 affected TBET and EOMES *in vitro*, we further analyzed their expression in tumor-infiltrating leukocytes.

We observed that IL-1R8-deficient CD8⁺ T and NK cells expressed higher TBET levels compared to wt NK cells. (Figure 11A, 11C). We further analyzed EOMES induction and distribution in total CTLs (Figure 11B, 11D), but we did not observe differences.
Figure 11. **TBET and EOMES expression in NK and αβ conventional T cells.** TBET\(^+\) and EOMES\(^+\) frequency of splenic (A, B) and tumor infiltrating (C, D) leukocytes in MC38 tumor-bearing mice (*p<0.05, **p<0.01, unpaired Student’s t test).

In agreement with results obtained in healthy mice, in *Il1r8\(^-\)\(^-\)* mice we found a significant increase of the frequency of TBET\(^+\)EOMES\(^+\) cells (Figure 12A, 12B), a cellular subset included in CD8\(^+\) T\(_{CM}\) subset and along T\(_{CM-EM}\) transition [268]. We observed that the majority of TBET\(^+\)EOMES\(^+\) cells (>80% in spleen, >50% in tumor) expressed central memory markers such as CD62L, CD44 and CD27 (Figure 12C, 12D).
**Figure 12.** Characterization of TBET⁺EOMES⁺ T cells in wt and IIIr8⁻/⁻ tumor-bearing mice. TBET⁺EOMES⁺ frequency calculated on total CD8⁺T cells (A) and on TCM (B). (C) TCM subset frequency on TBET⁺EOMES⁺T cells. (D) Layout from Flowjo of TBET⁺EOMES⁺T cells from spleen and tumor on CD44 and CD62L expression (*p<0.05, **p<0.01 unpaired Student’s t test).
5.3.5 IL-1R8 deficiency sustains CD25 induction in activated CD8\(^+\) T cells and enhances Granzyme B production in TBET\(^+\)EOMES\(^+\) expressing cells

To explain how IL-1R8 genetic silencing sensitized cells to IL-2, we tested whether IL-1R8 deficiency affected IL-2R expression. As shown in Figure 13, IL-1R8-deficiency was associated with higher expression of the IL-2R subunit α, the key IL-2R complex component, in response to stimulation with cytokines, suggesting an increased response to the positive feedback loop.
Figure 13. CD25 induction in freshly-isolated and activated CD8$^+$ T cells. CD25 protein expression in unstimulated and stimulated CD8$^+$ T cells. (*p < 0.05, **p < 0.01, ***p < 0.001, Mean ± SEM is shown, unpaired Student’s t test).

These results possibly explain the cell-autonomous mechanism regulating the TBET$^+$EOMES$^+$ cellular cluster enriched among IL-1R8-deficient CD8$^+$ T cells.

We also stimulated purified wt and IL-1R8-deficient CD8$^+$ TILs. IL-1R8 genetic deficiency favored EOMES expression (Figure 14A) and Granzyme B production (Figure 14B). Preliminary data suggested that TBET and EOMES co-expression conferred an increased cytotoxic activity (Figure 14C, 14D).
Collectively, these results indicate that at the time of sacrifice, no differences were observed in terms of maturation, or exhaustion between wt and Il1r8−/− TILs. However, increased functional activation was observed in IL-1R8-deficient TILs in terms of TBET/EOMES co-expression, responsiveness to IL-2, expression of cytotoxic molecules.

5.4 IL-1R8 as targetable molecule in cancer

5.4.1 IL-1R8 is expressed in CD8+ T cells of cancer patients

IL-1R8 expression is generally downregulated in innate immune cells after inflammatory stimulation. In contrast, recent data revealed that IL-1R8 expression was preserved in adaptive immune cells [269]. Since the therapeutic relevance of an immune checkpoint depends on the expression in targetable cells, we investigated the IL-1R8 expression profile in T cells from cancer patients. In particular, we first characterized circulating T cells of
Acut e Lymphoblastic Leukemia (ALL) patients (Table 1). In these patients we did not find differences in the frequency of CD4$^+$ and CD8$^+$ T cells (Figure 15A), but we observed enhanced T cell maturation as indicated by low frequency of naïve T cells in ALL samples (Figure 15B, 15C). Furthermore, in ALL samples CD8$^+$ T cells showed significantly higher expression of effector marker such as TBET, GZMK, GZMB and Granulysin Y (GNLY) (Figure 15D). CD8$^+$ T cells also upregulated immune checkpoints such as TIGIT and PD-1 (Figure 16D).

Table 1.

<table>
<thead>
<tr>
<th>Age at donation</th>
<th>Sex</th>
<th>Disease</th>
<th>Time point</th>
<th>Cytogenetic</th>
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<td>Primary</td>
<td>Exordium</td>
<td>46XY</td>
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<tr>
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<td>46.XY t(9;22)(q34;q11.2),Ph+</td>
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<tr>
<td>71</td>
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<td>Primary</td>
<td>Exordium</td>
<td>46.XY t(9;22)(q34;q11.2), Ph+</td>
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<td>66</td>
<td>F</td>
<td>Primary</td>
<td>Exordium</td>
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Figure 15. Immunophenotyping of T cells collected from ALL patients. (A) Frequency of CD4⁺, CD8⁺ and CD3⁺ CD4⁺ CD8⁻ circulating cells in healthy donors and B cell-ALL patients. (B) Frequency of CD8⁺ T cell subsets. (C) Layout from Flowjo of T cell subsets characterized on the basis of CD45RO and CCR7 expression. (D) Naïve, Central Memory, Effector Memory and Terminal Effector subset characterized according to the gating strategy showed in figure 1. Maturation, activation and exhaustion marker expression in CD8⁺ T cells (*p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student’s t test).

In these patients, we observed that CD4⁺ and CD8⁺ T cells expressed higher IL-1R8 levels in patients compared to healthy donors (Figure 16A), in particular in the CD45RO⁻ CCR7⁺ naïve-like population constituted by poorly mature T cells (Figure 16B). Indeed, in ALL patients circulating CD8⁺ T cells were mostly constituted by mature cells that expressed exhaustion/activation
markers. In addition, the analysis revealed that the immune checkpoint IL-1R8 is expressed by CD8+ T cells in ALL patients.

Figure 16. IL-1R8 is expressed in T cells of ALL patients. IL-1R8 protein levels in CD4+ and CD8+ T cells (A). IL-1R8 protein levels in CD8+ T cell maturation stages (B) (*p < 0.05, **p < 0.01, Mean ± SEM is shown, unpaired Student’s t test).

We also evaluated IL-1R8 expression in MC38 tumor-bearing mice. The immunogenic colon adenocarcinoma cell line MC38 was transplanted subcutaneously and tumor-bearing mice were sacrificed after 25 days. We first observed that immune cells in the spleen of tumor bearing mice showed lower IL-1R8 levels compared to healthy mice (Figure 17A, 17C). We also evaluated IL-1R8 expression in Tumor Infiltrating Leukocytes (TILs) that included most of the maturation stages. In MC38 primary lesion, IL-1R8 was mainly expressed by T lymphocytes compared to other immune cells such as NK and myeloid cells (Figure 17B). In agreement with data obtained in human, we
found that IL-1R8 expression was particularly enriched in antigen-experienced CD62L+ CD44+ T<sub>CM</sub> cells and CD62L− CD44+ PD-1+ T<sub>EM</sub> cells (Figure 17D).

**Figure 17. IL-1R8 expression in splenic T cells and TILs.** IL-1R8 expression in splenic immune cells from MC38 tumor-bearing mice (A) compared to healthy mice (C) and in
tumor infiltrating leukocytes (B and D). (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Mean ± SEM is represented, unpaired Student’s t test).

This analysis suggested that IL-1R8 was a candidate target molecule in CD8+ T cells in tumor bearing mice and patients.

5.4.2 IL-1R8 genetic silencing sustains human CD8+ T cell activity.

To confirm the relevance of our observations in human, we generated primary IL-1R8-deficient CD8+ T cells by adopting the CRISPR/Cas9 technology. The CRISPR/Cas9 system consists in a labeled gRNA and purified Cas9 protein that mediates site-specific DNA cleavage. Consequently, the CRISPR/Cas9 system is used to generate stable genetic knock-out cells by targeting specific genes [270]. Taking advantage of a fluorescent guide, we optimized the workflow in order to obtain a high transfection rate (>75% after 48h and > 95% after 72h). We then estimated the gene ablation persistency by FACS and qPCR analysis. Results obtained indicate that the approach was successful in generating stable IL-1R8-deficient CD8+ T cells (Figure 18A). Preliminary data indicated that both control and silenced primary CD8+ T cells up-regulated IL-1R8 in culture, which was significantly reduced in silenced cells (Figure 18A). Next, we quantified IFNγ- and GZMB- secreting cells
comparing IL-1R8-deficient cells to CD8\(^+\) T cells nucleofected with a control sgRNA. IL-1R8-deficiency potentiated the production of effector molecules after IL-18 stimulation (Figure 18B). Moreover, we found a significant increase of GZMB upon IL-2 stimulation, suggesting the conservation in human of the IL-2/EOMES-dependent mechanism observed in \(Il1r8^{−/−}\) mice (Figure 18B).

**Figure 18.** *IL-1R8 genetic blockade enhances human CD8\(^+\) T cell effector functions.* *IL-1R8* gene (A) and protein (B) expression in primary human CD8\(^+\) T cells nucleofected with a scramble (CTRL) and *IL-1R8* sgRNA (sgRNA\(^+\)). Layout from Flowjo of nucleofected cells that internalized fluorescently labeled gRNA after 48h in culture. IFN\(γ\)^+ and GZMB^+ cell frequency (B) among primary human stimulated CTRL and sgRNA^+CD8^+ T cells. (*p < 0.05, Mean ± SEM is shown, paired Student’s t test).
Finally, to investigate whether IL-1R8 silencing could represent a genetic tool to enhance the anti-tumor potential of T cells and CAR T cells from cancer patients, we analyzed IL-1R8 expression in lymphocytes and in CAR-T cells from Chronic Lymphocytic Leukemia (CLL) patients. In agreement with results obtained in ALL patients, CD8+ T cells expressed high level of IL-1R8 in CLL (Figure 19A); moreover, IL-1R8 expression was higher in mature T cells compared to the naïve subset (Figure 19B). Then, we characterized IL-1R8 expression along the CAR-T manufacturing process. Preliminary data indicated that IL-1R8 was downregulated in consequence of viral transduction, and then it was rescued by the recovery in vitro (Figure 19C, 19D). Next experiments will explore the possibility of generating stable IL-1R8-deficient CAR-T cells.
Figure 19. **IL-1R8 expression in CLL.** IL-1R8 protein expression in CD4+, CD8+ T cells (A) and in T cell subsets (B) from CLL patients. IL-1R8 protein expression in CLL-patient derived CD23+ CAR-T cells (C, D).
5.5 IL-1R8 regulation

5.5.1 Truncated forms of IL-1R8 are controlled by an independent intragenic promoter in immune cells

Part of my research activity was dedicated to enlighten IL-1R8 regulation in immune cells. As mentioned in results commented above, IL-1R8 gene possesses a well conserved regulatory area that encompasses the 1st exon (Figure 20A). In addition, this region is largely decorated by epigenetic modifications associated with along immune cell differentiation (Figure 20B). We characterized IL-1R8 epigenetic modifications in NK cells, in T cells and in monocytes/macrophages as representative myeloid populations. Histone modifications were demonstrated to tune gene transcription by influencing chromatin accessibility. Specific epigenetic markers such as H3K4 mono- and tri-methylation (H3K4me1 and H3K4me3), H3K9 acetylation (H3K9ac), H3K27 acetylation (H3K27ac), and H3K36 tri-methylation (H3K36me3) were shown to be enriched on active promoters and enhancers, while poised and repressed regulatory elements were associated with H3K27 tri-methylation (H3K27me3) and H3K9 tri-methylation (H3K9me3) [271]. Available Chip-sequencing data revealed a correlation between IL-1R8 expression and H4k27ac. In detail, NK cells showed higher acetylation levels compared to other immune cells (Figure 20B, 20D); H4k27ac increased in mature T cells both in CD4+ and in CD8+ T cells compared to blood naïve
cells (Figure 20C, 20D). In summary, our analysis indicated that the expression of IL-1R8 was associated with specific epigenetic modifications regulated during immune cell differentiation and maturation. Moreover, our analysis suggested the existence of an intragenic site in proximity of the 7th exon, characterized by epigenetic modification associated to promoters and enhancers (Figure 20B).
Figure 20. IL-1R8 epigenetic profile of immune cells. In silico analysis of sequence conservation performed with Genomatrix (A). Legend map: UTR (Yellow), Intron (Orange), Simple repeats elements (Green), Intergenic regions (Red). Chip-sequencing data of epigenetic modifications of human IL1R8 gene, from Ensembl. The height of picks represents the enrichment level of histone modifications in distinct leukocyte subsets and in T cell maturation stages; in red, histone H3K27ac (B).
Taking advantage of available RNA-seq and ChIP-seq datasets, we further characterized the intragenic locus in order to clarify its biological function. We found that the locus was characterized by TF binding sites recognized by CREB and EGR family members (Figure 21A), which are involved in driving myeloid cell activation and polarization. ChIP-seq data revealed that POL2A and its phosphorylated form pS2 POL2A were able to bind the intragenic site, hence revealing a Transcription Starting Site (TSS) (Figure 21B). We next analyzed RNA-seq libraries including IL-1R8-expressing cells. Our screening showed a significant enrichment of “RNA-seq reads” from the intragenic TSS to 3’UTR in most cell types (Figure 21C). Collectively, these data provide evidences about the existence of IL-1R8 truncated isoforms dependent on a dedicated promoter.
Figure 21: IL-1R8 gene has an intragenic TSS. (A, B) Chip-sequencing data of TF binding enrichment on the human IL-1R8 gene. The analysis shows a TFs enriched area associated with 7th, 8th, 9th and 10th exons. The enrichment score is represented by picks (A) or a greyscale (B) indicating increased independent experimental evidences of binding for a TF (A) and for POL2A subunit (B) at a particular genomic locus. Chip-seq analyzed from UCSC Genome browser. Representative cell lines: HCT-116, hESC, HL-60, GM12878, HepG2 (A-B). (C) RNA-sequencing analysis of IL-1R8 expression in human cell lines GM12878, H1-hESC, HeLa, HepG2, HSMM, HUVEC, K562, NHEK, NHLF. Picks indicates the enrichment of sequencing-reads from the 7th to 10th exon (C).
Since ChIP-seq data indicated that myeloid cells showed a strong transcriptional activity in the intragenic TSS, we further dissected IL-1R8 regulation by comparing full-length and truncated isoform expression in human monocyte-derived macrophages. First, we observed that IL-1R8 was downregulated in pro-inflammatory GM-Colony Stimulating Factor (GM-CSF)-induced “M1” macrophages, compared with resting M-CSF macrophages (Figure 22A-22C), suggesting that IL-1R8 expression was downregulated along monocyte to macrophage transition. In line with this observation, prototypical pro-inflammatory stimuli (e.g. LPS, IFNγ and TNFα) downregulated IL-1R8 transcription in M-CSF macrophages (Figure 22D). Since the intragenic promoter emerged as an independent regulatory region, we analyzed the expression of truncated isoforms in activated macrophages. We first analyzed CAGE-seq data in different conditions including M1-polarized macrophages. CAGE-seq introduces a tagged nucleotide on 5’UTR revealing where mRNA starts. Polarized macrophages showed transcripts generated in proximity of the intragenic TSS that lacked exon 1 to exon 4 sequence (Figure 22E). The putative Coding DNA Sequence (CDS) corresponded to the C-terminal region that includes the TIR intracellular domain. Indeed, we compared the mRNA level for the amplicon corresponding to the 440-bp sequence from ATG (exon 6) and the 878-bp sequence from ATG (exon 9-10). Despite full-length IL-1R8 expression was downregulated in M1 macrophages, we observed that the truncated isoforms
were upregulated upon polarizing stimuli. These results were confirmed by qPCR performed with couples of primers that amplify distinct exon sequences, which showed a strong enrichment of amplicons localized in the last 2 exons (Figure 22F, 22G).
Figure 22. Pro-inflammatory stimuli downregulate IL-1R8 expression in human macrophages while sustain the expression of IL-1R8 truncated isoforms. IL-1R8 mRNA (A) and protein (B and C) levels in FACS-sorted human monocytes and in human monocyte-derived macrophages. IL-1R8 gene expression in M-CSF human monocyte-derived macrophages stimulated with LPS, IFN-γ, and TNF-α (D). Cage-sequencing analysis of M1 polarized macrophages on FANTOM5. Violet picks indicated the 5’ mRNA of short IL-1R8 isoforms (E). qPCR of resting and stimulated macrophages with primers targeting the 6th exon or 9-10th exon junctions. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001, Relative Mean ± SEM is shown, paired Student’s t test).

Since the TSS contains 3 AUG sequences, we estimated the molecular weight of the truncated isoforms in about 37 KDa and performed a Western Blot analysis by incubating samples with two distinct anti-IL-1R8 antibodies that recognize the N-terminal and C-terminal domains. In line with our prediction, we detected a truncated protein (37-25 KDa) in macrophages using the mAb against the C-terminal domain, suggesting that the extracellular domain was not included in the truncated isoforms (Figure 23A). Intriguingly, truncated form of 37kDa showed a significant and specific upregulation in consequence of stimulation with LPS suggesting an unrevealed TLR4-mediated process (Figure 23B, 23C). Further experiments are required to clone IL-1R8 isoforms and to characterize their biological role.
Figure 23. The stimulation with LPS regulates a specific IL-1R8 truncated form. Western blot analysis in SDS-page reducing condition of resting and stimulated M-CSF macrophages (A). Western blot of resting and M1 polarized M-CSF and GM-CSF macrophages (B); time course of M1 M-CSF macrophages (*p < 0.05, ***p < 0.001, paired Student’s t test)
6. Discussion

A promising potential approach of personalized cancer therapy is the improvement of immune response by targeting immune checkpoints. IL-1R8 was described as a promising molecule because of its involvement in dampening key pathways responsible of NK cell activation. IL-1R8 genetic silencing was demonstrated to enhance NK cell anti-tumor and anti-viral response [32].

NK cells represent the innate counterpart of CD8+ T cells and share with them regulation and effector mechanisms. We thus investigated the biological role of IL-1R8 in adaptive immune cells.

Our results suggest that IL-1R8 is an immune checkpoint expressed in conventional T cells and upregulated during their maturation. Mirroring NK cells, IL-18 emerges as a target of IL-1R8 inhibitory activity through a cell-autonomous process. In addition, we demonstrated that IL-1R8 deficiency sensitizes CD8+ T cells to IL-2, thus sustaining maturation, proliferation and EOMES expression. IL-1R8 genetic silencing conferred protection against immunogenic transplantable tumor models and CD8+ T cell depletion abolished the antitumor resistance. Moreover, IL-1R8 deficiency sustained Type-1 responses and promoted TBET+ EOMES+ CD8+ T cells, a cellular subset secreting high GZMB levels. We demonstrated that IL-1R8 expression
is influenced by conserved epigenetic mechanisms and it is upregulated in T cells in cancer. Finally, IL-1R8 genetic silencing increased human CD8+ T cell effector functions, an evidence with promising clinical implications since CAR-T cells express IL-1R8.

The characterization of IL-1R8 expression highlights that IL-1R8 is acquired during T cell maturation, in particular during the transition from unexperienced naïve T cell to T_{CM-EM} cells. Since IL-1R8 has been shown to be upregulated during NK cell maturation, the regulation of IL-1R8 expression is similar between NK and CD8+T cells, emphasizing its relevance in cytotoxic cells.

Genetic silencing sustained CD8+ T cell activation through a cell-autonomous mechanism. In particular, IL-1R8 deficiency promoted IL-18-mediated pathways as previously emerged in NK cells [32]. In addition, we found that the enhanced Type-1 response was associated with TBET induction, a TF largely described as key player of CTL activation [272]. Notably, TBET was also shown to regulate NK cell effector functions.

We observed that \textit{Il1r8}^{-/-} mice were protected from MC38 and FS6 sc tumor transplantable models. The retrospective analysis of our results suggests that IL-1R8-deficient NK and CD8+ T cells cooperate in anti-tumor resistance depending on the immunogenicity of experimental models.
We observed increased frequency of TBET\textsuperscript{+}EOMES\textsuperscript{+}CD8\textsuperscript{+} T cells among TILs in \textit{Il1r8}\textsuperscript{-/-} mice. Moreover, we showed that EOMES-expressing cells produced higher GZMB levels compared with TBET\textsuperscript{+}EOMES\textsuperscript{-} T cells.

Both in basal conditions and in T cells from tumor bearing mice, we observed that IL-1R8 affects EOMES expression and activity. Since EOMES appears as a context- and timing-specific TF, recent studies characterized CD8\textsuperscript{+} T cells that co-express TBET and EOMES. It was demonstrated that tissue-resident TBET\textsuperscript{+}EOMES\textsuperscript{+}CD45RO\textsuperscript{-}CD69\textsuperscript{+} T cells were significantly enriched in melanoma patients that responded to immunotherapy [268]. Increased frequency of EOMES\textsuperscript{+} TBET\textsuperscript{+} CD8\textsuperscript{+} T cells also correlated with favorable prognosis in Cytomegalovirus (CMV), as suggested by comparing patients that controlled the infection versus relapsers [156].

We observed that IL-1R8 genetic silencing enhanced the responsiveness to IL-2. In particular, IL-1R8-deficient CD8\textsuperscript{+} T cells upregulated EOMES in consequence of the stimulation with this cytokine. Moreover, stimulated IL-1R8-deficient T cells showed higher expression levels of CD25, that is part of the IL-2R complex, which could explain the sensitization to IL-2. In line with these evidences, constitutive expression of EOMES in OT-1 CD8\textsuperscript{+} T was shown to increase cell survival, proliferation and effector functions. Moreover, EOMES-overexpressing T cells showed high level of CD25 and consequently enhanced responsiveness to IL-2 [157].
Further analyses will be necessary to elucidate the link between IL-1R8 and IL-2. Presently, ILR and TLR adaptor proteins are not included among IL-2 downstream regulators. However, the IL-2-mediated signaling pathway was shown to require tyrosine kinases JAK1 and JAK3, which in turn were described to drive PI3K-AKT-mTOR, STAT5 and ERK1/2 signaling axis [273]. CD8+ T cells were demonstrated to be influenced by the mTOR pathway, which affected both TBET and EOMES expression, as revealed by treating naïve T cell with rapamycin [274]. In other cellular context (e.g. NK cells, Th17 cells), IL-1R8 was shown to influence mTOR-mediated pathways [32, 39] thus indicating a downstream target possibly affected by IL-1R8 genetic silencing in consequence of the stimulation with IL-2. Future experiments of RNA-seq and ATAC-seq analyses of stimulated T cells will be necessary to explore the global effects of IL-1R8 genetic silencing, in order to elucidate the mechanisms that lead to EOMES and TBET co-expression.

Immune checkpoints are molecular targets because of their expression in immune cells from cancer patients. We demonstrated that IL-1R8 is expressed by circulating T cells collected by ALL, CLL patients and TILs from mouse tumors. Thus, IL-1R8 emerges as a promising candidate immune checkpoint of T cells; moreover, the expression of IL-1R8 in mouse TILs emphasizing the biological relevance of pre-clinical models to characterize its function in CD8+ T cells.
To assess the role played by IL-1R8 in human CD8\(^+\) T cells, we generated stable knock-out primary cells using CRISPR-Cas9. As observed in mouse, primary human IL-1R8-silenced T cells showed the enhancement of Type-1 responses upon the stimulation with IL-12 and IL-18 stimulation; in addition, we found a significant sensitization to IL-2, indicating conservation between mouse and human of IL-1R8 regulatory functions.

Recent publications demonstrated that T cell responses could be reinvigorated with IL-2 administration, activating specific cellular clusters. In a hepatitis B virus (HBV) infection model, T cell priming by Kupffer cells generated a defective subset, poorly mature, unresponsive to PD-L1 but oversensitive to IL-2 [275]. Notably, recombinant IL-2 composed of a tumor-targeting antibody (Ab) and a super mutant IL-2 (sumIL-2) were shown to selectively activate CTLs in tumors [276].

Application of CAR-T cells for neoplastic diseases and the augmentation of their anti-tumor activity is an important frontier in immunotherapy. A promising therapeutic tool consists in engineering immune checkpoint expression. IL-18 expression is one of the modifications suggested for 4th generation CAR-T cells. We reported that CAR-T cells express IL-1R8, which hence emerges as a promising candidate to promote IL-18- and IL-2-dependent CAR-T cell efficacy.
The last part of my thesis was dedicated to IL-1R8 regulation. In particular, we discovered an intragenic and independent promoter that transcribes IL-1R8 truncated forms. The existence of an intracellular soluble TIR domain-containing fragment appears as an intriguing question since truncated forms of other decoy receptors, such as IL-1R2, were shown to play relevant biological activities [12]. Future experiments will be aimed at cloning and overexpressing IL-1R8 isoform sequences to assess their effects in primary immune cells, including T cells. In addition, our data suggested that the inflammatory stimuli reduced IL-1R8 expression, as observed by polarizing macrophages, while on the contrary promoted truncated forms. The modulation of IL-1R8 isoforms could be itself a targetable mechanism to enhance immune cell activation.

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