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Fulminant viral hepatitis in human inherited IL-10RB deficiency

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Title: Fulminant viral hepatitis in human inherited IL-10RB deficiency

Abstract:

The key question within the infectious disease field remains elusive in its answer: why in a certain population where all individuals are exposed to the same pathogens only a certain amount of them will develop the disease after infection? The inter-variability within infected individuals can range in certain diseases from asymptomatic to life-threatening forms. The presence of pathogen is necessary to develop the disease but is not enough to explain these differences. One theory that could help us answer that question is the genetic theory of infectious disease. This theory postulates that susceptibility to severe forms of infectious disease is due to genetic defects affecting the immune response to the considered pathogen. This hypothesis has helped unveil hundreds of several primary immunodeficiencies such as defects in the genes *CIB1, RLTPR/CARMIL2*, *IRF4*, *IRF7*, etc. It is our theory that fulminant viral hepatitis (FVH) is no different.

My thesis work is mainly dedicated to the study of one consanguineous kindred with two affected individuals in which the index patient suffered from gastroenteritis and succumbed due to hepatic failure; and her sibling died from a combination of early-onset inflammatory bowel disease (EOIBD) and severe hepatitis due to hepatitis A virus (HAV). In the second patient I identified a homozygous W100G mutation in *IL10RB,* encoding the interleukin 10 receptor subunit beta. Autosomal recessive IL10RB deficiency is known to cause EOIBD. It is a severe, life-threatening disease, if not treated by bone marrow transplantation at early age. Over 30 patients have been reported with different *IL10RB* mutations and EOIBD with or without some other clinical manifestations. IL-10RB acts as a receptor for several cytokines, including IL-10, IL-22, IL-26 and IFNλs. Up to now, functional evaluations of IL-10RB have been focused on IL-10 signaling and rarely on other cytokines, leaving out the question if other pathways can also be altered and contribute to the disease pathogenesis. Therefore, I functionally characterized all reported *IL10RB* missense alleles and two stop-gain mutations in an overexpression system. I also tested patient cells carrying in homozygosity either the missense W100G IL-10RB or the stop-gain mutation W159X IL-10RB.

The IL-10RB W100G mutation carried by our second patient in an overexpression system generated: a decrease in total protein expression, an altered glycosylation, a lowered surface expression, a blunt response to IL-10 through STAT1 and STAT3 phosphorylation, and a normal response after IL-22, IL-26 and INF-λ1 stimulation. All other reported missense mutations shared the altered response to IL-10 stimulation, although the observed response patters in some cases differed from those seen with the W100G mutation. Homozygous W100G IL-10RB cells from another, unrelated EOIBD patient showed lowered surface IL-10RB expression accompanied with impaired responses to IL-10, IL-22, IL-26 and INF-λ1. Reconstitution with IL-10RB wild type in homozygous W100G IL-10RB cells corrected the phenotype, except for the impaired IL-26 response.

Our data are showing that the impact of each mutation might be different in function of the signaling pathway studied, suggesting a functional dissociation depending of the mutation. This might explain some clinical differences observed among all reported patients. Nevertheless, the EOIBD phenotype is probably caused by a defect downstream of IL-10, since all pathogenic mutations tested shared this phenotype. We previously reported IL-18BP deficiency as the first genetic cause of FVH following HAV infection. IL-18BP is an antagonist of IL-18, which is an inflammatory cytokine. Strikingly, IL-10 is an anti-inflammatory cytokine. Therefore both the lack of the first or second FVH disease causing genes function unleashes uncontrolled inflammation after hepatitis A infection. Inherited IL-10RB deficiency should be considered in patients with severe viral hepatitis and patients with EOIBD should be vaccinated against HAV.

Keywords: IL-10RB, IL-10, IL-22, IL-26, IFN-λ1, viral hepatitis, Inflammation, human genetics

Titre: Hépatite virale fulminante dans le déficit héréditaire en IL-10RB chez l'homme

Résumé:

La variabilité inter-individuelle dans l'expression clinique des maladies infectieuses est large allant de formes asymptomatiques ou bénignes à des formes sévères où le pronostic vital peut être engagé. L'agent pathogène est nécessaire pour développer la maladie mais il ne suffit pas à expliquer ces différences. La théorie génétique des maladies infectieuses postule que la sensibilité aux maladies infectieuses est due à des défauts génétiques affectant la réponse immunitaire en général ou à l'agent pathogène considéré. Cette hypothèse a permis de dévoiler des centaines de déficits immunitaires primaires tels que des défauts dans les gènes *CIB1, RLTPR / CARMIL2, IRF4, IRF7*, pour ne citer qu'eux. Notre hypothèse est que la théorie génétique peut aussi s'appliquer à l'hépatite virale fulminante (HVF).

Ma thèse a été consacrée à l'étude d'une famille consanguine dont le patient index a souffert de gastro-entérites et a succombé en raison d'une hépatite fulminante; sa sœur est décédée d'une combinaison de maladie inflammatoire intestinale à début précoce (EOIBD) et a développé une hépatite sévère au virus de l'hépatite A (HAV). J'ai identifié une mutation homozygote W100G dans *IL10RB*, gène codant la sous-unité bêta du récepteur de l'interleukine 10 (IL-10). Le déficit autosomique récessif en *IL10RB* est connu et est associé au phénotype d'EOIBD, de même que la mutation W100G. L'IL-10RB est aussi l'une des chaînes des récepteurs à l'IL-22, l'IL-26 et aux interférons (IFN)-λ. Jusqu'à présent, la caractérisation fonctionnelle des défauts en IL-10RB s'est focalisée sur la signalisation de l'IL-10, laissant de côté la question de savoir si la réponse aux autres cytokines pouvait également être modifiée. J'ai donc entrepris de caractériser tous les allèles faux-sens identifiés dans l'IL-10RB et deux mutations stop-gain dans un système de surexpression. J'ai également testé les cellules de patients déficients en IL10RB portant la mutation W100G ou la mutation W159X.

Dans le système de surexpression que j'ai utilisé, la mutation IL-10RB W100G se caractérise par une diminution de l'expression protéique totale ou transmembranaire, un profil de glycosylation modifié, une réponse à l'IL-10 réduite, et enfin une réponse normale après stimulation par l'IL-22, l'IL-26 et l'IFN-λ1. Les autres mutations faux-sens testées présentaient une réponse modifiée à la stimulation par l'IL-10, et des réponses variables en fonction des cytokines et des mutations. Des cellules homozygotes pour la mutation W100G ont montré une expression de surface réduite, accompagnée d'une réponse altérée en réponse à l'IL-10, l'IL-22, IL-26 et l'IFN-λ1. La reconstitution avec l'IL-10RB de type sauvage dans des cellules homozygotes W100G IL-10RB a corrigé le phénotype en réponse à l'IL-10, l'IL-22 et l'IFN-λ1.

Nos données montrent que l'impact de chaque mutation peut être différent en fonction de la voie de signalisation étudiée, suggérant une dissociation fonctionnelle en fonction de la mutation. Cela pourrait expliquer certaines différences cliniques observées chez tous les patients rapportés. Néanmoins, le phénotype EOIBD en lui-même est causé par un défaut en aval de l'IL-10, puisque toutes les mutations pathogènes testées partagent ce phénotype et que des mutations dans les gènes codant l'IL-10 ou la seconde chaine de son récepteur IL10-RA chez des patients avec un EOIBD ont aussi été rapportées. Nous avons précédemment rapporté un déficit en IL-18BP comme la première cause génétique de FVH. L'IL-18BP est un antagoniste de l'IL-18, qui est une cytokine inflammatoire. De manière frappante, l'IL-10 est une cytokine anti-inflammatoire. Ces deux défauts génétiques se caractérisent donc par une réponse inflammatoire incontrôlée, pouvant expliquer le phénotype d'hépatite fulminante. Un déficit héréditaire en IL-10RB doit être envisagé chez les patients atteints d'hépatite virale sévère et les patients atteints d'EOIBD doivent être vaccinés contre le HAV.

Mots clefs: IL-10RB, IL-10, IL-22, IL-26, IFN-λ1, Hépatite virale, Inflammation, génétique humaine

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Abbreviation list

AR: Autosomal recessive

CADD: Combined Annotation Dependent Depletion

CMV: Cytomegalovirus

DHF: Dengue hemorrhagic fever

EBV: Epstein–Barr virus

EOIBD: Early onset inflammatory bowel disease

EV: Empty vector

FVH: Fulminant viral hepatitis

HAV: Hepatitis A virus

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HEK293T: human embryonic kidney 293 cells expressing the SV40 large T antigen

HEV: Hepatitis E virus

HSV: Herpes simplex virus

IFN-: Interferon

IL-: Interleukin

NT: Non transfected

Pt: Patient

SIRS: Systemic inflammatory response syndrome

WT: Wild type

YFV: Yellow fever virus

Introduction

The germ theory brought by the works of Pasteur, John Snow, Francesco Redi and many others, postulates that pathogens are a *sine qua non* condition to infectious diseases[1]. Nevertheless, pathogen presence is not sufficient to develop the disease. As Charles Nicolle pointed out within a population exposed to the same pathogen only a part of its individuals will develop an infection and this infection will vary on its severity among the affected[1]. Why certain individuals are more affected than others? This has been the key question in the field of infectious disease unanswered for many years. At least some of this disparity of the pathogen effect on individuals can be explained by the genetic theory of diseases. This theory postulates that healthy individuals infected by a pathogen and developing a severe disease carry a defect in their genome[1]. This defect would affect selectively the immunity to a pathogen or a class of pathogens[1].

One of the main function of the immune system is to protect the organism against pathogens[2]. The immune system is constituted by physical barriers, soluble particles and cells[2]. After a pathogen has managed to go inside the organism through one of the physical barriers such as the skin, the intestinal mucosa, or the pulmonary epithelium; the innate immune response is triggered first followed by the adaptive one[2].

The innate immune response is constituted by many different cells such as the NK cells, and myeloid cells, such as monocytes, macrophages, dendritic cells and granulocytes, including neutrophils, basophiles, and eosinophils[2]. All of them can be further classified and it's out of the scope of this introduction to even attempt to exhaustively do so. Soluble factors are also components of the innate immune response: the complement cascade that can degrade microorganism by pore formation on their membranes and opsonization; and the specialized chemical mediators that are called cytokines that can both induce and repress the immune response, and where a subset of them can have chemotactic qualities and be equally called chemokines[2]. Many cells that we do not typically think as immune cells also play a crucial role in the innate immune response: for example fibroblasts and hepatocytes, after being stimulated/infected, can secrete certain cytokines and also have expressed some cytokines/chemokines receptors[2,3]. Once the pathogen has entered the

organism, the immune cells that it may encounter or the ones attracted by chemokines could recognize the pathogen, internalize it by phagocytosis or destroy it through lysis, oxidative burst, or by antimicrobial peptides[2]. Parallel to this function, they can also trigger the adaptive immune response particularly through antigen presentation[2]. The adaptive immune response is also constituted by cells and soluble agents: mainly B and T cells; and antibodies as soluble agents[2]. The adaptive immune response is far more specific than the innate immune response[2].

Any defect on any of the components of either the innate or the adaptive immune response will lead to immunodeficiency[1]. Immunodeficiency will be acquired if it is secondary to another condition, such as an immunosuppressive treatment or a disease that compromises its components such as the human immunodeficiency virus[1]. Primary immunodeficiencies are generated when there is a genetic affecting the immune response: any soluble component, any receptor, any transcription factor, any signaling protein that are involved in the fight against pathogens theoretically can fail –if not lethal in utero- and therefore cause immunodeficiency[1,4]. This work will try to find one of such immunodeficiencies, particularly to severe hepatitis infection.

STUDY OF A NOVEL MONOGENIC CAUSE OF FULMINANT VIRAL HEPATITIS

FULMINANT VIRAL HEPATITIS

DEFINITION

Fulminant viral hepatitis (FVH) is a rare syndrome associated with massive necrosis of the liver parenchyma and an acute atrophy produced by a viral infection in a patient without previously known hepatopathy. The definition also includes an altered coagulation and hepatic encephalopathy manifesting in the eight weeks after the onset of symptoms. Jaundice alongside other clinical signs can be also present[5–8]. The overall outcome is poor, with some studies showing less than 25% survival rate without liver transplantation, depending of co-morbidities[5,6,9–12]. The systemic inflammatory response syndrome (SIRS) to the hepatocyte death and liver injury caused by the virus could be behind the two most common lethal complications: cerebral edema and multi-organ system failure[13]. SIRS is manifested by the release of pro-inflammatory cytokines such as interleukin (IL)-6, IL-18, TNFα, IL-1β[14–16] and anti-inflammatory cytokines such as IL-10[16,17].

The most common causes of FVH are primary infections with hepatitis A virus (HAV); hepatitis B virus (HBV); or, in pregnant women and immunosuppressed individuals, hepatitis E virus (HEV)[18–20]. Less common FVH causing viruses are: cytomegalovirus (CMV), herpes simplex virus (HSV), Epstein–Barr virus (EBV), hepatitis C virus (HCV), dengue hemorrhagic fever (DHF) and yellow fever virus (YFV)[21–27]. In the vast majority of cases, FVH strikes otherwise healthy individuals, normally resistant to other infectious agents[5–8]. The incidence of FVH is not well known but has been estimated at about 0.5% of symptomatic HAV individuals[28]. Since the prevalence of HAV infection varies greatly from country to country, as does the HAV vaccination coverage, the estimations of FVH have varied from ~0.6 cases per million individuals in Scotland[29] to ~4 cases per million in Thailand[30].

WHY STUDY MONOGENIC CAUSES OF FVH?

No FVH epidemic has been reported, suggesting that FVH is not the consequence of the emergence of a new, high-virulent viral strain. Specifically for HAV, particular HAV genotypes were not found to account for FHV[31,32]. Although most cases are sporadic, some families are multiplex and/or consanguineous, suggesting that genetic factors might be involved, at least in some individuals[33–35]. Our laboratory has shown that a variety of life-threatening infectious diseases striking otherwise healthy individuals, including a number of organ-specific viral illnesses, can be caused by single-gene inborn errors of immunity[1,36–40]. Such as the work I participated in unveiling defects in type I interferons (IFN) immunity as a cause of severe COVID-19[41]. We thus hypothesize that inborn errors of liver immunity may underlie FVH in at least some individuals. Interestingly, patients with primary immunodeficiencies affecting T and/or B lymphocytes do not develop any FVH following infections, suggesting that the inborn errors may affect the innate immune response. Finally our group has described the first monogenic cause of FVH with interleukin 18 binding protein (IL-18BP) deficiency[42], which, as predicted, is part of the innate immune response. The discovery of autosomal recessive (AR) IL-18BP deficiency has validated the hypothesis that inborn errors could lead to FVH, but also the feasibility of studying monogenic causes of FVH.

Another reason to embark in this work is that despite the fact that vaccination has proven to eradicate FVH and deaths due to HAV in countries such as Argentina[43,44], many countries still do not include HAV vaccination on their routine immunization calendar[28,45]. Vaccinations for other viruses causing FVH such as for HBV and YFV are still not universally applied[46,47]. Despite progress being made, there are no commercial available vaccinations for EBV, CMV and DHF (only available after first infection)[48–50]. The lack of vaccination propels the resulting FVH cases, costly treatments and deaths. Therapeutics for FVH are mainly supportive, meaning you can only treat the symptoms, and can include liver transplantation[51]. Thus unveiling new genetic etiologies for the disease could disclose new pharmacological targets to investigate.

IL-18BP DEFICIENCY: FIRST MONOGENIC CAUSE OF FVH

As previously mentioned, we have identified IL-18BP deficiency as the first genetic etiology of FVH following HAV infection in one consanguineous family[47]. The index case from this family presented a homozygous deletion on IL-18BP causing splicing errors leading to non-functional proteins. IL-18BP is a constitutively secreted, naturally circulating neutralizer of the cytokine IL-18[52]. IL-18 is a pleiotropic inflammatory cytokine, which was first discovered in mouse liver and described as an activator of NK cells and T cells in addition to its potent IFN-γ-inducing activity[53]. The deletion in *IL18BP* on the patient generated three aberrant splice variants, that were incapable of neutralizing IL-18 and therefore inhibit IFN-γ secretion. This work also showed how IL-18BP blocked IL-18 and how this could stop NK cells from killing HAV infected hepatocytes. Thus non-functional IL-18BP generated elevated IL-18 signaling that could cause a hyper-inflammatory state responsible for the liver symptomatology due to hepatocyte death.

RESULTS

IL-10RB deficiency: Second monogenic cause of FVH

DISCOVERY OF THE VARIANT W100G *IL10RB* IN AN AFFECTED FAMILY

We studied a 6 year old female from a consanguineous family who died from hepatic failure (P1, Figure 1), with only the relevant clinical antecedent of diarrhea. One of her sisters, P2, developed chronic diarrhea and failure to thrive attributed to celiac disease. Celiac disease was diagnosed based on positive serology and findings of moderate chronic inflammation and subtotal to total villous atrophy on intestinal biopsy. She was admitted to the hospital three times for pneumonias and suppurative otitis media. At 2 years of age, she presented with lethargy, yellow discoloration of sclera and skin and elevated liver enzymes. Serology was positive for hepatitis A. P2 recovered from HAV, but her bowel disease worsened with multiple perforations in the colon, requiring total colectomy and ileostomy.

At 3 years old the patient developed hypotension and hypothermia resembling septic shock, progressing to multi-organ failure and died due to cardiac arrest.

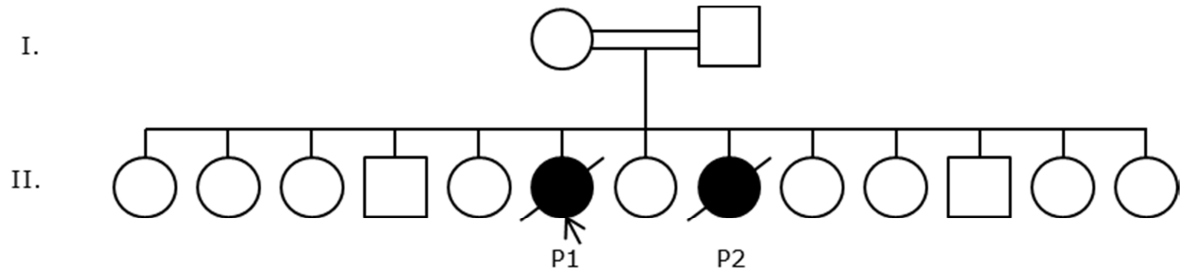

Figure 1: Pedigree of the family. Circles are females, squares are males. Double horizontal line indicates consanguinity. The patients are shown in black, whereas healthy individuals are shown in white. Crossed individual signifies deceased. Arrow indicates index patient.

There was no genetic material available from patient 1. A materials and methods section for all the following results can be found on the Annexe 1. We performed whole exome sequencing on P2 DNA. Since the parents of both sisters are first degree cousins and P2 homozygosity rate[54] was 4.71%, we hypothesized that the genetic defect causing their disease could be most likely autosomal recessive. Therefore, we only analyzed the homozygous mutations. We discarded variants with <50 genotype quality, <5 reads, no consensus coding sequence, >13.84 gene damage index (GDI)[55], >0.001 allele frequency in any population present in GnomAD, combined annotation dependent depletion (CADD) score V3.1<Mutation Significance Cutoff (MSC) at 99%[56] and present in our in-house blacklist[57]. We also discarded variants that were not included in the following categories: indel-inframe, indel-frameshift, start-lost, essential splicing, missense, nonsense, stop-lost, and splicing. All variants left were manually checked on Alamut 2.9.0 to discard artifacts. Finally only 18 variants were left (Table 1).

Table 1: Homozygous mutations present on patient 2.

Chr	Pos	Ref	Alt	Function	Gene	AAChange	Sift_Pred	Polyphen2HVAR_Pred	CADD_Phred	MSC	GDI	Known PID?
21	34649025	T	G	missense	*IL10RB*	p.Trp100Gly	D,D	D,D,D,D	29.8	23.8	10.34	Yes
2	208725979	T	C	missense	*PLEKHM3*	p.Glu653Gly	D,D	P	29.7	2.31	3.26	No
16	88926306	C	G	missense	*TRAPPC2L*	p.Phe100Leu	D	P,P	25.9	23.84	5.19	No
6	41303876	C	T	missense	*NCR2*	p.Thr35Met	D,D,D	D,D,D	23.3	2.31	1.78	No
20	47269910	C	T	missense	*PREX1*	p.Glu779Lys	T,T	B,B	20.9	2.31	13.43	No
7	15725797	ATGG	A	indel-inframe	*MEOX2*	p.His77del			20.5	4.78	5.44	No
4	8307852	G	T	missense	*HTRA3*	p.Val451Leu	T	B	15.35	2.31	2.85	No
2	238737852	T	A	splicing	*RBM44*				10.96	2.31	4.67	No
2	238737860	A	G	splicing	*RBM44*	p.Arg868Arg			10.79	2.31	4.67	No
13	38320594	TA	T	splicing	*TRPC4*				9.94	2.31	1.08	No
2	238737854	T	G	splicing	*RBM44*				8.267	2.31	4.67	No
16	12009530	C	CCCG	indel-inframe	*GSPT1*	p.Gly16dup			7.76	2.31	10.44	No
7	131241029	G	GGGCGAC	indel-inframe	*PODXL*	p.Ser29_Pro30dup			5.726	2.31	3.35	No
20	32664864	C	CCAG	indel-inframe	*RALY*	p.Ala230_Gly231insSer			4.967	2.31	5.17	No
19	49657889	T	TTCC	indel-inframe	*HRC*	p.Glu202dup			4.004	2.31	10.86	No
13	25355965	GT	G	splicing	*RNF17*				3.637	2.31	9.75	No
3	158537415	CTTTTT	C	splicing	*MFSD1*				2.493	2.31	5.61	No
17	78081526	A	AGCAGCGG	splicing	*GAA*				0.341	0.01	7.92	No

From all the variants the one with the highest CADD score and the only one already described as deleterious[58] was IL-10RB W100G, making it the most likely candidate disease causing variation. Allele segregation also matched our theory since both parents are heterozygous (see paper).

In addition, in the general population no homozygous predicted loss of function variations in *IL10RB* are present with a CADD score above the MSC (Figure 2).

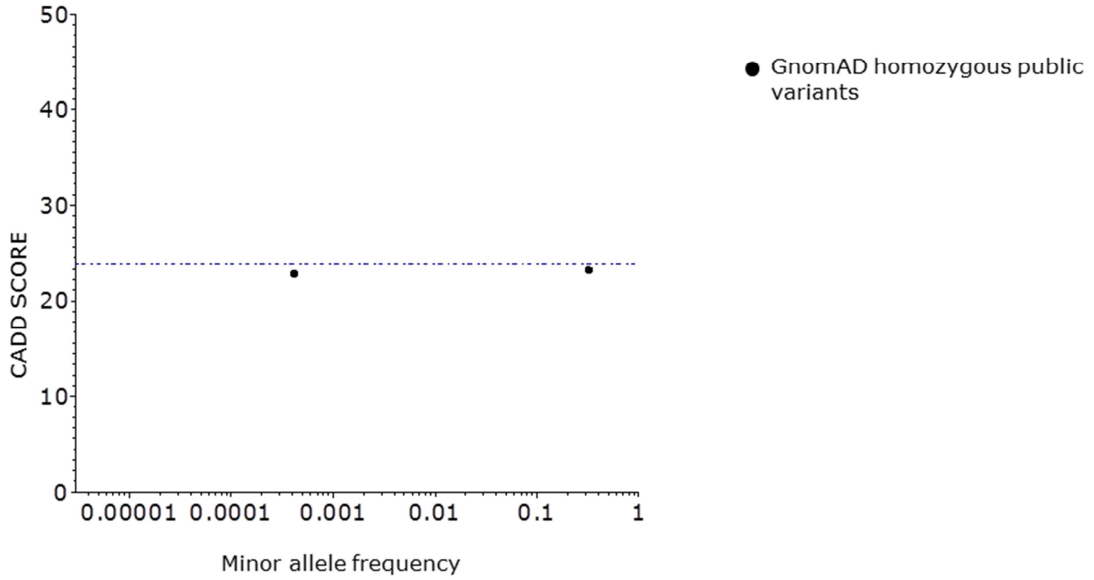

Figure 2: CADD and minor allele frequency of public homozygous IL10RB variants.

AGONISTS OF IL-10RB

The known function of IL-10RB is to be a receptor for IL-10, IL-22, IL-26 and IFN-λs[59,60]. The function of these cytokines is diverse. IL-10 is mainly acting as an anti-inflammatory cytokine, which limits tissue damage during infections but it might be also an immunostimulator[61]. IL-22 has many biological properties, including tissue repair and proliferation, such as liver regeneration[62] but it is also known to induce pro-inflammatory acute phase reactants in the liver[60,63,64]. The function of IL-26 is still in debate but it seems to have pro-inflammatory and also antiviral properties[65,66]. Finally, the IFN-λs have mainly antiviral properties, including inhibition of HBV and HCV replication[67,68]. The binding of the cytokine to the first chain of its receptor leads to the interaction with IL-10RB and the activation of signaling cascade through TYK2 and STATs proteins, including STAT3 and STAT1 depending of each cytokine[60] (Figure 3). IL-22 is also able to activate MAP kinase pathway[69].

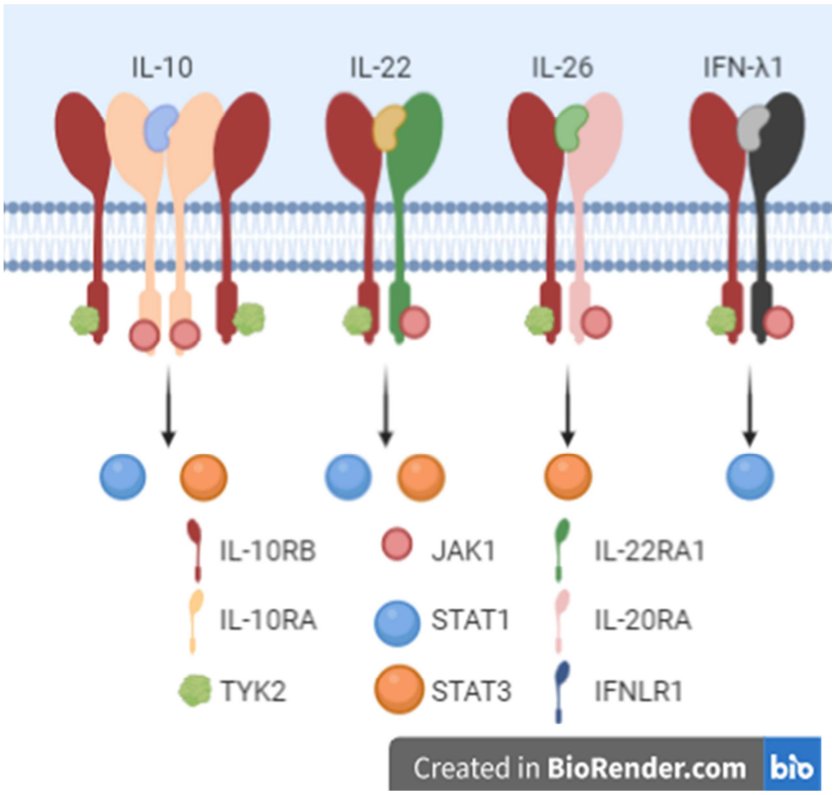

Figure 3: Graphical representation of signaling trough IL-10RB

All cytokines signaling trough IL-10RB are good candidates to be behind the pathogenesis of FVH. The variant IL-10 C627A, generates low IL-10 levels leading to increased risk of advanced liver disease in heavy drinkers[70]; and IL-10 treatment in chronic HCV patients could decrease hepatic inflammation score and increase HCV viral burden[71]. There is also evidence of IL-10 involvement in liver damage coming from mice models: in the concanavalin A model (T-cell mediated hepatitis), IL-10 neutralization can worsen liver damage[72], and on the contrary giving IL-10 can prevent it[73]; in the LPS or staphylococcal enterotoxin B (SEB) in D-galactosamine (GalN)-sensitized mice, IL-10 treatment can prevent liver damage[74]; and finally on the ischemia-reperfusion liver injury model IL-10 pretreatment can prevent liver damage[75]. Rats can be also used as an animal model for the cycloheximide-induced liver injury model where it was seen that neutralization of IL-10 can worsen liver damage[76].

A defective IL-22 function can also be linked to liver damage. In humans there are high IL-22 levels and producing cells in livers of HCV or chronic HBV patients[77,78]; and IL-22 treatment seems to improve liver regeneration in alcoholic hepatitis[79]. In mice in the concanavalin A model, the IL-22 neutralization and *IL22*^{KO} present worsened liver

damage[80,81], on the contrary both IL-22 pretreatment and IL-22-overexpressing mice prevented liver damage[80]; and finally in partial hepatectomy[82], non-alcoholic fatty liver disease[83], alcohol induced[84], in both bacterial[85] and parasitic infection[86], and ischemia-reperfusion[87] hepatic injury models IL-22 treatment prevented liver damage.

IL-26 is not that well studied, but still we can find links to viral hepatitis since IL-26 is enhanced in chronically HCV-infected patients and IL-26-stimulated NK cells kill HCV-infected hepatocytes in a TRAIL-dependent manner[88]. IFN-λ1 being more studied we can find more data related to liver damage: both HCV and HBV infection in humans presents with enhanced IFN-λ1 production in infected patients or in infected cells, IFN-λs inhibit viral replication[89–91], and polymorphisms in the *IFNL* region are related to HCV virus clearance[92,93]. In the Hep3B (HBV-Hepatocellular carcinoma) cell xenograft mouse model, IFN-λ1 treatment suppressed tumor growth and hepatitis B surface antigen secretion, and induced tumor cell apoptosis[94]. Finally in Chimpanzees subjected to HCV infection, IFN-λs were strongly induced and these levels correlated with IFN-stimulated gene (ISG) expression and viremia[91].

AUTOSOMAL RECESSIVE IL-10RB

IL10RB mutations have been reported to underlie early-onset inflammatory bowel disease (EOIBD), which could explain the gastrointestinal symptoms of both patients. We show the distribution of the CADD score and minor allele frequency of all reported mutations (Table 2 and Figure 4) and the distribution within the protein domains (Figure 5). Almost all pathogenic mutations' CADD scores clustered over the MSC with minor allele frequencies lower than 10^{-4}, as expected from a rare AR disease. And while reported pathogenic mutations are all over the protein, missense mutations are only located on the extracellular domain.

Table 2: IL-10RB mutations published with CADD 1.3 score when applicable.

Mutation	Allele frequency	CADD Score	Publication
p.W3X	Private	27.6	Shouval 2014[95]-2017[96]
c.50-2A>T	Private	16.92	Shouval 2016[97]-2017[96]
p.W18fsX12	0.00001988	24.7	Engelhardt 2013[98]/Shouval 2017[96]
p.S31F	Private	33	Yazdani 2019[98]
c.174-3300_5483del	Private	-	Neven 2013[99]/Pigneur 2013[58]
p.S58R	Private	22.6	Shouval 2014[95]-2016[97]-2017[96]
p.Y59C	Private	23.3	Neven 2013[99]/Pigneur 2013[58]
p.Y59_D110del	Private	-	Shouval 2014[95]-2017[96]
p.Y59_D110del_insCT	Private	-	Charbit-Henrion 2018[58]
p.C66Y	Private	24.4	Kotlartz 2012[100]
p.W100G	Private	29.8	Pigneur 2013[58]/This work
c.331+1G>C	Private	25.2	Engelhardt 2013[98]/Shouval 2017[96]/Mukhopadhyay 2020[101]
c.331+907_574del	Private	-	Kotlartz 2012[100]
c.332-1356_5148del	Private	-	Shouval 2014[95]-2017[96]
p.E141X	Private	36	Begue 2011/Neven 2013[99]/Pigneur 2013[58]
p.W159X	0.000003977	43	Glocker 2009/Kotlartz 2012[100]/Shouval 2014[95]-2017[96]/Beser 2015[101]/Karaca 2016[101]
p.G193R	Private	32	Engelhardt 2013[98]
p.W202X	Private	-	Shouval 2014[95]-2016[97]-2017[96]
p.W204X	0.000007953	43	Kotlartz 2012[100]
p.W204C	0.00002386	31	Neven 2013[99]/Pigneur 2013[58]/Gong 2019[102]
p.C209X	Private	36	Petersen 2017[102]
c.646+2T>C	Private	22.9	Petersen 2017[102]
p.S230X	Private	35	Kotlartz 2012[100]
p.W246X	0.000003976	36	Huang 2017[102]/Zheng 2019
p.F269fsX275	Private	26.3	Neven 2013[99]/Pigneur 2013[58]
c.*52C>T	Private	7.461	Kotlartz 2012[100]/Shouval 2014[95]-2016[97]-2017[96]

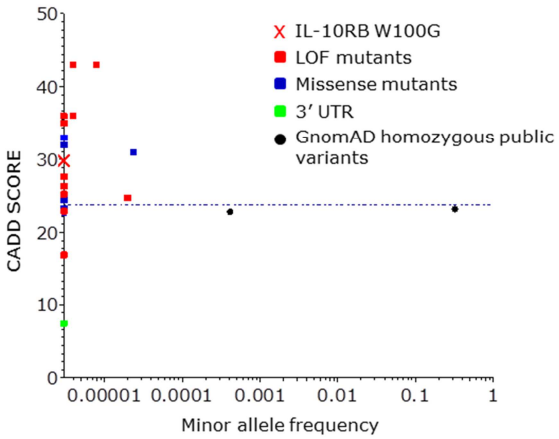

Figure 4: CADD score and minor allele frequency of our patients' mutation, all reported mutations, and public homozygous IL10RB *variants.*

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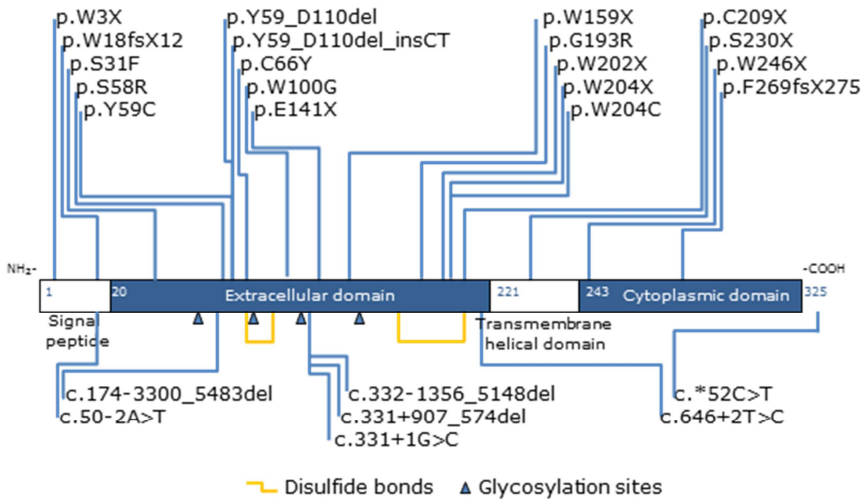

Figure 5: Distribution of all reported mutations of IL10RB *within the protein domains.*

Our main hypothesis is that, as for IL-18BP deficiency, the HAV infection induces an uncontrolled inflammation, which will lead to a massive destruction of hepatocytes. As the known functions of all IL10RB-dependent cytokines might be responsible of the clinical phenotype, in this thesis I have studied the impact of each missense mutation in all IL-10RB-dependent signaling and tried to connect it to FVH pathogenesis.

STUDY OF THE W100G IL-10RB ALLELE IN ISOLATION

PROTEIN EXPRESSION AND LOCALIZATION

In order to investigate the possible role of W100G in the development of FVH, we first studied the allele in overexpression system. Additionally, we included all reported missense *IL10RB* variants (Table 3) for comparison and two stop-gain variants (E141X and W159X) as negative controls.

Table 3: AR IL10RB *patients carrying at least one missense mutation.*

Mutation	Number of patients	Molecular mechanism	Patients' country of origin	Onset of disease (days)
S31F	1	Homozygous	Iran	60
S58R	1	Compound heterozygous	USA	<30
Y59C	1	Homozygous	Italy	14
C66Y	1	Homozygous	Bangladesh	45
W100G	2	Homozygous	France/Saudi Arabia	84/?
G193R	1	Homozygous	Turkey	10
W204C	2	Compound heterozygous/Homozygous	France/China	14/?

We started out by transfecting human embryonic kidney 293 cells expressing the SV40 large T antigen (HEK293T) cells with a pCMV6 plasmid encoding the cDNAs corresponding to all the aforementioned variants and measured the *IL10RB* mRNA expression after 24h of transfection. All missense variants tested were comparable to the *WT* allele, while the two stop-gains alleles (E141X and W159X) showed markedly decreased mRNA levels (Figure 6).

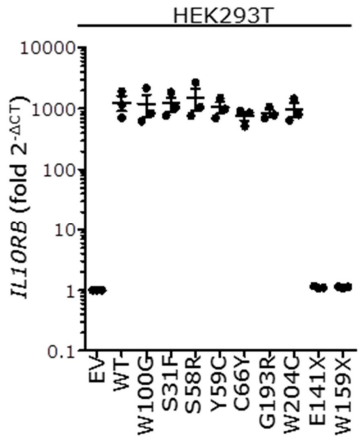

Figure 6: RT-qPCRs results from HEK293T transfected 24 hours with the indicated IL-10RB alleles. IL-10RB mRNA expression is shown corrected by the endogenous expression of HPRT1 mRNA and normalized to the first column set as 1. Values shown are the means of three independent experiments performed in duplicate ± SEM.

Using the same cell type, we additionally evaluated protein expression after 24 hours of transfection of all missense and the two stop-gain variants. We observed that the WT protein presented two bands at 50 kDa and 60 kDa, indicating post-translational modifications (Figure 7). All missense variants assayed presented a similar molecular weight with the exception of W100G presenting with a higher molecular weight in both bands. The

predicted stop gain mutations E141X and W159X both expressed a single lower band, indicating the presence of truncated proteins. Interestingly, two missense variants (C66Y and W204C) showed only one band. All alleles showed a lowered molecular weight post treatment with PNGase-F, an enzyme which cleaves between the innermost N-Acetylglucosamine and asparagine residues of high mannose, hybrid, and complex oligosaccharides. Therefore, PNGase-F treatment removes almost all N-linked oligosaccharides from glycoproteins. The higher molecular weight of W100G was corrected after de-glycosylation treatment, indicating that the mutation altered the glycosylation pattern. Furthermore six of the nine alleles tested (W100G, S31F, Y59C, C66Y, E141X and W159X) showed reduced or altered protein expression compared to WT IL-10RB.

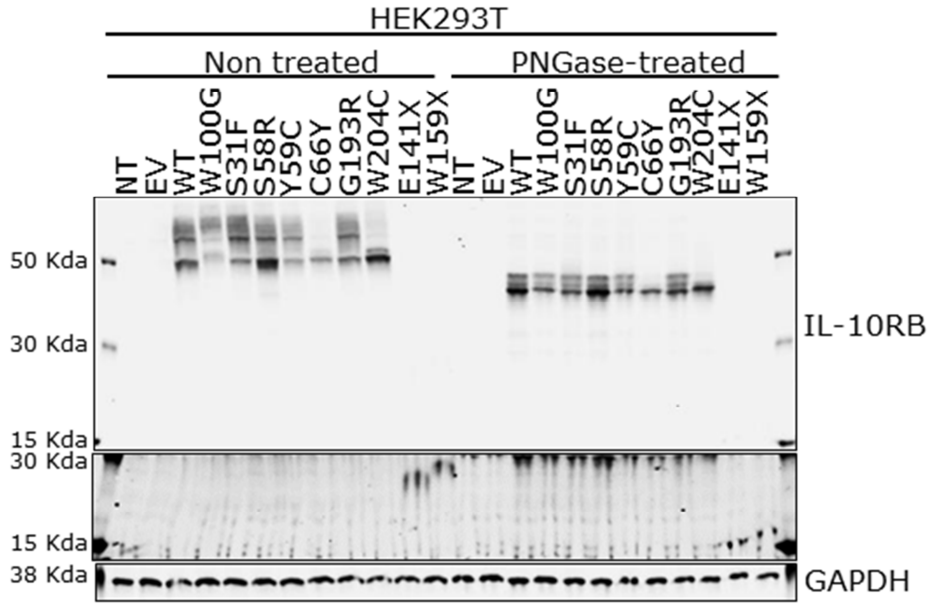

Figure 7: Altered molecular weight of W100G IL-10RB protein and correction post de-glycosylation treatment. Representative western blot showing IL-10RB protein expression in HEK293T cells either left non transfected (NT), transfected with the empty vector (EV), or a plasmid encoding the IL-10RB wild-type (WT) or the mutant alleles. Cell lysates were subjected to western blotting with or without PNGase F treatment. GAPDH is shown as a loading control.

IL-10RB is a surface receptor. Therefore it is of utmost importance for its functionality to be able to reach the surface to allow engagement of its co-receptors and agonists. The N-terminal part of the IL-10RB is extracellular, while the C-terminal part remains intracellular. All IL-10RB constructs were tagged C-terminally with a myc tag. To test the surface expression we made use of simian virus 40 (SV40) transformed fibroblasts from a patient with complete IL-10RB deficiency (IL-10RB^{KO})[103]. We transfected the SV-40 fibroblasts for 24 hours and stained both extra- and intracellular using the same antibody clone of IL-10RB in

different colors. Additionally, intracellular staining of the myc tag was performed (Figure 8). We could observe that all missense alleles were expressed in the cytosol comparable to WT IL-10RB by the myc antibody, while E141X and W159X were expressed at very low levels compared to WT IL-10RB. We could observe the same for the cytosolic staining of IL-10RB, with the exception of the alleles W204C, E141X and W159X that are not recognized by the used antibody (Monoclonal Mouse IgG1 Clone # 90220). Surface expression results for IL-10RB showed a more interesting picture: W100G and G193R showed a much decreased surface presence, while S31F, C66Y signals were indistinguishable from the cells transfected with the EV. W204C, E141X and W159X showed no positive signal concordantly with the cytosolic staining results, since both antibodies are from the same clone. Taking all these results in consideration we might conclude that the mutations W100G, S31F, and G193R allow for protein expression, but lead to reduce or no surface localization. Finally the only variants with intact surface expression are S58R and Y59C.

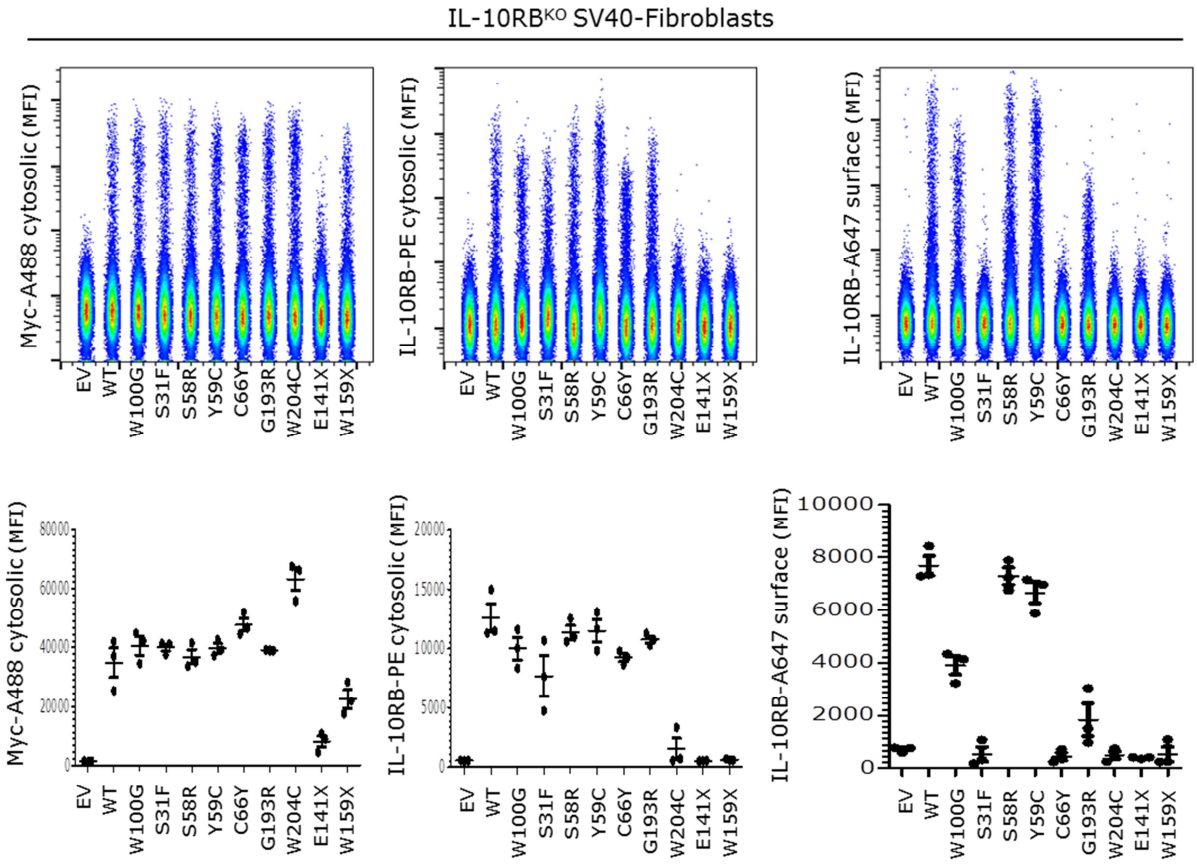

Figure 8: Altered surface expression of W100G IL-10RB protein. Flow cytometry of the cytosolic tag Myc; and surface and cytosolic IL-10RB on transfected IL-10RB^{KO} SV-40 fibroblasts 24 hours with indicated IL-10RB-Myc alleles tagged on the cytosolic C-terminal. We show dot-plot of one representative experiment of each staining and the accumulated three independent experiments by mean fluorescent intensity (MFI) on IL-10RB+ gate ±SEM.

Since we could not evaluate surface expression of W204C with the anti-IL-10RB clone at our disposal, we created N-terminally myc tagged plasmids using the same backbone as before. We were not certain, if the IL-10RB epitope recognized by the antibody clone we used for the previous experiments could be masked on the cell surface by an altered tertiary structure due to the mutations S31F and C66Y, therefore we also decided to make new N-terminally myc tagged constructs for S31F and C66Y. When we transfected IL-10RB^{KO} SV-40 fibroblasts for 24 hours and surface stained for myc, we could now observe that actually S31F had a higher surface expression than WT IL-10RB, while C66Y and W204C had a lower one, but still higher in comparison to EV transfected cells.

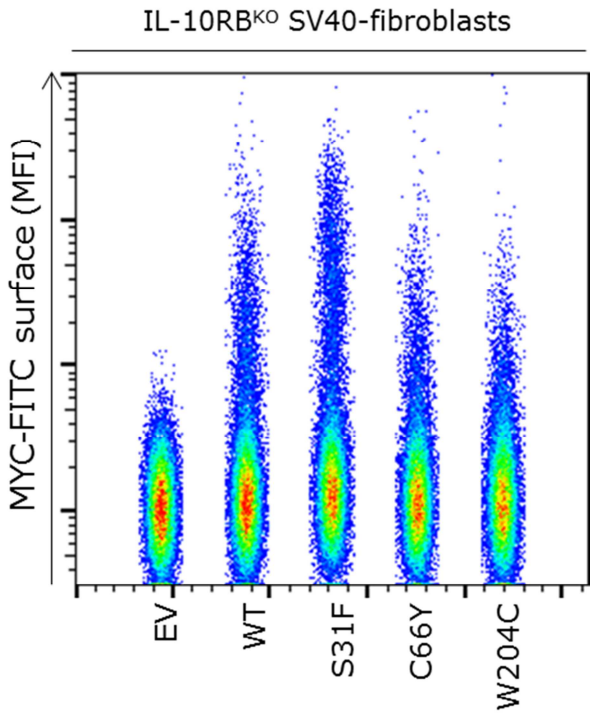

Figure 9: Surface expression of S31F, C66Y and W204C IL-10RB proteins. Flow cytometry of the surface tag Myc on transfected IL-10RB^{KO} SV-40 fibroblasts after 24 hours with indicated IL-10RB N-terminally Myc-tagged alleles. We show one dot-plot of one representative experiment.

RESPONSE TO IL-10

We tested the response to IL-10 by transfecting with *WT* or mutant *IL10RB* (W100G, S31F, S58R, Y59C, C66Y, G193R, W204C, E141E or W159X) for 24 hours in IL-10RB^{KO} SV-40 fibroblasts. As fibroblasts do not express the required co-receptor of IL10-RB for IL-10 signaling, we also co-transfected *WT IL-10RA* V5. We could observe the same altered glycosylation pattern and differences in molecular weight as observed in the HEK293T cells,

with the difference that the expression levels were more comparable. This may possibly due to the higher transfection efficiency in HEK293T cells in comparison to SV-40 fibroblasts (Figure 10A). The alleles W100G, C66Y, and W204C showed hypomorphic responses in terms of STAT1 and STAT3 phosphorylation after IL-10 stimulation, while S31F, S58R, Y59C, G193R, E141X and W159X showed a completely impaired response in comparison to IL-10RB WT. When we assayed the response of W100G, Y59C, E141X, and W159X to IL-10 stimulation in terms of *CXCL9* expression we found that W100G was hypomorphic and Y59C, E141X and W159X had abrogated responses, concordantly with the results of STATs phosphorylation (Figure 10B).

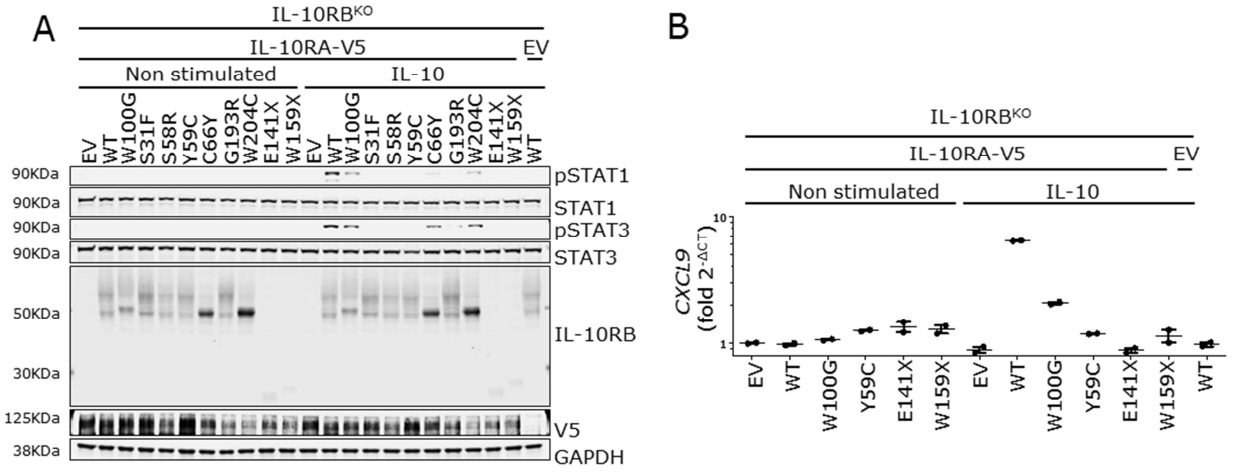

Figure 10: The response of all assayed IL-10RB pathogenic mutations is altered after IL-10 stimulation. A- Response to IL-10 stimulation 40ng/ml for 30 minutes on IL-10RB^{KO} SV-40 fibroblasts transfected with indicated IL-10RB alleles and IL-10RA-V5 on representative immunoblot image showing the analysis of STAT1 and STAT3 phosphorylation (pSTAT1; pSTAT3). STAT1, STAT3, GAPDH, V5 (IL-10RA) and IL-10RB levels were also assessed. B- Response to IL-10 stimulation 40ng/ml for 2 hours on IL-10RB^{KO} SV-40 fibroblasts transfected with indicated IL10RB alleles and IL10RA-V5 analyzed RT-qPCR showing the mRNA levels of CXCL9*. mRNA expression is shown corrected by the endogenous expression of HPRT1 mRNA. Results are normalized to the empty vector. Values shown are the means of two independent experiments performed in duplicate ± SEM.*

RESPONSE TO IL-22

We proceeded to test the response to IL-22 in similar way as to IL-10, with the exception that now we were co-transfecting *IL22RA1* C-terminally his tagged alongside WT *IL10RB* or the pathogenic variants (W100G, S31F, S58R, Y59C, C66Y, G193R, W204C, E141E or W159X). The alleles W100G, S58R, and Y59C responded comparable to WT after IL-22 stimulation in terms of both STAT1 and STAT3 phosphorylation; and *CXCL9* mRNA induction (Figure 11A and B). On the contrary, the alleles C66Y and W204C were hypomorphic and the alleles S31F, G193R, E141X and W159X had completely abrogated responses.

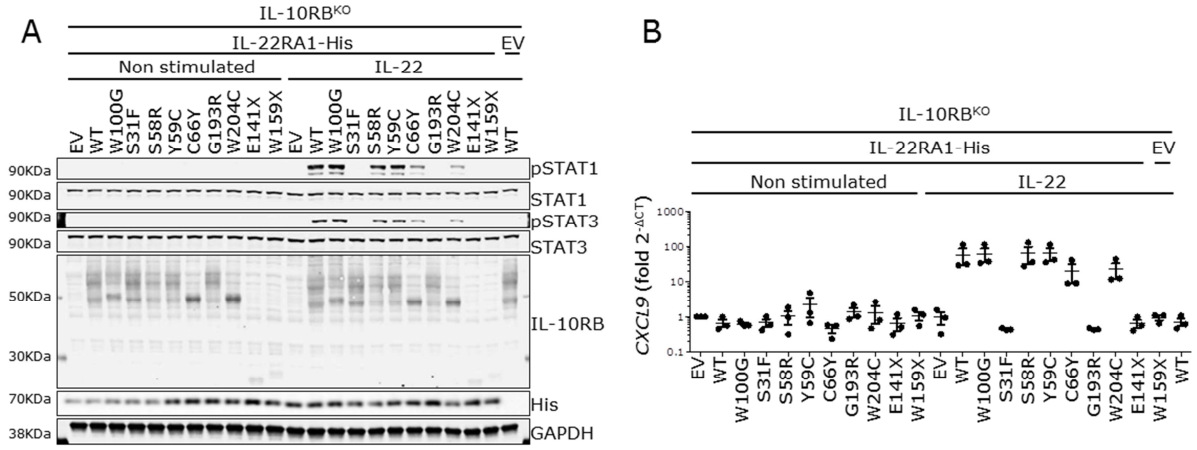

Figure 11: The response of IL-10RB pathogenic mutations after IL-22 stimulation. A- Response to IL-22 stimulation 100ng/ml for 30 minutes on IL-10RB^{KO} SV-40 fibroblasts transfected with indicated IL-10RB alleles and IL-22RA1-His on representative immunoblot image showing the analysis of STAT1 (pSTAT1) and STAT3 (pSTAT3) phosphorylation. STAT1, STAT3, GAPDH, His (IL-22RA1) and IL-10RB levels were also assessed. B- Response to IL-22 stimulation 100ng/ml for 2 hours on IL-10RB^{KO} SV-40 fibroblasts transfected with indicated IL10RB alleles and IL-22RA1-His on RT-qPCR showing the mRNA levels of CXCL9. *mRNA expression is shown corrected by the endogenous expression of HPRT1 mRNA. Results are normalized to the first column set as 1. Values shown are the means of three independent experiments performed in duplicate ± SEM.*

RESPONSE TO IL-26

We proceeded to test the response to IL-26 in similar fashion as to IL-10 and IL-22, but the transient co-overexpression of IL-20RA did not let to robust results (data not shown). So we decided to stably transduce SV-40 fibroblasts with IL-20RA and afterwards transfect IL-10RB WT or the pathogenic variants. We could observe that W100G, S58R, Y59C, C66Y, and W204 responded in terms of STAT3 phosphorylation comparable to WT IL-10RB; while the variants S31F and G193 were hypomorphic and W141X and W159X had abrogated responses.

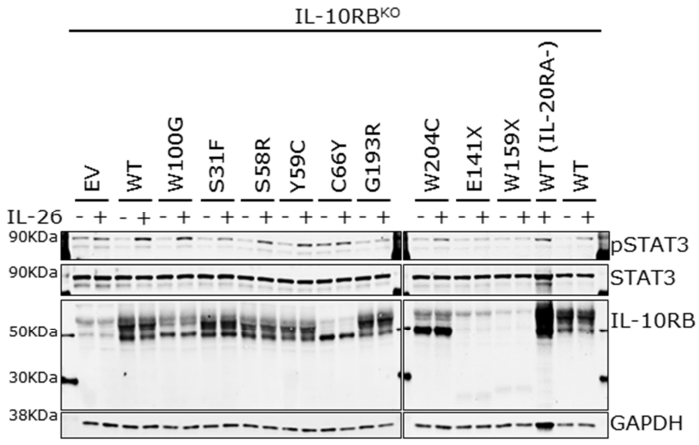

Figure 12: The response of IL-10RB pathogenic mutations after IL-26 stimulation. Western blot showing IL-26 stimulation 100ng/ml for 45 minutes on IL-10RB^{KO} SV-40 fibroblasts transfected with indicated IL-10RB alleles and stably transduced

with IL-20RA on representative immunoblot image showing the analysis of STAT3 (pSTAT3) phosphorylation. STAT3, GAPDH, and IL-10RB levels were also assessed.

RESPONSE TO IFN-λ1

We proceeded to test the response to IFN-λ1 in similar manner as to IL-10 and IL-22, but co-transfecting in this case WT *IFNLR1 DDK tagged*. As a result we could observe that W100G and S58R responded as WT IL-10RB in terms of STAT1 phosphorylation and *CXCL9* mRNA induction, while Y59C, C66Y, and W204C had hypomorphic responses and there was no response for the alleles: S31F, G193R, E141X and W159X (Figure 13).

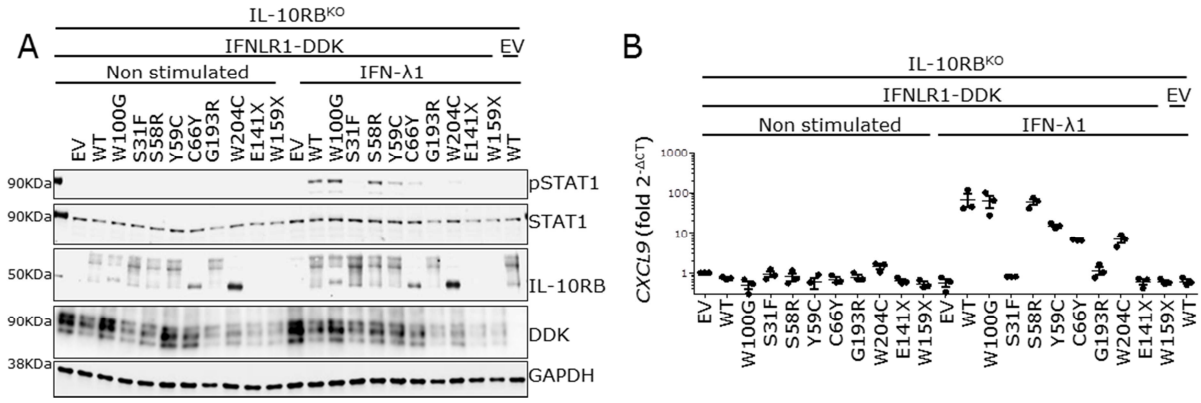

Figure 13: The response of IL-10RB pathogenic mutations after IFN-λ1 stimulation. A- Response to IFN-λ1 stimulation 100ng/ml for 30 minutes on IL-10RB^{KO} SV-40 fibroblasts transfected with indicated IL-10RB alleles and ILFNLR1-DDK on representative immunoblot image showing the analysis of STAT1 (pSTAT1) phosphorylation. STAT1, GAPDH, DDK (IFNLR1) and IL-10RB levels were also assessed. B- Response to IFN-λ1 stimulation 100ng/ml for 2 hours on IL-10RB^{KO} SV-40 fibroblasts transfected with indicated IL10RB alleles and IFNLR1-DDK on RT-qPCR showing the mRNA levels of CXCL9*. mRNA expression is shown corrected by the endogenous expression of HPRT1 mRNA. Results are normalized to the first column set as 1. Values shown are the means of three independent experiments performed in duplicate ± SEM.*

We summarize all the results from the study of the W100G IL-10RB allele in comparison with all published IL-10RB missense variants in the table below (Table 4). We can conclude from this series of experiments that there is a disassociation between the responses to the IL10RB-dependent cytokines of the different disease-causing alleles but all of the variants tested on this work are defective in their response towards IL-10. Interestingly the allele carried by the family which is object of this study is the only one showing a defective response towards IL-10 while maintaining a completely normal response to IL-22, IL-26 and IFN-λ1, at least in overexpression.

Table 4: Summary of results on the overexpression system. +++ equals the intensity of the WT allele, ++ means slightly decreased response compared to WT, + means much decreased response compared to WT: no response, and ND: not done.

Stimulation	Read out	IL10RB overexpression system									
		WT	W100G	S31F	S58R	Y59C	C66Y	S193R	W204C	E141X	W159X
IL-10	WB-pSTAT1	+++	+	-	-	-	+	-	+	-	-
	WB-pSTAT3	+++	++	-	-	-	++	-	++	-	-
	qPCR-CXCL9	+++	-	ND	ND	-	ND	ND	ND	-	-
IL-22	WB-pSTAT1	+++	+++	-	+++	+++	+	-	+	-	-
	WB-pSTAT3	+++	+++	-	+++	+++	+	-	+	-	-
	qPCR-CXCL9	+++	+++	-	+++	+++	+	-	+	-	-
IL-26	WB-pSTAT3	+++	+++	-	+++	+++	+++	-	+++	-	-
IFN-λ1	WB-pSTAT1	+++	+++	-	+++	++	+	-	+	-	-
	qPCR-CXCL9	+++	+++	-	+++	+	+	-	+	-	-

STUDY OF IL-10RB$^{W100G/W100G}$ PATIENT CELLS

PROTEIN EXPRESSION AND LOCALIZATION

We were unfortunate in that there was neither tissue nor cells available from both the patients of this study. In order to study the consequence of the homozygous W100G IL-10RB in cells in endogenous conditions, we made use of SV-40 fibroblasts from another IL-10RB$^{W100G/W100G}$ patient[58] (referred onwards as patient cells). We started by measuring *IL10RB* mRNA by qPCR (Figure 14A) where patient cells showed completely normal levels, differently from the IL-10RB^{KO} patient cells with markedly decreased levels. This data was in agreement with the overexpression results. We tried several IL-10RB antibodies in western blot, but unfortunately we were unable to detect endogenous protein levels (data not shown). However, we were able to measure IL-10RB protein expression on the cell surface by flow cytometry (Figure 14B). As a result, we could observe that patient cells have lower surface protein when compared to controls, at very similar levels in comparison to the IL-10RB^{KO} patient cells. These results were also congruent with the overexpression results of the W100G allele in isolation.

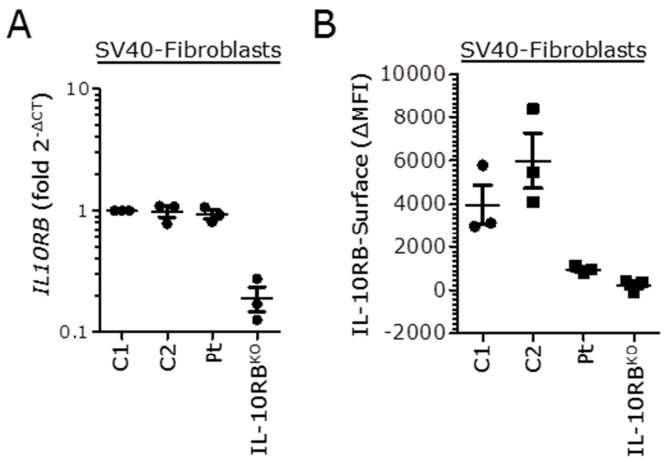

Figure 14: IL-10RB mRNA measurement by RT-qPCR and cell surface expression in patient (Pt) and control (C1, C2) cells. A- Graph shows the copy numbers of IL-10RB mRNA corrected by the endogenous expression of HPRT1 mRNA and relative to the mean copy number of the first control set as 1 ± SEM. Values shown are the means of three independent experiments performed in duplicate. B- Cell surface expression of IL-10RB was assessed in SV-40 fibroblasts from healthy controls, patient, and an IL-10RB–deficient patient (IL-10RBKO) by flow cytometry. The results are expressed as mean fluorescence intensity (MFI) minus the MFI of the isotype control for each (ΔMFI) ± SEM.

RESPONSE TO IL-10

We tested the response to IL-10 by using patient and control cells stably transduced with IL-10RA-V5 WT. Patient cells had a heavily decrease response to IL-10 both in terms of STAT1 and STAT3 phosphorylation, and *CXCL9* mRNA induction (Figure 15).

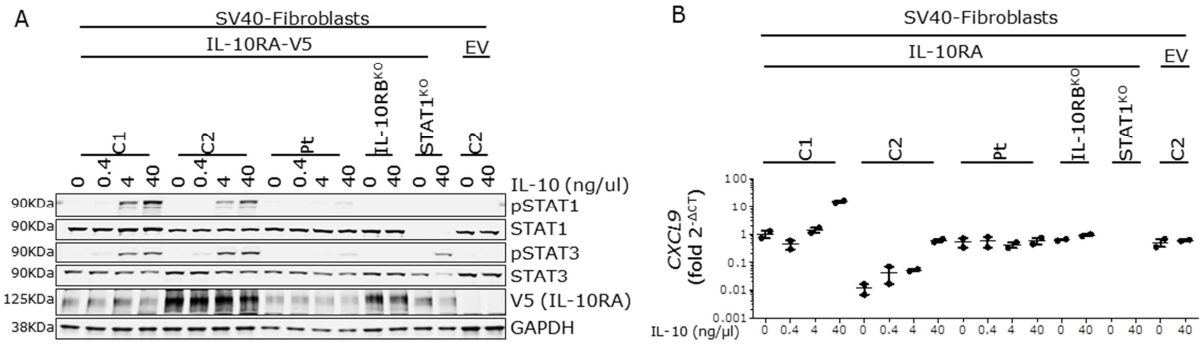

Figure 15: Patient and control cells response to IL-10. A- Response to IL-10 stimulation at the indicated concentrations for 30 minutes on controls, patient, STAT1^{KO} and IL-10RB^{KO} SV-40 fibroblasts stably transduced with IL-10RA-V5 when indicated on representative immunoblot image showing the analysis of STAT1 (pSTAT1) and STAT3 (pSTAT3) phosphorylation. STAT1, STAT3, GAPDH and V5 (IL-10RA) levels were also assessed. B- Response to IL-10 stimulation at the indicated concentrations for 6 hours on controls, patient, STAT1^{KO} and IL-10RB^{KO} SV-40 fibroblasts stably transduced with IL-10RA-V5 when indicated, showing the analysis of CXCL9 mRNA levels. HPRT1 mRNA levels were used as endogenous loading controls.

We proceeded to test if complementation with transient transfection of IL-10RB WT and IL-10RA in patient and IL-10RB^{KO} cells could complement the defect on STAT1 and STAT3 phosphorylation; we indeed saw that it was the case (Figure 16).

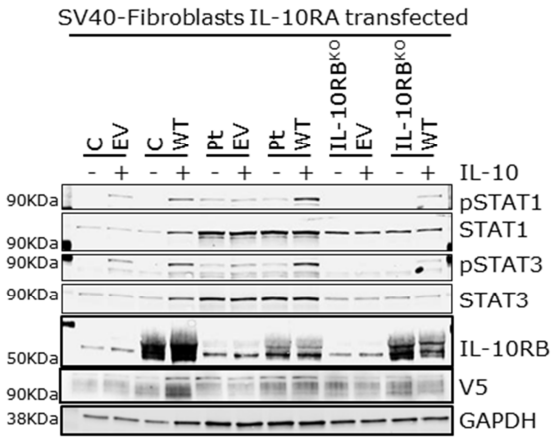

Figure 16: Effect of IL-10RB WT reconstitution on patient and control cells after IL-10 stimulation. Western blot depicting phospho-STAT1 (pSTAT1) and phospho-STAT3 (pSTAT3) on reconstituted with WT IL-10RB or EV: patient, IL-10RB^{KO} and control SV-40 fibroblasts, with IL-10 stimulation of 40ng/ml for 30 minutes. GAPDH was used as a loading control, STAT1, STAT3 and V5 (IL-10RA1) levels were also depicted.

RESPONSE TO IL-22

We also tested the response to IL-22 on patient cells, but this time transducing IL-22RA-His on patient and control cells. We found that patient cells responded as controls for the phosphorylation of STAT3, but response in regards to STAT1 phosphorylation and *CXCL9* mRNA induction was impaired (Figure 17A and B). We proceeded to test if IL-10RB WT complementation would rescue the defect of STAT1 phosphorylation (Figure 17C). We could observe that after IL-10RB WT and IL-22RA transfection both patient and IL-10RBKO cells augmented their response in terms of STAT1 phosphorylation, providing evidence that the observed phenotype was caused by the IL10RB deficiency.

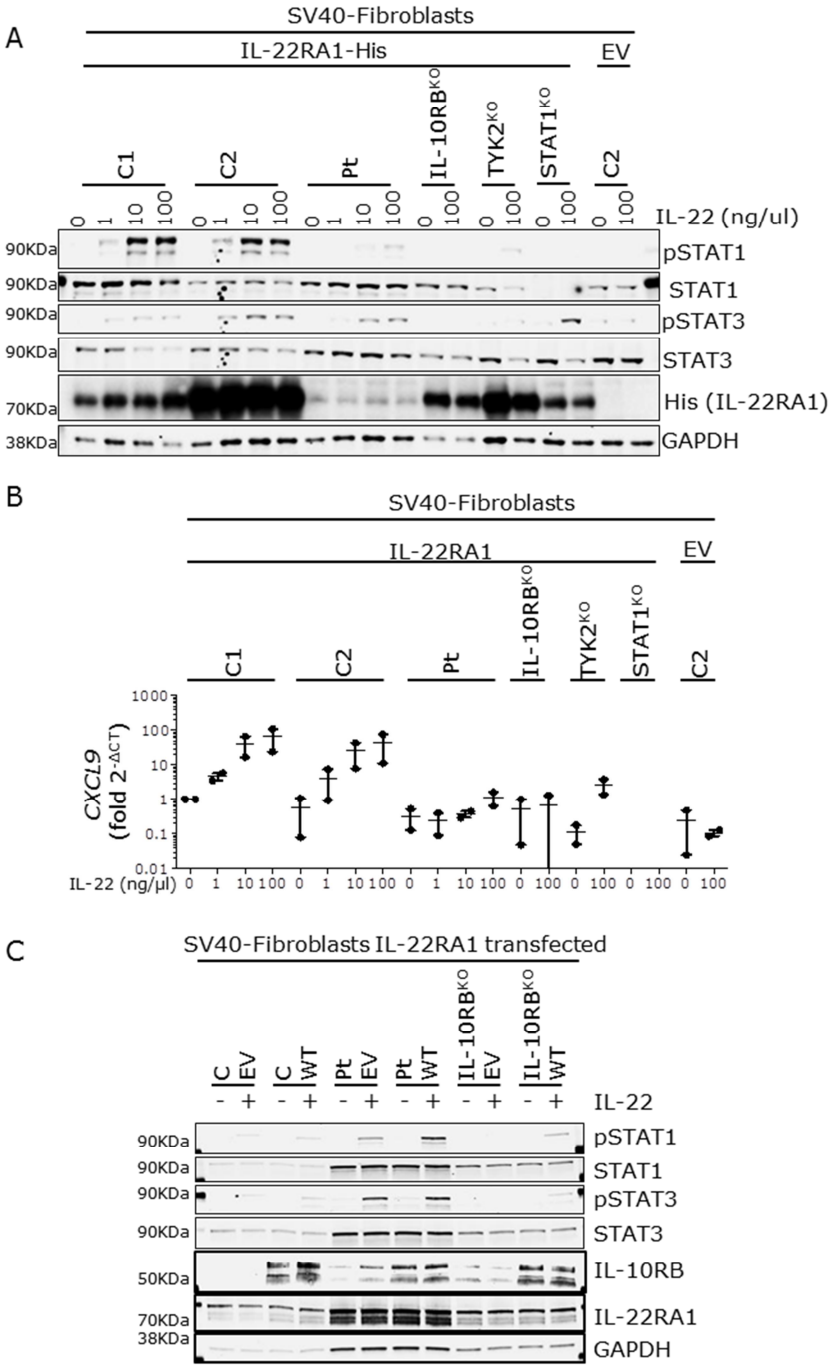

Figure 17: Patient and control cells response to IL-22. A- Response to IL-22 stimulation at the indicated concentrations for 30 minutes on controls, patient, STAT1^{KO}, TYK2^{KO} and IL-10RB^{KO} SV-40 fibroblasts stably transduced with IL-22RA1-His when indicated on representative immunoblot image showing the analysis of STAT1 (pSTAT1) and STAT3 (pSTAT3) phosphorylation. STAT1, STAT3, GAPDH and His (IL-22RA1) levels were also assessed. B- Response to IL-22 stimulation at the indicated concentrations for 2 hours on controls, patient, STAT1^{KO}, TYK2^{KO} and IL-10RB^{KO} SV-40 fibroblasts stably transduced with IL-22RA1-His when indicated, showing the analysis of CXCL9 mRNA levels.CXCL9 mRNA levels for STAT1KO cells were undetectable. HPRT1 mRNA levels were used as endogenous loading controls. C- Effect of IL-10RB WT reconstitution on patient and control cells after IL-22 stimulation. Western blot depicting phospho-STAT1 (pSTAT1) and phospho-STAT3 (pSTAT3) on reconstituted with WT IL-10RB or EV: patient, IL-10RB^{KO} and control SV-40 fibroblasts, with IL-10 stimulation of 40ng/ml for 30 minutes. GAPDH was used as a loading control, STAT1, STAT3 and V5 (IL-10RA1) levels were also depicted.

RESPONSE TO IL-26

We also tested the response to IL-26 on patient cells, but this time transducing IL-20RA-DDK on patient and control cells. As a result we could observe that the patient had no augmentation in the phosphorylation of STAT3 after IL-26 stimulation (Figure 18). We could not test if complementation of the defect in the STAT3 phosphorylation response with WT IL-10RB, since we cannot get control cells to respond to IL-26 with IL-20RA in transient transfection (Data not shown).

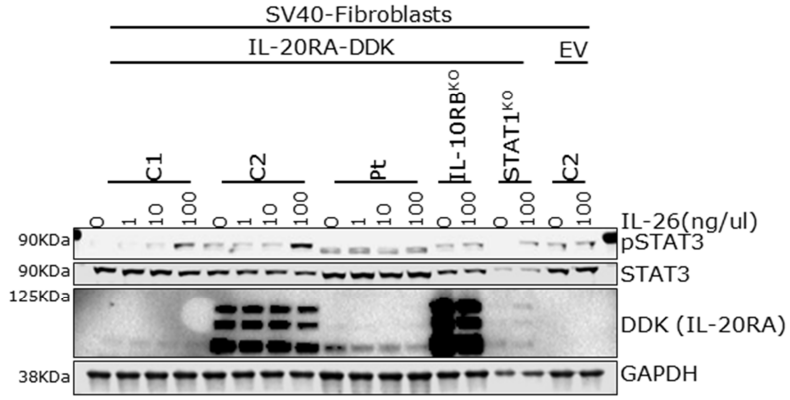

Figure 18: Effect of IL-10RB WT reconstitution on patient and control cells after IL-22 stimulation. Western blot depicting phospho-STAT3 (pSTAT3) on reconstituted with WT IL-10RB or EV: patient, IL-10RB^{KO}, STAT1^{KO} and control SV-40 fibroblasts, with IL-26 stimulation of 100ng/ml for 45 minutes. GAPDH was used as a loading control, STAT3 and DDK (IL-20RA) levels were also depicted.

RESPONSE TO IFN-λ1

Finally, we tested the response on patients and controls cells after IFN-λ1 stimulation with cells transduced with IFNLR1-DDK WT. We could observe a reduced response compared to controls in patient cells both in terms of STAT1 phosphorylation and *CXCL9* mRNA induction (Figure 19).

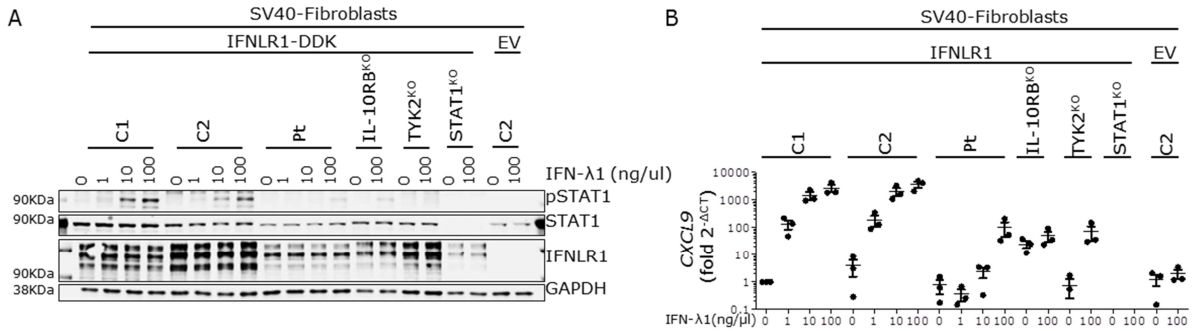

Figure 19: Patient and control cells response to IFN-λ1. A- Response to IFN-λ1 stimulation at the indicated concentrations for 30 minutes on controls, patient, STAT1^{KO}, TYK2^{KO} and IL-10RB^{KO} SV-40 fibroblasts stably transduced with IL-22RA1-His when indicated on representative immunoblot image showing the analysis of STAT1 (pSTAT1) and STAT3 (pSTAT3) phosphorylation. STAT1, STAT3, GAPDH and IFNLR1 levels were also assessed. B- Response to IFN-λ1 stimulation at the indicated concentrations for 6 hours on controls, patient, STAT1^{KO}, TYK2^{KO} and IL-10RB^{KO} SV-40 fibroblasts stably

transduced with IFNLR1-DDK when indicated, showing the analysis of CXCL9 mRNA levels. HPRT1 mRNA levels were used as endogenous loading controls.

We tested if we could complement the defect in the STAT1 phosphorylation response with WT IL-10RB. As a result we could observe that there was complementation for STAT1 phosphorylation in both patient and IL-10RB^{KO} cells with the transfection of WT IL-10RB and IFNLR1 (Figure 20).

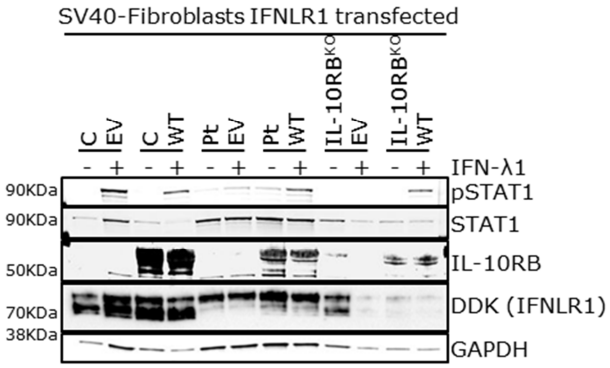

Figure 20: Effect of IL-10RB WT reconstitution on patient and control cells after IFN-λ1 stimulation. Western blot depicting phospho-STAT1 (pSTAT1) on reconstituted with WT IL-10RB or EV: patient, and control SV-40 fibroblasts, with IFN-λ1stimulation of 100ng/ml for 30 minutes. GAPDH was used as a loading control, STAT1 and DDK (IFNLR1) levels were also depicted.

STUDY OF IL-10RB AXIS COMPONENTS IN LIVER CELLS

mRNA EXPRESSION OF IL-10RB SIGNALING COMPONENTS IN LIVER CELLS

We were interested in knowing which cells in the liver presented the IL-10RB components and therefore would be involved in FVH pathology. To achieve this goal, we studied mRNA from human liver and selected the genes related to IL-10 response (Figure 21). We could observe that *IL10RB* mRNA is present in all liver cell types, while *IL10RA* mRNA expression is restricted mainly to macrophages/monocytes, and lymphoid cells; with only a few cells positive for the mRNA on all other cell types. *IL10* mRNA can be mostly found in macrophages/monocytes, lymphoid cells, cholangiocytes, endothelial cells and hepatocytes. *IL22RA1* can only be found in hepatocytes and a few cholangiocytes. *IL22* mRNA is practically not detectable in basal state in all investigated cell types. Cholangiocytes are the main source of *IL20RA* mRNA in the liver; while *IL26* mRNA is only present on lymphoid cells. On the contrary the presence of *IFNLR1* mRNA covers most liver cell types: lymphoid cells,

hepatocytes, endothelial cells, mononuclear phagocytes, cholangiocytes, and plasma cells. Finally, *IFNL1* mRNA expression is restricted to lymphoid cells.

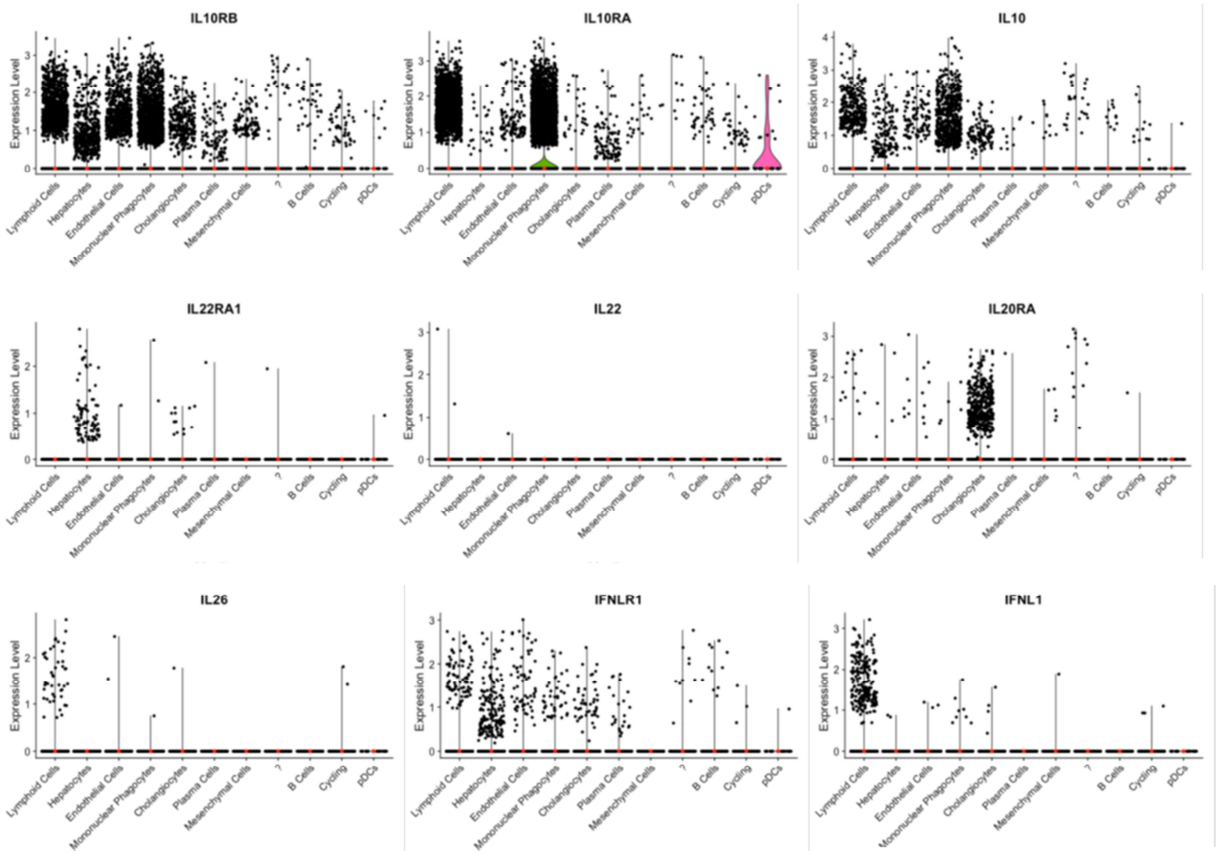

Figure 21: Human liver expression of IL-10RB axis components Single cell RNA-sequencing data showing differential expression of Il-10RB, its co-receptors and agonists in different liver cell types.

HEPATOCYTES RESPONSE TO IL-10RB DEPENDANT CYTOKINES

We wanted to check if the previous results were accurate and, given the importance of hepatocytes in the liver, also check their response to IL-10RB dependent cytokines. To that objective we took human primary hepatocytes and stimulated them with IL-10, IL-22, IL-26 and IFN-λ1 (Figure 22). Response in terms of phosphorylation of STAT1 was positive for IL-22 and IFN-λ1. STAT3 phosphorylation was strongly present after IL-22 stimulation and almost no detectable for IFN-λ1 stimulation. IL-10 and IL-26 stimulation showed no response.

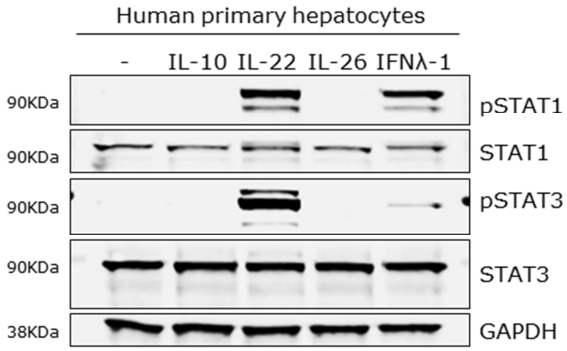

Figure 22: Response of human hepatocytes to IL-10RB cytokines. Western blot showing stimulation with IL-10 (40ng/ml), IL-22, IL-26, and IFNλ-1 (all 100ng/ml) and posterior phosphorylation of STAT1 and STAT3, representative western blot of n=2. GAPDH was use as a housekeeping control.

Discussion and Conclusion

This is the first report of a family with patients suffering from FVH caused by AR *IL10RB* deficiency. In accordance with previously published cases, both affected family members suffered from EOIBD[103]. AR *IL10RB* deficiency has also been linked to skin folliculitis, B cell lymphoma and arthritis, all absent in this family[58,96,99,100,104]. It is interesting to remark the variability in the clinical presentation of reported patients. Not all patients presented with B cell lymphoma, arthritis or skin folliculitis. But all up to date published patients presented with EOIBD. Nevertheless, even within the gastrointestinal manifestations and between patients carrying the same mutations, we observe variability in clinical presentation. In the first published family with two affected members carrying in homozygosity the W159X *IL10RB* allele, the male sibling presented with stronger clinical manifestations than his sister[103]. Therefore, FVH might be added as another variable clinical manifestation due to AR IL-10RB. But this opens the question if there are other pathologies that could be escaping discovery? I think it is highly likely since most, if not all, reported patients, with AR *IL10RB* deficiency, were from studies in EOIBD disease cohorts[58,98,100,103–107], while the patients we investigated are the first ones identified from a FVH cohort. This opens the question whether in the future, with WES being more readily available, will AR *IL10RB* patients be found without, or with very minor gastrointestinal manifestations? In that direction we think worthy to study *IL10RB* as a possible cause of liver damage in not only other hepatic viral cohorts, but also where there is higher injury to the liver than expected. For example, cohorts of patients with enhanced drug or alcohol liver injury where it is suspected that there are genetic causes remaining to be unveiled[108–110].

To our knowledge, this work is the first study to test AR *IL10RB* deficiency causative allele responses in isolation. Meaning all other studies have been directly performed on patient cells or transfecting those with the WT *IL10RB* allele to assess reconstitution. EOIBD is known to be a consequence of altered IL-10 signaling, since there are EOIBD patients described with AR *IL10RA*[103,105] and *IL10*[100] deficiencies. We confirm this, as in our results, all mutations tested shared a defective response after IL-10 stimulation.

From the analysis of the different reported missense variants, there is a pattern emerging (Figure 23) with alleles such as *IL10RB* S31F and G193R not responding to any tested cytokine. *IL10RB* C66Y and W204C are both hypomorphic in terms of response to IL-10, IL-22, IL-26 and IFNλ-1, and both share the lack of an upper band when overexpressed. We hypothesized that both mutations may change the protein 3D structure by disrupting the disulfide bonds, therefore leading to an altered protein expression. The first disulfide bond on IL-10RB uses cysteine residue 66 that disappears on C66Y IL-10RB. The second disulfide bond includes the cysteines at positions 188 and 209, which are maintained with the W204C mutation. However IL-10RB W204C mutation may create a new sulfhydryl donor site close enough to position 209 that may generate the shift of the disulfide bond between positions 188 and 209 to 188 and 209, modifying the protein 3D structure. But this is still speculation and will require further experiments to prove. Also, IL-10RB S58R and Y59C are not responding to IL-10, but respond to IL-22, IL-26 and IFN-λ1. This is concordant with previous publications that showed that the second extracellular loop, in which both mutations lie, is important for the IL-10RB-IL-10 interaction[111], but one of IL-10RB and IL-22. IL-10RB W100G is an outlier in this analysis, by being the only IL-10RB mutant affecting solely the response to IL-10 in overexpression system. It could be clinically relevant to see if this discrepancy in response of the different IL-10RB disease causing alleles can be associated to a characteristic clinical phenotype, which could be used as prognostic once new patients are diagnosed. But to be able to do so, we need to increase the number of patients studied and a better characterization of the clinical phenotype of the patients harboring these mutations.

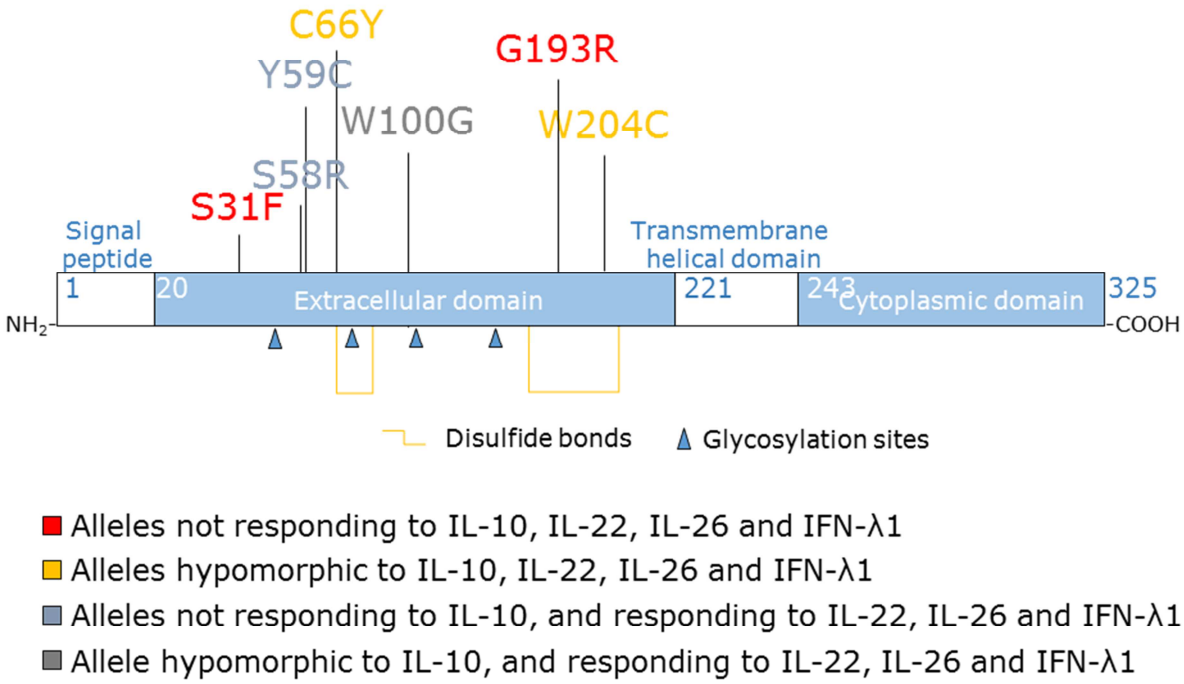

Figure 23: Missense mutations in IL10RB show different cytokine-dependent function patterns.

The response to IL-22 has only been tested in one patient in the paper of Begue *et al*. in 2011[105]. The authors demonstrated that the response to IL-22 was lower in terms of STAT3 phosphorylation and induction of *HBD2, HBD3, MUC1* and *MUC13* for the patient carrying in homozygosity the E141X *IL10RB* allele. In our study, we show pathogenic *IL10RB* variants that respond normally to IL-22 in terms of STAT3 phosphorylation. These results emphasize how variable the effect in terms of protein function of different mutations might be, even when giving a similar EOIBD phenotype to the affected patients. Even though the overexpression results for the W100G *IL10RB* variant showed conserved functionality to IL-22, we could observe upon testing IL10RB$^{W100G/W100G}$ SV40-fibroblasts that their response in terms of STAT1 phosphorylation and *CXCL9* induction were diminished. In overexpression, the much higher amounts of IL-10RB could be saturating the response, thereby masking a hypomorphic effect, unveiled under lower, more physiological, amounts of W100G IL-10RB as can be found in patient cells. The defect on patient cells was resolved with the transfection of IL-10RB WT probing that the observed defect is caused by the W100G IL-10RB mutation. These data highly suggest that the defect towards IL-22 is a purely quantitative defect, in opposition to the defect of W100G IL-10RB towards IL-10 that would not be only quantitative, but also qualitative.

We could see that W100G IL-10RB had a normal response to IL-26 in overexpression, but the patient cells harbored a defect that we could not technically complement with the addition of IL-10RB WT. Therefore, we think the defect towards IL-26 could be intrinsic of the patient cells or it could be related to the AR *IL10RB* deficiency. We don't possess whole exome/genome data of these patient cells, therefore it is possible there could be other mutations in the IL-26 pathway. IL-26 has also been characterized as a DNA binding pro-inflammatory protein more akin to a soluble pattern recognition receptor[112,113]. For this effect IL-26 does not require IL-10RB and can signal trough TLR9[113]. I think that probably the action of IL-26 trough IL-10RB/IL-20RA is not as important as its action as a soluble sensor to DNA. For all these reasons we could think IL-26 does not have a significant role to play in the pathogenesis of our patient's symptomatology or in other AR *IL10RB* deficient patients.

Both the W100G *IL10RB* allele and the IL10RB$^{W100G/W100G}$ patient cells responded to IFNλ-1 stimulation, although patient cells did so in much lower capacities compared to control cells. Thus, as in the case towards IL-22 stimuli, the defect in W100G IL-10RB cells must be purely quantitative. Since complementation with IL-10RB did improve the response on patient cells and IFN-λs are part of the immunological response to liver tropic viruses such as HBV[114] and HCV[68], we cannot exclude the possibility that even this tenuous defect could contribute to the disease onset or progression.

Previously our laboratory has published a monogenic defect in *IL18BP* as the first genetic cause for susceptibility to FVH following HAV infection. IL-18BP binds IL-18, thereby blocking its function[115]. Thus non-functional IL-18BP generates elevated IL-18 signaling causing a hyper-inflammatory state responsible for the liver symptomatology, manifested by the high activation of liver macrophages in the reported patient. Lack of IL-10 signaling can also bring an inflammatory state, since IL-10 is an anti-inflammatory cytokine[116]. Consequently, a hyper-inflammatory state can be also produced by lack of a functional IL-10RB protein. This has been demonstrated as IL-10RB deficient patient M1 macrophages have a marked inflammatory signature by higher secretion of pro-inflammatory cytokines[95]. Moreover IL-10RB$^{-/-}$ mice have chronic colitis[117] and their regulatory T cells are incapacitated to suppress inflammation[118]. Therefore both monogenic deficiencies mimic each other in terms of converging into a pro-inflammatory signature. For these reasons and that the response of the W100G allele in isolation towards IL-10 was the only one affected; I think IL-10 the most likely candidate to be behind the pathogenesis in our patients' disease. But I

cannot strictly exclude the contribution of IL-22 and IFNλ-1 as modifiers or contributors to the FVH pathogenesis since patient cells response is impaired and we were able to complement the defect with IL-10RB WT.

It is my opinion that all patients that have been diagnosed as harboring mutations in the IL-10 axis should be preemptively vaccinated against all liver tropic viruses when available, but especially for HAV. Since HAV is not included in most countries mandatory vaccination calendar and coverage is very low even in individuals were it is strongly recommended, as for example patients with chronic liver disease[119]. Mortality of AR IL-10RB diagnosed patients is very high, if they do not undergo BMT[100,120,121]. The last statement in conjunction with the low number of reported cases potentially explains the fact that this is the first report of patients with AR IL-10RB deficiency suffering from FVH and EOIBD.

The main limitation of the present work is the lack of multiple patients from different families sharing the FVH phenotype due to *IL10RB* mutations. Nevertheless reporting single patients helps identify others in the future and around 21% of monogenic primary immunodeficiencies (PIDs) were first identified on single patient studies[122]. Autoimmunity has not been widely reported in IL-10RB-deficient patients with only three patients published, two presenting arthritis[58,100] and one with autoimmune hepatitis[100]. Thus there are two IL10RB-deficient patients with affected livers. Hence probably AR IL-10RB deficiency leads to a more susceptible liver to injury be it by means of autoimmune complexes or by viral infection.

This is the first work linking AR *IL10RB* as the second monogenic cause to FVH trough a probable enhanced pro-inflammatory milieu. A diagnosis of AR IL10RB deficiency should be considered in patients with EOIBD specially if accompanying liver manifestations. Crucially all patients suspected with AR *IL10RB* should undergo vaccination to liver-tropic viruses particularly HAV.

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Annexe 1

RESUME SUBSTANTIEL EN FRANÇAIS

Les agents les plus courants de l'hépatite virale humaine sont le virus de l'hépatite A (VHA), le virus de l'hépatite B (VHB), le virus de l'hépatite C (VHC) et le virus de l'hépatite E (VHE)[18–20]. Les virus VHB et VHC sont responsables d'hépatite chronique pouvant aller jusqu'au développement d'une cirrhose puis d'un hépato-carcinome[123]. En revanche, les infections par le VHA et le VHE sont aiguës et généralement bénignes[124]. Dans de rares cas, une primo-infection par le VHA, le VHB ou chez la femme enceinte et les personnes immunodéprimées par le VHE, peut entraîner une hépatite virale fulminante (HVF), caractérisée par une insuffisance hépatique mettant en jeu le pronostic vital. Dans la grande majorité des cas, l'HVF frappe des individus par ailleurs en bonne santé, normalement résistants à d'autres agents infectieux[5–8]. L'incidence de l'HVF n'est pas bien connue, mais a été estimée à environ 0,5% des individus symptomatiques du VHA[28]. Étant donné que la prévalence de l'infection à VHA varie considérablement d'un pays à l'autre, tout comme la couverture vaccinale contre le VHA, les estimations de l'HVF ont varié d'environ 0,6 cas par million d'individus en Écosse[29] à ~ 1,6 cas par million en Thaïlande[30]. Le pronostique de l'HVF est dramatique, avec moins de 25% de survie sans transplantation hépatique[5,6,9–12].

Il n'y a pas été identifié de génotypes particuliers du VHA responsables d'HVF [31,32], et aucune épidémie n'a été rapportée, ce qui suggère que l'HVF n'est pas causée par des souches virales plus virulentes. Bien que presque toujours sporadiques, quelques formes familiales d'HVF ont été signalées[33–35]. Ces observations suggèrent que l'HVF pourrait résulter d'erreurs monogéniques affectant la réponse immunitaire antivirale. Cela a déjà été décrit pour de nombreuses maladies virales graves[1,36–40,125–129]. Nous avons récemment rapporté le déficit autosomique récessif (AR) de l'*IL18BP,* comme première cause monogénique de l'HVF due au HAV chez un patient[42]. IL-18BP est un antagoniste de la cytokine IL-18[130], qui active la cytotoxicité des lymphocytes NK et T et leur production d'IFN-γ[53,131]. L'absence d'IL-18BP libère l'activité de l'IL-18, ce qui conduit à une cytotoxicité incontrôlée, tuant ainsi à la fois les hépatocytes infectés par le VHA et les autres

hépatocytes[42]. Nous avons étudié une famille multiplexe et consanguine, dans laquelle le cas index (P1) présentait avec une forme de maladie précoce inflammatoire chronique de l'intestin (EOIBD) et est décédée d'une hépatite fulminante alors que sa sœur (P2) atteinte d'une forme plus grave d'EOIBD a développé une hépatite sévère due au HAV et est décédée de la combinaison des deux conditions.

Avec une approche de séquençage haut débit sur P2, nous avons confirmé la consanguinité parentale, avec un taux d'homozygotie de 4,71%[54]. Sous l'hypothèse d'une transmission autosomique récessive (AR), nous avons sélectionné les variations homozygotes non synonymes, rares (fréquence allélique <0,01) et les avons hiérarchisées en fonction de l'impact délétère prédit in silico. Nous avons trouvé la variation p.Trp100Gly (W100G) sur le gène *IL10RB*, avec un score CADD de 29,8 au-dessus du score de 99% du seuil de signification de la mutation (MSC) de 23,8, comme étant la meilleure variante candidate car elle avait le score CADD le plus élevé et avait déjà été rapportée dans une famille avec EOIBD[58]. La ségrégation familiale était compatible avec un trait AR, puisque les parents étaient hétérozygotes et une sœur en bonne santé était WT/WT. Des mutations bi-alléliques dans *IL10*, *IL10R*A et *IL10RB* ont été précédemment décrites comme provoquant EOIBD[100,103]. De plus, il n'y a pas de variations homozygotes *IL10RB* prédites pour être LOF dans la population générale avec un score CADD supérieur au MSC. En outre, *IL10RB* code la deuxième chaîne du récepteur pour IL-10 en association avec IL-10RA, pour l'IL-22 en association avec IL-22RA1, pour l'IL-26 en association avec IL-20RA et enfin pour les interférons (IFN)-λs en association avec IFNLR1[59,60]. Les mutations *IL10RB* LOF rapportées présentent principalement un score CADD supérieur au MSC et sont privées ou avec une fréquence inférieure à 10^{-4} (tableau 2). Collectivement, ces données suggèrent fortement que la mutation homozygote W100G soit la cause de l'EOIBD, et de l'hépatite sévère, chez les deux sœurs.

Aucun des patients déficients en IL-10RB précédemment rapportés n'a développé d'HVF; aucun n'a été signalé non plus comme infecté par le HAV[58,59,97–101,103,105,132,133]. Un patient homozygote pour W159X a développé une hépatite auto-immune[100]. Nous avons entrepris la caractérisation moléculaire de tous les allèles IL-10RB faux-sens pathogènes signalés et de deux allèles stop-gain (E141X et W159X) par des expériences de surexpression.

Ces variations ont toutes été décrites précédemment pour provoquer EOIBD[58,95,96,98–100,103,105,134]. Toutes les mutations perte de fonction (pLOF) décrites dans *IL10RB* sont réparties dans sur l'ensemble des différents domaines de la protéine, tandis que les mutations pathogènes faux-sens sont regroupées dans le domaine extracellulaire[58,95,96,98–100,134]. Dans les expériences de surexpression dans les cellules HEK293T, tous les variants faux-sens testés ont montré une expression d'ARNm normale, par rapport à WT *IL10RB*, tandis que les deux variants pLOF testés (E141X et W159X) ont montré une forte diminution, suggérant une dégradation de l'ARNm (nonsence mediated decay). Au niveau des protéines, WT IL-10RB présentait deux bandes principales (environ 50 et 60 kDa), suggérant des modifications post-traductionnelles. Comme attendu, les protéines E141X et W159X avaient à la fois un poids moléculaire (PM) plus petit et un niveau d'expression plus faible, par rapport au WT. Quatre protéines faux-sens, y compris W100G, sont exprimées plus faiblement que la forme WT. Fait intéressant, nous avons observé un PM supérieur à WT pour W100G, tandis que C66Y et W204C affichent uniquement la plus petite bande observée avec la forme WT, suggérant des modèles de glycosylation en fonction des mutations. Suite au traitement à la PNGase-F, qui supprime tous les oligosaccharides liés à N, la protéine WT et tous les allèles faux-sens sont passés de deux bandes principales à un triplet avec une bande majeure d'environ 45 kDa, à l'exception de C66Y et W204C affichant uniquement la partie moléculaire inférieure. Nous avons normalisé le poids moléculaire de W100G par rapport à WT, confirmant que son poids moléculaire supérieur initial était dû à une glycosylation aberrante. Avec et sans traitement à la PNGase-F, les protéines C66Y et W204C ont un profil de PM différent de la protéine WT, ce qui suggère que ces deux mutations pourraient également altérer d'autres modifications post-traductionnelles.

Nous avons utilisé des fibroblastes transformés par SV40 provenant d'un patient présentant un déficit complet en IL-10RB (IL-10RB^{KO})[103]. Par des expériences de surexpression et en utilisant un anticorps spécifique de l'IL-10RB, nous avons montré que quatre protéines faux-sens (W100G, S58R, Y59C et G193R) sont exprimées à la surface cellulaire, mais pour deux avec une intensité de fluorescence médiane (MFI) inférieure à celle de WT (W100G et G193R). En revanche, les trois autres protéines faux-sens testées (S31F, C66Y, W204C) et les deux protéines avec les variants pLOF E141X et W159X ne sont pas exprimées à la surface cellulaire. Dans les mêmes expériences, nous avons également

analysé l'expression intracellulaire avec des anticorps contre l'IL-10RB ou une étiquette (Cter-Myc). Avec l'anticorps anti-Myc, nous avons observé une expression intracellulaire normale de toutes les protéines faux-sens et une expression diminuée des protéines codées par les deux variants pLOF, par rapport à WT. De plus, avec l'anticorps anti-IL-10RB, nous avons observé une expression cytosolique normale des protéines S58R, Y59C et G193; une légère diminution du MFI de W100G, S31F et C66Y; et aucune expression détectable de W204C, E141X et W159X, suggérant que ces protéines mutées ne sont pas reconnues par l'anticorps anti-IL-10RB. Les variations W100G, S31F, C66Y et G193R qui, par rapport à WT, ont des niveaux cytosoliques similaires et des niveaux de surface fortement diminués. Ceci peut s'expliquer par soit une rétention cytosolique ou une dégradation augmentée du récepteur soit par des modifications post-traductionnelles qui peuvent masquer l'épitope reconnu par l'anticorps. Nous avons conclu que le variant *IL10RB* W100G génère une diminution de l'expression protéique totale et transmembranaire, et un profil de glycosylation modifié. Fait intéressant, c'est le seul variant d'*IL10RB* pathogène connu qui code une protéine avec un poids moléculaire accru.

En association avec l'IL-10RA, l'IL-22RA1, l'IL-20R et l'IFNLR1, IL-10RB est impliquée dans la signalisation de l'IL-10, l'IL-22, l'IL-26 et des IFNλ, respectivement[60,135–139]. A l'exception de l'IL-10, la réponse aux cytokines dépendantes de l'IL-10RB n'a pas été vraiment étudiée. Pour déterminer si toutes les variations ont le même impact, nous avons transfecté de manière transitoire des fibroblastes IL-10RB^{KO} SV40 avec WT ou mutant *IL10RB* (W100G, S31F, S58R, Y59C, C66Y, G193R, W204C, E141E ou W159X), avec WT *IL10RA*. Un modèle de glycosylation modifié similaire pour les protéines W100G, C66Y et W204C a été observé dans ce système, par rapport aux cellules HEK293T. Suite à la stimulation avec IL-10, nous avons observé une diminution de la phosphorylation de STAT1 et STAT3 pour les mutations W100G, C66Y et W204C. Toutes les autres mutations (S31F, S58R, Y59Y, G193R, E141X et W159X) présentaient une perte de fonction en termes de phosphorylation à la fois STAT1 et STAT3. Nous avons également analysé l'induction de *CXCL9*, en aval de la phosphorylation de STAT1[140], et l'induction de *SOCS3*, en aval de la phosphorylation de STAT3[141]. L'induction de *CXCL9* et *SOCS3* corrèlait avec les résultats de phosphorylation de STAT1 et STAT3 respectivement pour les allèles testés. Dans l'ensemble, nous avons constaté que les variations S31F, S58R, Y59Y, G193R, E141X et W159X ont aboli toutes les réponses à

l'IL-10, tandis que W100G, C66Y et W204C ont une réponse altérée mais pas abolie. Ces données suggèrent que les variations faux-sens décrites n'ont pas le même impact sur la réponse cellulaire à l'IL-10, malgré une expression clinique apparemment similaire, en terme d'EOIBD.

Nous avons ensuite étudié les réponses cellulaires à l'IL-22, l'IL-26 et l'IFN-λ1 en co-transfectant l'ADN complémentaire WT ou muté, conjointement avec l'*IL22RA, l'IL20RA* ou l'*IFNLR* respectivement dans des cellules IL-10RB^{KO}. IL-10RB W100G, S58R et Y59C ont répondu normalement à la stimulation par l'IL-22, en termes de phosphorylation STAT1 et STAT3. Les protéines IL-10RB C66Y et W204C ont une réponse diminuée, alors que S31F, G193R, E141X et W159X ne présentent aucune réponse. L'induction de *CXCL9* corrèlait les résultats de la phosphorylation de STAT1. La stimulation par l'IL-26 a montré une réponse normale pour les protéines W100G, S58R, Y59C, C66Y et W204C en comparaison à la protéine WT pour la phosphorylation de STAT3, tandis que S31F, G193R, E141X et W159X ont eu une réponse abolie. Suite à la stimulation de l'IFN-λ1, S58R et W100G ont répondu normalement, tandis que Y59C, C66Y et W204C ont une réponse altérée et toutes les autres protéines mutées n'ont pas répondu, en terme de phosphorylation STAT1 et *CXCL9*. Surtout, la mutation W100G s'est comportée normalement, en termes de réponses à l'IL-22, l'IL-26 et l'IFN-λ1 dans ce système expérimental. Toutes les autres variations testées dans ce système in vitro ont présenté soit des réponses normales, soit des réponses abolies. Ces résultats confirment que l'IL-22, l'IL-26 et l'IFN-λ1 ne sont pas cruciaux pour le phénotype EOIBD, comme observés par les patients avec des mutations dans les gènes *IL10* et *IL10RA*. En revanche, on ne peut exclure qu'ils peuvent impacter l'expression clinique, sur d'autres manifestations observées chez les patients, même si cela va nécessiter d'autres investigations expérimentales.

Nous avons utilisé les fibroblastes d'un autre patient IL-10RB$^{W100G / W100G}$[58] (désigné à partir de maintenant comme cellules de patient). Nous avons montré que les cellules du patient ont un niveau d'expression normale en terme d'ARNm d'*IL10RB*, tandis que l'expression est fortement diminuée dans des cellules IL-10RB^{KO}. En terme d'expression protéique, nous avons trouvé une expression fortement réduite de l'IL-10RB à la surface des cellules du patient et des cellules IL-10RB^{KO}, par rapport aux cellules témoins. Nous avons

ensuite transduit de manière stable individuellement chacun des quatre co-récepteurs de l'IL-10RB dans les cellules du patient, d'un patient IL-10RB^{KO} et les cellules témoins. En réponse à l'IL-10, la phosphorylation des protéines STAT1 et STAT3 a été diminuée, et l'induction d'ARNm de *SOCS3* et *CXCL9* est abrogée, dans les cellules du patient. Comme prévu, les cellules STAT1^{KO} n'ont pas répondu à la phosphorylation de STAT1 ou à l'induction de l'ARNm de *CXCL9*. Les cellules IL-10RB^{KO} n'ont pas répondu à la stimulation par IL-10. Par une expérience de complémentation en transfecter l'ADN complémentaire sauvage de *IL10RB* et *IL10RA* dans les cellules de patients a restauré le phénotype en terme de phosphorylation STAT1 et STAT3. Les réponses cellulaires à l'IL-22 sont également altérées dans les cellules du patient W100G, avec une phosphorylation STAT1 plus faible et une induction *CXCL9*, mais avec une phosphorylation STAT3 normale. La reconstitution des cellules du patient avec WT IL-10RB a complémenté le défaut de réponse à l'IL-22 ainsi que sur les cellules IL-10RB^{KO} transfectées avec *IL10RA*. Lors de la stimulation à l'IL-26, il n'y avait pas de phosphorylation STAT3 dans les cellules du patient. Nous n'avons pas pu évaluer la complémentation de l'IL-10RB et la stimulation de l'IL-26. La stimulation par l'IFN-λ1 des fibroblastes du patient a entraîné une diminution de la phosphorylation de STAT1, en comparaison avec les cellules témoins. La reconstitution de l'*IL10RB* et la transfection d'*IFNLR1* dans les cellules du patient ont augmenté la phosphorylation de STAT1 après stimulation par l'IFN-λ1. Dans l'ensemble, les tests fonctionnels dans les cellules du patient IL-10RB$^{W100G\ /\ W100G}$ présentent un défaut plus profond que ce que nous avons observé par les expériences de surexpression de l'allèle W100G. Sur le système de surexpression, nous avons observé un défaut lors de la stimulation de l'IL-10, tandis que les cellules du patient sont également déficientes lors de la stimulation par IL-22, IL-26 et IFN-λ1. Cela peut être une conséquence de la différence d'expression de la surface cellulaire de la protéine IL-10RB dans les deux contextes expérimentaux, la surexpression par rapport au niveau endogène, respectivement. Compte-tenu de la complémentation avec WT IL-10RB sur les réponses IL-10, IL-22 et IFN-λ1, nous concluons que les cellules des patients IL-10RB$^{W100G/W100G}$ présentent un défaut uniquement dans ces voies en raison de la mutation IL-10RB W100G. Les défauts des cellules des patients concernant la stimulation de l'IL-26 pourraient également être dus à la mutation IL-10RB W100G, mais nous ne pouvons pas le dire avec certitude car nous ne sommes pas capables de créer un test suffisamment robuste pour le tester.

Nous avons profité des données de RNAseq réalisés sur des foies d´individus différents (Vilarinho, données non publiées) pour étudier l'expression des transcripts des gènes liés à l'axe IL-10RB à l'état basal. L'ARNm d'*IL10RB* est exprimé dans la plupart des types cellulaires présents dans le foie humain, y compris les hépatocytes, les cholangiocytes, les cellules endothéliales, ainsi que les cellules de Kupffer et lymphoïdes. En revanche, l'expression de l'ARNm d'*IL10RA* est limitée aux cellules immunitaires, y compris les macrophages/monocytes et les cellules lymphoïdes; bien qu'une légère expression de l'ARNm soit détectable sur l'ensemble des types de cellules. L'ARNm de l'*IL22RA1* n'est détecté que dans les hépatocytes et quelques cholangiocytes. Les cholangiocytes sont la principale source d'ARNm d'*IL20RA* dans le foie, tandis que l'ARNm d'*IFNLR1*, est détecté dans la plupart des types de cellules hépatiques, mais dans une moindre mesure. En terme de cytokines, l'ARNm de l'*IL10* est principalement exprimé dans les macrophages/monocytes, les cellules lymphoïdes, mais aussi dans les hépatocytes, les cholangiocytes et les cellules endothéliales. L'ARNm de l'*IL22* n'est pratiquement pas détecté dans le foie, tandis que l'ARNm de l'*IL26* n'était présent que dans les cellules lymphoïdes, et seulement dans une faible proportion de cellules. Enfin, l'expression de l'ARNm de l'*IFNL1* est limitée aux cellules lymphoïdes. Pour valider ces résultats d'ARNm, nous avons stimulé les hépatocytes humains avec IL-10, IL-22, IL-26 et IFN-λ1. Les hépatocytes ont fortement répondu à l'IL-22 et à l'IFN-λ1 en termes de phosphorylation de STAT1 et STAT3. En revanche, nous n'avons pas détecté de réponse, suite à des stimulations par IL-10 et IL-26. Ces résultats corroborent l'expression des récepteurs à chaque cytokine détectée dans les données de RNAseq au niveau basal.

Discussion and conclusion

Nous rapportons une déficience en AR IL-10RB comme une cause monogénique de FVH. Un diagnostic de déficit en IL-10RB doit être envisagé chez les patients atteints de l'HVF, en particulier, mais pas uniquement, chez les patients présentant une EOIBD préexistante. Nos résultats indiquent que la perturbation de la signalisation de l'IL-10 pourrait être responsable de la FVH lors d'une infection par le HAV, seule ou en association avec la signalisation altérée de l'IL-22 et / ou de l'IFN-λ1. En effet, la reconstitution de l'*IL10RB* WT dans les cellules du patient ne complète le défaut que dans l'IL-10, l'IL-22 et

l'IFN-λ1. Les patients présentant un déficit en IL-10 ou IL-10RA pourraient donc être également probablement sujets à l'HVF. Pour prévenir l'HVF, les patients avec un diagnostic de déficit en IL-10, IL-10RA et IL-10RB, avec EOIBD, avant et après une greffe de moelle osseuse (BMT), doivent être vaccinés contre l'HAV et l'HBV. On ne sait pas si le mécanisme de la FVH est hématopoïétique ou non chez ces patients. Nous avions précédemment signalé un déficit en IL-18BP comme la première cause génétique de l'HVF après une infection par le HAV[42]. L'IL-18BP est un antagoniste de l'IL-18, qui est une cytokine inflammatoire induisant l'IFN-γ[115]. L'absence de l'IL-18BP libère l'IL-18, augmentant l'activation de NK, au moins en terme de cytotoxicité, et probablement des cellules T CD8. Ceci pourrait conduire à une production incontrôlée d'IFN-γ et par conséquence à une activation massive des macrophages. De manière frappante, l'IL-10 est une cytokine anti-inflammatoire, et un inhibiteur de la production d'IFN-γ[116]. L'absence de réponse à l'IL10 pourrait conduite à une activité accrue et non régulée de l'IFN-γ, conduisant là aussi à une activation incontrolée des macrophages du foie. Il est tentant de spéculer que la production augmentée et non contrôlée d'IFN-γ pourrait être un mécanisme dans la pathogénie de la FVH.

Les patientes souffraient de formes légères ou sévères d'EOIBD. L'une d'elles avait un retard de croissance, ce qui a nécessité une iléostomie et une colostomie. L'expression clinique de l'EOIBD, associé à un déficit en AR IL-10RB, est variable et peut également être à l'origine de lésions périanales, de folliculite cutanée, de lymphome à cellules B et d'arthrite, [58,96,99,100,104]. Tous les patients rapportés n'avaient pas de lymphome à cellules B, d'arthrite ou de folliculite cutanée. En revanche, tous les patients publiés présentaient une IBD[58,95,96,98–100,103,105,134]. Ces différences cliniques ne semblent pas liées à la mutation, car les patients partageant une même mutation peuvent avoir des phénotypes cliniques différents[103]. Ceci a été illustré avec la première famille publiée avec deux individus affectés porteurs de l'allèle homozygote W159X, dans laquelle le frère présentait des manifestations cliniques plus sévères que sa sœur[103]. Des études antérieures se sont concentrées sur les dosages fonctionnels en réponse à l'IL-10. La réponse à l'IL-22 a été rapportée chez un seul patient[105], mais à notre connaissance, c'est la première fois que des cellules de patients ont été testées en réponse à l'IL-26 et à l'IFN-λ1. Notre étude des conséquences du déficit en AR *IL10RB* en termes de réponses cellulaires à l'IL-10, IL-22, IL-26 et IFN-λ1, a des implications

intéressantes pour la pathogenèse des EOIBD. Le phénotype IBD est connu pour être la conséquence des mutations de l'IL-10RB sur les réponses à l'IL-10. En effet, les déficits héréditaires en IL-10 et IL-10RA sont des phénocopies cliniques du déficit en IL-10RB, au moins pour le phénotype intestinal[100,103]. Nous confirmons que c'est le cas, car la plupart des mutations altèrent uniquement la réponse à l'IL-10 et pas à l'IL-22, l'IL-26 et l'IFN-λ1 dans notre système de surexpression au moins. Les patients ayant reçu une BMT se rétablissent en termes de manifestations cliniques intestinales, suggérant que le phénotype EOIBD est principalement le fait de cellules hématopoïétiques, même si nous pouvons exclure un rôle d'autres types cellulaires[100,120,121].

Dans un système de surexpression, nos résultats suggèrent que les mutations faux-sens dans l'IL-10RB impactent différemment la réponse aux différentes cytokines IL10RB-dépendantes. Il est intéressant de noter que le défaut de réponses cellulaires à l'IL-22, à l'IL-26 et à l'IFN-λ1 par les mutations S31F et G193R, et par les mutations stop, ne semble pas se traduire par un phénotype clinique spécifique, du moins à l'âge de la transplantation ou du décès des patients[98,134]. De plus, C66Y et W204C, hypomorphes en terme de réponse à l'IL-10, l'IL-22 et à l'IFN-λ1, partagent un profil d'expression protéique identique. Ces mutations peuvent modifier la structure 3D de la protéine en perturbant ou en créant de nouvelles liaisons disulfure. Les mutations S58R et Y59C ne répondent pas à l'IL-10, mais répondent normalement ou partiellement à l'IL-22, à l'IL-26 et à l'IFN-λ1. Ceci est concordant avec les travaux qui ont montré que le résidu Y59, localisé sur la boucle 2 de l'IL-10RB, important essentiel pour l'interaction IL-10/IL-10RB[111], mais pas pour l'interaction IL-22/IL-10RB[111]. Malheureusement, nous n'avons pas eu accès aux fibroblastes de patients porteurs d'autres mutations faux-sens dans IL-10RB pour tester leur réponse à d'autres cytokines comme pour la mutation W100G. D'autres études, en plus d'une description clinique plus détaillée, seraient nécessaires pour disséquer et corréler toutes les manifestations cliniques et la réponse à l'IL-10, à l'IL-22, à l'IL-26 et à l'IFN-λ1 chez les patients présentant un déficit en IL-10RB. Notre étude suggère que l'altération ou la perturbation des réponses cellulaires à l'IL-22, à l'IL-26 et à l'IFN-λ1 n'est pas nécessaire pour le développement du phénotype clé observé chez les patients déficients en *IL10RB*, à savoir l'EOIBD.

Annexe 2

Fulminant viral hepatitis in siblings with inherited IL-10RB deficiency

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# Equal contribution

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Abstract

Fulminant viral hepatitis (FVH) caused by hepatitis A virus (HAV) is a life-threatening disease striking otherwise healthy individuals, normally resistant to other infections. The only known genetic etiology of FVH is inherited IL-18BP deficiency, which unleashes IL-18-dependent lymphocyte cytotoxicity and IFN-γ production. We report a multiplex family in which two siblings died from a combination of early-onset inflammatory bowel disease (EOIBD) and FVH by HAV. The sibling tested is homozygous for the W100G mutation in *IL10RB,* like another unrelated patient with EOIBD. We show that homozygosity for loss-of-expression *IL10RB* variants, seen in other EOIBD patients, disrupts cellular responses to IL-10, IL-22, IL-26, and IFNλs. Surprisingly, missense mutations reported in EOIBD patients did not behave equally in their response to all stimuli. The W100G variant tested in isolation impairs cellular response to IL-10, but not IL-22, IL-26, and IFNλ1. In contrast, a lower expression in patient cells results in a more general impairment including IL-10, IL-22, IL-26 and IFNλ1. Moreover, we also show that IL-10RB mRNA is expressed in all liver resident cells, with hepatocytes responding predominantly to IL-22 and IFNλs, while mononuclear phagocytes respond more strongly to IL-10. Inherited IL-10RB deficiency may account for FVH, especially in patients with EOIBD, who should be vaccinated against HAV. EOIBD in patients with IL-10RB deficiency seems to be caused primarily by the disruption of IL-10 immunity, consistent with its occurrence in patients with IL-10 or IL-10RA deficiency. The disruption of IL-22, IL-26, and IFNλs immunity in patients without IL-10RB expression does not seem to bear detectable clinical consequences. Finally, IL-10 being a potent antagonist of IFN-γ, this experiment of nature suggests that molecular basis of FVH in patients with IL-18BP or IL-10RB deficiency involves excessive IFN-γ activity in the course of HSV infection.

Introduction

The most common agents of human viral hepatitis are hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis E virus (HEV)[1–3]. HBV and HCV typically cause chronic infections, which can underlie fibrosis, cirrhosis, and hepatocellular carcinoma[4]. In contrast, infections with HAV and HEV are always acute and in most cases benign[5]. In rare cases, primary infection with HAV, HBV, or in pregnant women and immunosuppressed individuals HEV, can lead to fulminant viral hepatitis (FVH), which is characterized by life-threatening hepatic failure. In the vast majority of cases, FVH strikes otherwise healthy individuals, normally resistant to other infectious agents[6–9]. The incidence of FVH is not well known but has been estimated at about 0.5% of symptomatic HAV individuals[10]. Since the prevalence of HAV infection varies greatly from country to country, as does the HAV vaccination coverage, the estimations of FVH have varied from ~0.6 cases per million individuals in Scotland[11] to ~1,6 cases per million in Thailand[12]. The outcome of FVH is poor, with less than 25% of survival without liver transplantation[6,7,13–16].

Particular HAV genotypes were not found to account for FHV[17,18], which is not epidemic, suggesting that FVH is not caused by more virulent viral isolates. Although almost always sporadic, a few familial forms of FVH have been reported, yet with years of interval between cases[19–21]. These observations suggested that FVH might result from monogenic inborn errors of liver immunity to viruses, more often with incomplete than complete penetrance. Severe viral diseases other than FVH can result from such single-gene inborn errors of immunity[22,23,32,24–31]. We recently reported autosomal recessive (AR) IL-18BP deficiency as the first monogenic cause of FVH due to HAV in an otherwise healthy patient[33]. IL-18BP is an antagonist of the IL-18 cytokine[34], which activates NK and T cytotoxicity and their production of IFN-γ[35,36]. The lack of

IL-18BP unleashes IL-18 activity, which leads to uncontrolled cytotoxicity, thereby killing both HAV-infected and other hepatocytes [33]. We studied another, multiplex family, in which the index patient with a mild form of early-onset inflammatory bowel disease (EOIBD) died of fulminant hepatitis while her sister with a more severe form of EOIBD developed severe hepatitis due to HAV and died of the combination of both conditions.

Results

Identification of an homozygous missense mutation in IL-10RB

We studied a 6-year-old girl who presented a mild form of early-onset inflammatory bowel disease (EOIBD) and died from fulminant hepatitis without being studied for the etiology (P1, Figure 1A). Her sister, P2, developed a more severe form of EOIBD. At 2 years of age, she was hospitalized with lethargy, jaundice, and elevated liver enzymes due to HAV infection. She recovered from HAV infection, but her IBD worsened with multiple perforations of the colon. Total colectomy and ileostomy were performed at age 3. At 3 years she suddenly died of multi-organ failure possibly due to a septic shock. The two siblings were born to first-cousin parents originating from and living in Saudi Arabia. Eleven other siblings were healthy. We performed whole exome sequencing on P2 and confirmed parental consanguinity, with a rate of homozygosity of 4.71%[37]. No DNA was available for P1. We hypothesized that the genetic disorder in this family may display autosomal recessive (AR) inheritance. We thus selected very rare homozygous non-synonymous variations, and prioritized them according to their predicted deleteriousness and potential impairment of liver immunity. There were 18 homozygous candidate variants (Supplementary table 1). We found the homozygous variant p.Trp100Gly on the *IL10RB* gene, with a CADD score of 29.8 above the mutation significance cutoff (MSC) 99%

score of 23.8, to be the best candidate variant as it had the highest CADD score and it was previously reported in a family with EOIBD (Figure 1B)[38]. Its familial segregation was consistent with an AR trait, since the parents were heterozygous and one healthy sister was WT/WT. We do not have available genetic material from the remaining siblings. Moreover, there are no homozygous *IL10RB* variations predicted to be LOF in the general population with a CADD score higher than the MSC (Figure 1C). Furthermore, *IL10RB* is encoding the second chain of the receptor for IL-10 in association with IL-10RA, IL-22 in association with IL-22RA1, IL-26 in association with IL-20RA and IFNλs in association with IFNLR1[39,40]. Finally, bi-allelic mutations in *IL10*, *IL10RA*, and *IL10RB* have previously been described as causing EOIBD[41,42]. Reported *IL10RB* LOF mutations present mostly with a CADD score higher than the MSC and are private or with a frequency lower than 10^{-4} (Supplementary table 2). The W100G variation itself has even been reported in one patient[38]. Collectively, these data strongly suggested that homozygosity for W100G in *IL10RB* was the cause of EOIBD, and perhaps also that of severe hepatitis, in the two siblings.

Impaired expression and glycosylation pattern of W100G protein

None of the previously reported IL-10RB-deficient patients had FVH; none was either reported to have been infected with HAV[38,39,49,41–48]. One patient homozygous for W159X developed autoimmune hepatitis[42]. We undertook the molecular characterization of all reported pathogenic missense *IL10RB* alleles (Table 1) and two stop-gain alleles (E141X and W159X) by overexpression experiments. These variants were all previously described to cause EOIBD[38,41–44,46,50–53]. Predicted loss-of-function (pLOF) mutations in *IL10RB* have been reported to cause premature termination of translation in different domains of the protein, whereas missense pathogenic mutations are clustered in the extracellular domain[38,42,46,50–53] (Figure 1D). In

overexpression experiments in HEK293T cells, all missense variants tested showed normal mRNA expression, when compared with WT *IL10RB*, while the two pLOF variants tested (E141X and W159X) showed a strong decrease, suggesting nonsense-mediated mRNA decay (Figure 2A). At the protein level, WT IL-10RB displayed two major bands (about 50 and 60 kDa), suggestive of post-translational modifications. As expected, E141X and W159X proteins had both smaller molecular weight (MW) and lower expression level, when compared with WT. Four missense proteins, including W100G, were expressed at lower levels than WT (Figure 2B). Interestingly, we observed a higher than WT MW for W100G, while C66Y and W204C displayed only the smaller WT band, suggestive of glycosylation patterns depending of the mutations (Figure 2B). Following PNGase-F treatment, which removes all N-linked oligosaccharides, the WT and all missense alleles went from two major bands to a triplet with a major band around 45 kDa, with the exception of C66Y and W204C displaying only the lower molecular part. We normalized the MW of W100G in comparison to WT, confirming that its initial higher molecular weight was due to aberrant glycosylation. With and without PNGase-F treatment, C66Y and W204C proteins are a singlet whereas WT protein is a triplet, suggesting that these two mutations might also alter other post-translational modifications.

Impaired surface expression of W100G protein

We used SV40-transformed fibroblasts from a patient with complete IL-10RB deficiency (IL-10RB^{KO})[41]. By overexpression experiments and using an antibody specific to IL-10RB, we showed that four missense proteins (W100G, S58R, Y59C, and G193R) are expressed at the cell surface, albeit for two with a lower median fluorescent intensity (MFI) than WT (W100G and G193R) (Figure 2C). In contrast, the three other missense proteins tested (S31F, C66Y, W204C) and the two proteins potentially encoded by pLOF variants E141X and W159X are not expressed at the cell surface (Figure 2C). In the same experiments, we also analyzed intracellular expression

with antibodies against IL-10RB or a tag (Cter-Myc). With the anti-Myc antibody, we observed a normal intracellular expression of all missense proteins and a decreased expression of the proteins encoded by the two pLOF variants, when compared with WT (Supplementary figure 1A). In addition, with the antibody to IL-10RB, we observed a normal cytosolic expression of S58R, Y59C and G193; a slight decrease in MFI of W100G, S31F and C66Y; and no detectable expression of W204C, E141X, and W159X, suggesting that these last mutant proteins are not recognized by the anti-IL-10RB antibody. The variants IL-10RB W100G, S31F, C66Y, and G193R that in comparison to WT, have cytosolic levels similar and surface levels heavily diminished may suffer from cytosolic retention, or enhance receptor degradation or post-translational modifications that may mask the epitope recognized by the antibody clone once the membrane surface is reached. We concluded that the *IL10RB* W100G variant generates a decrease in total protein expression, an altered glycosylation pattern, and a lowered surface expression. Interestingly, it is the only known pathogenic *IL10RB* variant that encodes a protein with an increased MW.

Impaired response to IL-10 for all missense variants

In association with IL10RA, IL22RA1, IL20R, and IFNLR1, IL-10RB is involved in the signaling downstream IL-10, IL-22, IL-26, and IFNλs, respectively[40,54–58]. The function of the IL-10RB pathogenic proteins, in terms of their response to IL-10RB-dependent cytokines, has not been reported. To determine if all variants have the same impact: we transiently transfected IL-10RB^{KO} SV40-fibroblasts with WT or mutant *IL10RB* (W100G, S31F, S58R, Y59C, C66Y, G193R, W204C, E141E or W159X), together with WT *IL10RA*. A similar altered glycosylation pattern for W100G, C66Y and W204C proteins was observed in this system, when compared with HEK293T cells (Figure 2D). Following stimulation with IL-10, we observed a decreased

phosphorylation of STAT1 and STAT3 for W100G, C66Y, and W204C IL-10RB (Figure 2D). All other variants (S31F, S58R, Y59Y, G193R, E141X and W159X) were loss-of-function in terms of both STAT1 and STAT3 phosphorylation. We also analyzed CXCL9 induction, downstream of STAT1 phosphorylation[59], and SOCS3 induction, downstream of STAT3 phosphorylation[60]. Patterns of CXCL9 and SOCS3 induction mirrored the patterns of STAT1 and STAT3 phosphorylation respectively for the alleles tested (Supplementary figure 1B). Altogether, we found that *IL10RB* variants S31F, S58R, Y59Y, G193R, E141X and W159X abolished all responses to IL-10, while W100G, C66Y, and W204C impaired but did not abolish responses to IL-10. These data suggest that the various missense variants did not have equal impacts on cellular responses to IL-10, despite having apparently similar clinical impacts, in terms of EOIBD.

Impaired or normal response to IL-22, IL-26, and IFN-λ1 activation in overexpression system

We then studied cellular responses to IL-22, IL-26, and IFN-λ1 by co-transfecting WT or mutant *IL10RB,* together with *IL22RA*, *IL20RA*, or *IFNLR* respectively in IL10RB^{KO} cells. IL-10RB W100G, S58R, and Y59C responded normally to IL-22 stimulation, in terms of STAT1 and STAT3 phosphorylation (Figure 2E). The IL-10RB C66Y and W204C proteins responded poorly, while S31F, G193R, E141X, and W159X did not respond at all. CXCL9 induction mirrored the results of STAT1 phosphorylation (Supplementary figure 1C). IL-26 stimulation resulted in IL-10RB W100G, S58R, Y59C, C66Y, and W204C responding like WT for STAT3 phosphorylation, while S31F, G193R, E141X and W159X had an abolished response (Figure 2F). Following IFN-λ1 stimulation, S58R and W100G responded normally, while Y59C, C66Y, and W204C responded poorly and all other mutants did not respond at all, in terms of

STAT1/STAT3 phosphorylation (Figure 2G) and CXCL9 (Supplementary figure 3D). Importantly, our family's variant (W100G) behaved normally, in terms of responses to IL-22, IL-26, and IFN-λ1 in this experimental system. All other variants tested in this *in vitro* system ranged in responses from normal to abolished, suggesting that IL-22, IL-26, and IFN-λ1 are not crucial for the EOIBD phenotype, while not excluding that they may operate as modifiers of this phenotype while underlying other, as yet unknown clinical manifestations.

Impaired response to IL-10, IL-22, IL-26, and IFN-λ1 in patient's cells

No cells were available from the two deceased siblings. Nevertheless we took advantage of the availability of fibroblasts from another IL-10RB$^{W100G/W100G}$ patient[38] (referred onwards as patient cells). We showed that patient cells have normal levels of *IL10RB* mRNAs, as measured by qPCR, while their levels were decreased in IL-10RB^{KO} cells (Figure 3A). We tested the expression of IL-10RB on the cell surface by flow cytometry. We found a strongly reduced expression of IL-10RB in patient and IL-10RB^{KO} cells, when compared with control cells (Figure 3B). We then stably transduced individually each of the four co-receptors of IL-10RB in patient, IL-10RB^{KO}, and control cells. In response to IL-10, STAT1 and STAT3 protein phosphorylation were decreased, and SOCS3 and CXCL9 mRNA induction abrogated, in the patient cells (Figure 3C and supplementary figure 2A). STAT1^{KO} cells as expected did not respond for the phosphorylation of STAT1, or the induction of CXCL9 mRNA. IL-10RB^{KO} cells did not respond in any way tested to IL-10 stimulation. Transfecting IL-10RB WT and IL-10RA in patient cells rescued the phenotype in terms of STAT1 and STAT3 phosphorylation (Figure 3D). Cellular responses to IL-22 were also impaired in patient SV40-fibroblasts, with lower STAT1 phosphorylation and CXCL9 induction, but with a normal STAT3 phosphorylation (Figure 3F

and supplementary figure 2B). Reconstitution of patient cells with WT IL-10RB rescued the IL-22 response defect as well as on IL-10RB^{KO} cells transfected with IL-10RA (Figure 3F). Upon IL-26 stimulation, there was no STAT3 phosphorylation in the patient cells (Figure 3G). We could not assess IL-10RB complementation and IL-26 stimulation. IFN-λ1 stimulation of patient SV40-fibroblasts resulted in diminished STAT1 phosphorylation, in comparison with control cells (Figure 3I and supplementary figure 2C). IL-10RB reconstitution and IFNLR1 transfection in patient cells increased STAT1 phosphorylation after IFN-λ1 stimulation (Figure 3I). Altogether, functional assays on IL-10RB$^{W100G/W100G}$ patient cells have a more profound defect than what we observed on the overexpression of the W100G IL-10RB allele in isolation. On the overexpression system, we observed a defect upon IL-10 stimulation, whereas patient cells are also deficient upon IL-22, IL-26 and IFN-λ1 stimulation. This may be a consequence of the difference of cell surface expression of IL-10RB protein in both experimental settings, overexpression versus endogenous level, respectively. Therefore making the defect towards IL-22 and IFN-λ1 stimulation a purely quantitative defect, since at higher concentrations of the W100G IL-10RB protein, as in the overexpression system there is no defect observed. Considering the complementation with WT IL-10RB on IL-10, IL-22 and IFN-λ1 responses, we conclude that the IL-10RB$^{W100G/W100G}$ patient cells present a defect only in those pathways due to IL-10RB W100G mutation. The defects of patient cells regarding IL-26 stimulation could be due to the IL-10RB W100G mutation also, but we cannot say for sure since we are not capable of creating an assay robust enough to test it.

mRNA expression in human liver cells and functional response of hepatocytes of IL-10RB axis components

We took advantage of mRNAseq liver data obtained from X different individuals (Vilarinho unpublished data) to study the abundance of transcripts related to the IL-10RB axis in a basal state (Figure 4A). *IL10RB* mRNA is expressed in most cell types present in the human liver, including hepatocytes, cholangiocytes, endothelial cells, also Kupffer and lymphoid cells. In contrast, *IL10RA* mRNA expression is mainly restricted to immune cells, including macrophages/monocytes, and lymphoid cells; despite a slight detection of mRNA expression on all other cell types. *IL22RA1* is only detected in hepatocytes and a few cholangiocytes. Cholangiocytes are the main source of *IL20RA* mRNA in the liver, while *IFNLR1* mRNA, as *IL10RB*, is detected in most liver cell types but in a lower extent. In term of cytokines, *IL10* mRNA is mostly expressed in macrophages/monocytes, lymphoid cells, but also in hepatocytes, cholangiocytes and endothelial cells. *IL22* mRNA was practically not detected in the liver, while *IL26* mRNA was present only in lymphoid cells, and only in a weak proportion of cells. Finally, *IFNL1* mRNA expression is restricted to lymphoid cells. To validate these mRNA results, we stimulated human hepatocytes with IL-10, IL-22, IL-26 and IFN-λ1 (Figure 4B). Hepatocytes responded strongly to IL-22 and IFN-λ1 in term of STAT1 and STAT3 phosphorylation. In contrast, we did not detect a response following IL-10 and IL-26 stimulations. These results corroborate the expression the receptors to each cytokine detected in RNAseq data at basal level.

Discussion

We report AR IL-10RB deficiency as a monogenic cause of FVH. A diagnosis of IL-10RB deficiency should be considered in patients with FVH, especially but not only in patients with pre-existing EOIBD. Our findings indicate that the disruption of the IL-10 signaling might

be responsible for FVH upon HAV infection, alone or in association with the impaired signaling to IL-22, and/or IFN-λ1. Indeed, the reconstitution of IL10RB WT in patient cells complements the defect only in IL-10, IL-22 and IFN-λ1. Patients with IL-10 or IL-10RA deficiency might be therefore also probably prone to FVH. To prevent FVH, patients with a diagnosis of IL-10, IL-10RA, and IL-10RB deficiency, whether asymptomatic or with EOIBD, prior to and after bone marrow transplantation (BMT), should be vaccinated against liver-tropic viruses, particularly HAV and HBV. It is unknown if the mechanism of FVH is hematopoietic or not in these patients. We previously reported IL-18BP deficiency as the first genetic cause of FVH following HAV infection[33]. IL-18BP is an antagonist of IL-18, which is an inflammatory IFN-γ-inducing cytokine[61]. The lack of IL-18BP unleashes IL-18, enhancing activation of NK, at least in terms of cytotoxicity, probably T cells, and of macrophages, probably by uncontrolled IFN-γ production. Strikingly, IL-10 is an anti-inflammatory cytokine, a potent de-activator of phagocytes, and as such a natural antidote to IFN-γ[62]. Increased, unregulated IFN-γ activity can thus result from abolished cellular responses to IL-10. Both IL-18BP and IL-10RB deficiencies result in enhanced inflammation, including enhanced IFN-γ production. It is tempting to speculate that enhanced IFN-γ production may be a core mechanism underlying FVH.

Our patients suffered from mild or severe forms of EOIBD. One of them had failure to thrive, which required ileostomy and colostomy. AR IL-10RB deficiency can also underlie perianal lesions, skin folliculitis, B cell lymphoma, and arthritis, which were not seen in our patients[38,42,44,53,63]. Not all reported patients had B cell lymphoma, arthritis, or skin folliculitis. In contrast, all published patients presented with IBD[38,41–44,46,50–53]. These clinical differences do not seem to be related to the mutation, as patients sharing a same mutation could have different outcomes[41]. This has been exemplified with the first published family with two affected members carrying in homozygosity the W159X *IL10RB* allele, in which the male sibling presented a

stronger clinical manifestations than his sister[41]. Previous studies have focused on functional assays in response to IL-10. The response to IL-22 has been reported in only one patient[43], but to our knowledge is the first time patient cells have been assayed in response to IL-26 and IFN-λ1. Our study of the consequences of AR IL10RB deficiency in terms of cellular responses to IL-10, IL-22, IL-26 and IFN-λ1, has interesting implications for the pathogenesis of IBD. The IBD phenotype was known to be caused by the impact of IL-10RB mutations on responses to IL-10, because inherited IL-10 and IL-10RA deficiencies are clinical phenocopies of inherited IL-10RB deficiency, at least in terms of their intestinal phenotypes[41,42]. We confirm that this is the case, as most mutations impair IL-10 but not IL-22, IL-26 and IFN-λ1 responses in our overexpression system at least (Table 2). It is not proven that the EOIBD phenotype is a purely and solely hematological disease, but it is likely as patients who underwent BMT recovered in term of intestinal clinical manifestations[42,64,65].

There are different patterns emerging in the response of the studied missense IL-10RB mutations in overexpression system (Figure 5). It is interesting that the disruption of cellular responses to IL-22, IL-26, and IFN-λ1 by S31F and G193R mutations, and by stop mutations, does not seem to underlie any particular clinical phenotype, at least by the age of death or transplantation of the patients[46,51]. Moreover, IL-10RB C66Y and W204C are both hypomorphic in terms of response to IL-10, IL-22 and IFN-λ1, and share the lack of an upper band when overexpressed. These mutations may change the protein 3D structure by disrupting or creating disulfide bonds. There are also IL-10RB S58R and Y59C not responding to IL-10, but responding to IL-22, IL-26 and IFN-λ1. This is concordant with previous publications that have remarked on how residue Y59 is located on the loop 2 of IL-10RB, important to IL-10RB/IL-10 interaction[66], but not for IL-10RB/IL-22 interaction. At any rate, IL-10RB W100G is the only IL-10RB reported missense mutant affecting only the response to IL-10 in our overexpression

system in IL-10RB^{KO} cells. Unfortunately, we did not have access to fibroblasts from patients carrying other missense mutations in IL-10RB to validate their response to other cytokines as for W100G. These different patterns of response could also accompany a distinct natural history of disease. Further studies, in addition of more detailed clinical description, would be necessary to dissect and correlate all clinical manifestations and response to IL-10, IL-22, IL-26 and IFN-λ1 in patients with IL-10RB deficiency. Our study suggests that the impairment or disruption of cellular responses to IL-22, IL-26, and IFN-λ1 is not required for the development of the key phenotype seen in IL10RB-deficient patients, namely EOIBD. They do not exclude the possibility that these deficits operate as modifiers for both phenotypes. They do not either exclude that hitherto unknown phenotypes, infectious or otherwise, are revealed with the diagnosis of more patients and the study of new phenotypes, as exemplified by this study of FVH.

Materials and methods

Whole exome sequencing (WES) and Sanger sequencing

DNA was isolated from blood with the iPrep PureLink gDNA Blood Kit and iPrep Instruments by the manufacturer's instructions (Life Technologies). Whole exome sequencing and annotation of the variants was previously described in Belkaya et al[33]. Filtering of the variant calls was done taking out the ones with a genotype quality <50, depth of coverage <5, no consensus coding sequence, gene damage index>13.84[67], allele frequency in any population present in GnomAD >0.001, CADD score V3.1>MSC[68] and present in the blacklist[69]. Only variants annotated as indel-inframe, indel-frameshift, start-lost, missense, nonsense, stop-lost, and splicing were retained and manually checked in Alamut 2.9.0 to verify that they actually were present.

IL-10RB W100G mutation was checked with the Sanger method using the forward primer TGTCTCATAAATCACATGCCCC and reverse primer CTTGTGCAAGGCCATCCATTT; for the IL-10RB W159X mutation, forward primer AGCAGTGTACTTCCGTGGAC and reverse primer ACCAGTCCATAAGGTGCTGC were used with Taq DNA polymerase (Applied Biosystems, Invitrogen) to amplify genomic DNA. 1% agarose gel was used to analyze PCR products by electrophoresis. Afterwards, purification was done with Sephadex G-50 Superfine resin (GE Healthcare). Sequencing was performed by dideoxynucleotide termination, with the BigDye Terminator kit (Applied Biosystems). Purification was done again by Sephadex G-50 Superfine resin before sequencing on a 3700 apparatus (Applied Biosystems). Results were analyzed with DNA Baser 4.36.0.2 software.

Cell culture

SV40-fibroblasts from the published patients $STAT1^{c.1928insA/c.1928insA}$ ($STAT1^{KO}$)[70], Tyk2 ($Tyk2^{KO}$), $IL10RB^{W100G/W100G}$ (patient)[38] and $IL10RB^{W159X/W159X}$ ($IL\text{-}10RB^{KO}$) were used in this study. SV40-transformed fibroblasts (SV40-fibroblasts) were cultured in DMEM medium supplemented with 10% fetal calf serum (FCS Thermo Fisher). Cells were cultured at 37°C, under an atmosphere containing 5% CO2.

Human primary hepatocytes were purchased (Cytes biotechnologies), thawed, and seeded by the providing company instructions with the accompanying media: Hepatocyte Thawing Medium, Hepatocyte Plating Medium and Hepatocyte Maintenance Medium (all from Cytes biotechnologies). Seeding was performed 24 hours before stimulation.

Plasmids, transfections and transductions

Cells were transfected by Lipofectamine LTX (Invitrogen) following instructions by the manufacturer. Plasmids for pLX302-IL10RA-V5 and pLX302 empty vector were purchased from Addgene. pCMV3-IL22RA1-C-His, pCMV3-SP-Myc-IL-10RB and pCMV3 empty vector were purchased from Sinobiological. All other vectors were purchased from Origene: pCMV6-IL10RB-Myc-DDK, pCMV6-IFNLR1-Myc-DDK (transcript variant 1), pCMV6-IL20RA-Myc-DDK and pCMV6 empty vector. To obtain the mutant alleles for S31F, S58R, Y59C, C66Y, W100G, and G193R; and the elimination of the DDK tag on pCMV6-IL10RB-Myc-DDK we performed mutagenesis with a site-directed mutagenesis kit (QuikChangeII XL; Agilent Technologies), according to the manufacturer's instructions. For the preparation of pCMV6-E141X-IL10RB-Myc and pCMV6-W159X-IL10RB-Myc we designed reverse primers on the plasmid from the stops codons and forward from the start of the Myc tag. We amplified using CloneAmp HiFi Premix (Takara) following the protocol provided by the manufacturer. Afterwards we purified the PCR products with DNA PCR purification kit (Qiagen) and used both the Quick Blunting Kit (New England Biolabs) and Quick Ligation Kit (New England Biolabs), finally transforming the NEB 10-beta Competent *E. coli* (High Efficiency, New England Biolabs) following all steps from manufacturer's recommendations. All plasmids were inspected by sequencing the full length of the ORF and the parts where it inserts to the backbone and tags. All primers are available upon request.

Transduction on SV40-fibroblasts of IL-10RA, IL-20RA, IL-22RA1 and IFNLR1 was carried out by following the protocol described elsewhere[71] from the plasmids mentioned on the previous paragraph while keeping the tags. The only difference to the protocol published is that we added a step of double purification of the virus first by a 5nm filter (Millex, Merckmillipore) and afterwards on a 0.2nm filter (Pall Corporation).

For all experiments requiring transient transfection, half a million cells were seeded on 12 well plates. The next day, cells were transfected with lipofectamine LTX plus (Thermofisher) under the manufacturer's instructions, using the highest recommended amount of lipofectamine LTX. Cells were harvested or stimulated 24 hours afterwards.

Real time quantitative PCRs

Total RNA was extracted with the RNeasy Plus Mini Kit (Qiagen). RNA was reverse transcribed directly, with the High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Q-PCR was performed with the Applied Biosystems Assays-on-Demand probes/primers specific for CXCL9 (Hs00171065_m1), SOCS3 (Hs02330328_s1) and IL-10RB (Hs00175123_m1). GUS (Hs99999908_m1) was used as endogenous control for SOCS3; and HPRT1 (HGPRT, Hs99999909_m1) for CXCL9 and IL-10RB. Results are expressed according to the ΔCt method, as described by the manufacturer.

Flow cytometry

Cells were trypsinized (Thermofisher), washed with staining buffer (PBS plus 0.5% FCS), to prepare the samples for staining. For the experiments on the overexpression system IL-10RB Alexa fluor 647 (BD Mouse IgG1, κ Clone #90220), Myc-FITC (Miltenyi Biotec) and IL-10RB IL-10RB PE (Biothechne, Mouse IgG1 Clone #90220) were used in conjunction with the BD Cytofix/Cytoperm kit following the manufacturer's instructions to perform the staining. For the patient cells, staining was made in 100 µl staining buffer and IL-10RB PE or isotype control (Biothechne, Mouse IgG1 Clone #90220 and mouse IgG1 PE isotype) over ice for one hour. Acquisition was done on a Gallios cytometer (Beckman Coulter) and analysis was performed on FlowJo.

PNGase treatment and cytokine stimulationsPNGase treatment was carried out with PNGase F (New England Biolabs) as per manufacturer instructions. Stimulations were carried out with, IL-10, IL-22, IL-26 and IL-29/IFNλ1 all from R&D Systems at the time and concentrations indicated on each figure. In all cases cells were plated the day before stimulation with fresh media. All experiments were performed at least 3 times.

Immunobloting

Total protein was extracted from the cells with a lysis buffer containing 1% NP-40 (Fluka), 20 mM Tris-HCl pH 7.4 (Tris MP Biomedicals, HCl Sigma), 140 mM NaCl (VWR), and 2 mM EDTA (MP Biomedicals); supplemented with 100 mM orthovanadate (Sigma), 200 mM PMSF (Sigma), proteinase inhibitor cocktail mix (Roche), phosSTOP (Roche), and 0.1 mM DTT (Invitrogen). Protein fractions were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes (Biorad). The following primary Abs were used: mouse anti–phosphorylated Y701 STAT1 (BD), mouse anti-STAT1 (BD), rabbit anti–phosphorylated Y705 STAT3 (Cell Signaling Technology), rabbit anti-STAT3 (Cell Signaling Technology), goat anti-IL-10RB (Biothecne), mouse HRP-conjugated anti-His (Santa Cruz Biotechnology), mouse anti-V5 (Invitrogen), mouse anti-GAPDH (Santa Cruz Biotechnology) or rabbit anti-GAPDH (Santa Cruz Biotechnology) and mouse HRP-conjugated anti-DDK (Origene). Antibody binding was detected by incubation with HRP-conjugated anti–mouse, anti-goat or anti–rabbit secondary Abs (GE Healthcare), with the ECL system (Thermo Fisher Scientific). Antibodies were also revealed by fluorescence on the Licor system with secondary antibodies conjugated to IRDye800 or IRDye680 anti-mouse, anti-goat or anti-rabbit (all from Licor Biosciences Proteomics).

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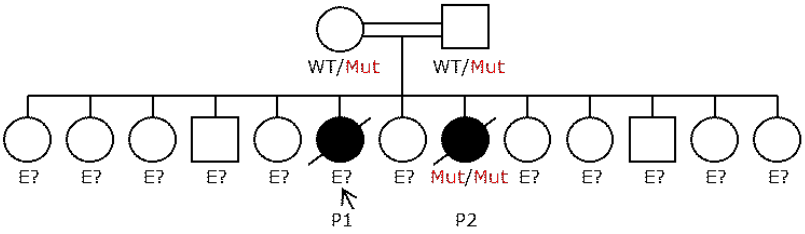
The authors have no conflict of interest to declare.

Figures

Figure 1 *Homozygous tryptophan to glycine replacement at position 100 (W100G) of* IL-10RB. *A.* ***Pedigree of the family. The patients are shown in black, whereas healthy individuals are shown in white. IL-10RB*** **W100G status is indicated in red when available.** *B.* ***Confirmation by Sanger sequencing of the mutation and its homozygous state in the patient and heterozygous in parents.*** *C.* ***Graph showing the CADD 1.3 scores of the published pathogenic*** **IL-10RB** ***variants –where possible to apply- versus their minor allele frequency on GnomAD. W100G*** **IL-10RB** ***is marked as a red cross, loss of function (LOF, including stop-gain, indel-frameshift and essential splicing) published variants are red squares, missense mutants are blue squares, and a 3*UTR mutant is marked with a green square; finally public homozygous variants –both missense- are marked in black dots. Mutation significance cutoff (MSC) score 99% signaled with a dashed line.*** *D.* ***Schematic diagram of IL-10RB protein with pathogenic mutations marked.***

Figure 1

A



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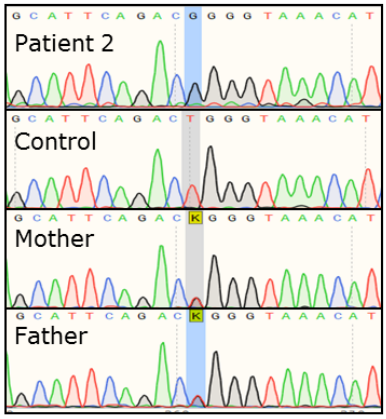

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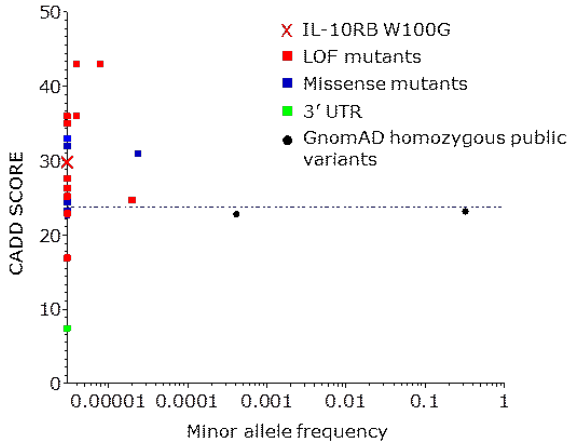

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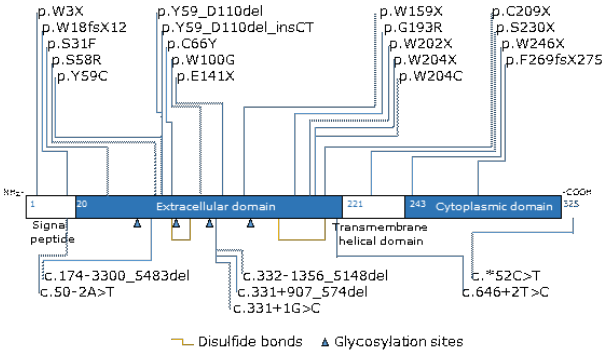


Figure 2 *W100G* IL-10RB *allele characterization and functional assessment to IL-10, IL-22, IL-26 and IL-29 stimulation. A.* ***RT-qPCRs results from HEK293T transfected 24 hours with the indicated IL-10RB alleles. IL-10RB mRNA expression is shown corrected by the endogenous expression of HPRT1 mRNA and normalized to the first column set as 1. Values shown are the means of three independent experiments performed in duplicate ± SEM.*** *B.* ***Representative western blot showing IL-10RB alleles expression level on HEK293T cells post 24-hour transfection with and without PNGase treatment.*** C. ***Flow cytometry of surface IL-10RB on transfected IL-10RB^{KO} SV-40 fibroblasts 24 hours with indicated*** **IL-10RB** ***alleles, showing mean fluorescent intensity (MFI) on IL-10RB+ gate ± SEM. Results from three independent experiments are shown.*** *D.* ***Response to IL-10 stimulation 40ng/ml for 30 minutes on*** **IL-10RB^{KO}** ***SV-40 fibroblasts transfected with*** i***ndicated*** **IL10RB** ***alleles and IL10RA-V5 on representative immunoblot image showing the analysis of STAT1 (pSTAT1) and STAT3 (pSTAT3) phosphorylation. STAT1, STAT3, GAPDH, V5 (IL-10RA) and IL-10RB levels were also assessed. E. Western blot depicting phospho-STAT1 (pSTAT1) and phospho-STAT3 (pSTAT3) on IL-10RB^{KO} SV-40 fibroblasts transfected with indicated*** **IL-10RB** ***alleles and*** **IL-22RA1-***His with IL-22 stimulation 100ng/ml for 30 minutes. GAPDH was used as a loading control, STAT1, STAT3, His (IL-22RA1) and IL-10RB levels were also depicted.*** *F.* ***Western blot of IL-10RB^{KO} SV40-fibroblasts transfected with the indicated IL-10RB alleles and WT IL-20RA-DDK after treatment with IL-26 100ng/ml for 45 minutes. The membrane has been probed with an antibody specific for phosphorylated STAT3 (pSTAT3). An antibody against GAPDH was used as a loading control; STAT3 and IL-10RB levels were also shown.*** *G.* ***Western blot showing the detection of phospho-STAT1 (pSTAT1) in response to IFN-λ1 100ng/ml 30 minute stimuli on IL10RB^{KO} SV-40 fibroblasts transfected with indicated IL10RB alleles and IFNLR1-DDK.GAPDH was used as loading control. We show also STAT1, DDK***

(IFNLR1) and IL-10RB. EV = empty vector, WT = wild type, SEM = standard error of the mean and p = phosphorylated. All western blots performed at least three times, with the exception of 2F performed twice.

Figure 2

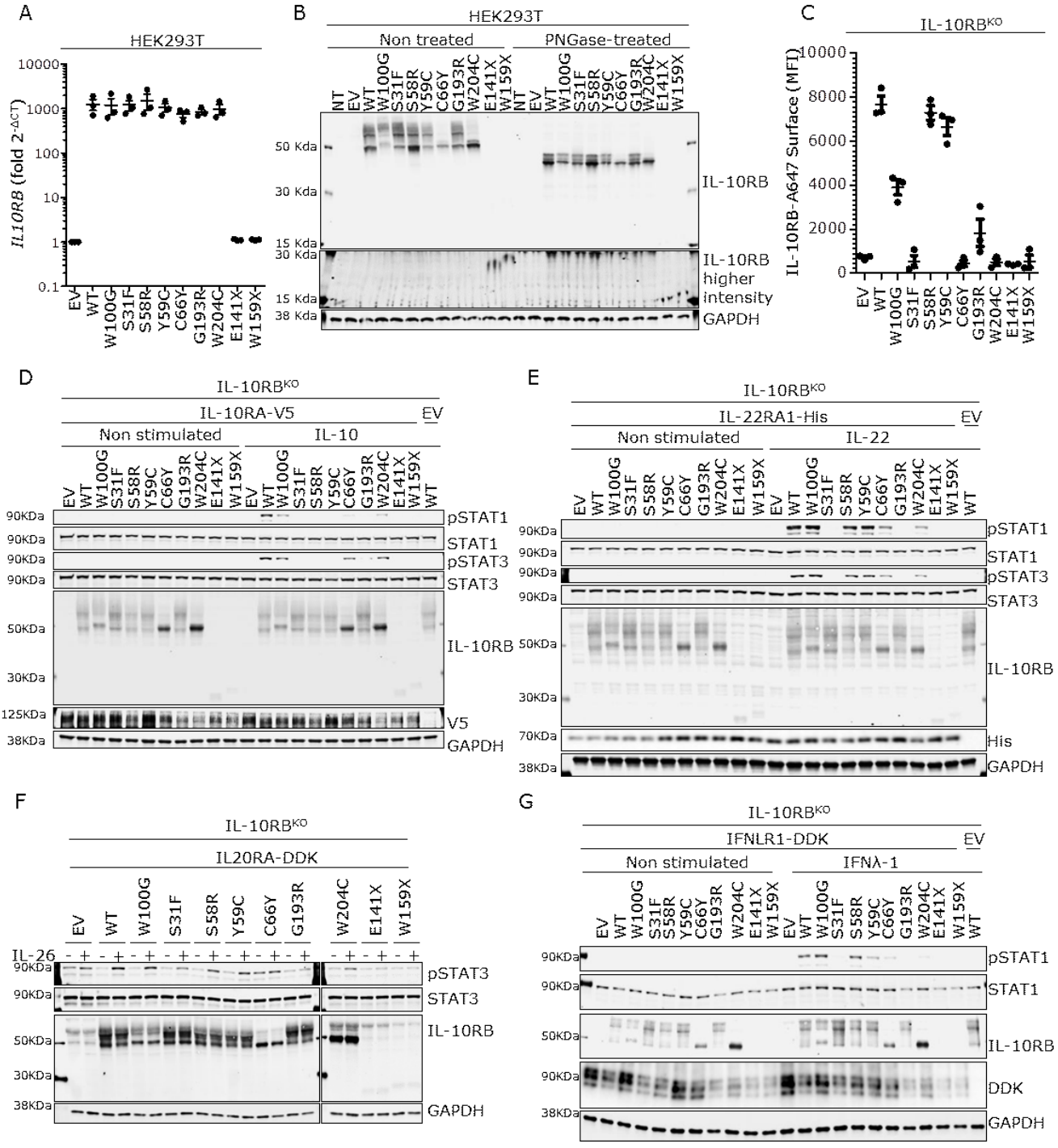
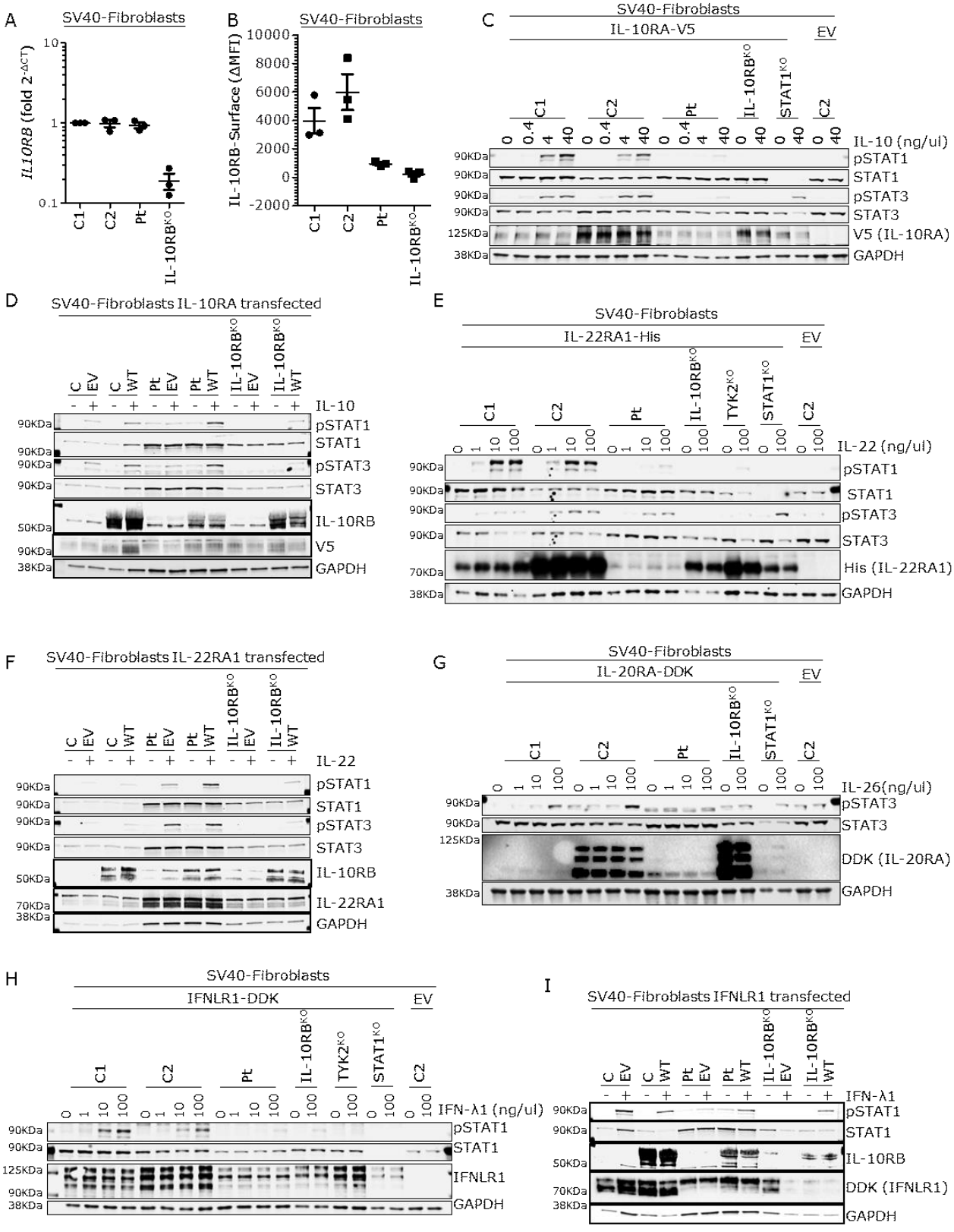


Figure 3. *Analysis of patient cells. A.* ***IL10RB mRNA measurement by RT-qPCR in patient and control cells. Graph shows the copy numbers of IL-10RB mRNA corrected by the endogenous expression of HPRT1 mRNA and relative to the mean copy number of the first control set as 1 ± SEM. Values shown are the means of three independent experiments performed in duplicate.*** *B.* ***Cell surface expression of IL-10RB was assessed in SV-40 fibroblasts from healthy controls, patient, and an IL-10RB–deficient patient (IL-10RB^{KO}) by flow cytometry. The results are expressed as mean fluorescence intensity (MFI) minus the MFI of the isotype control for each (ΔMFI) ± SEM.*** *C.* ***Response to IL-10 stimulation at the indicated concentrations for 30 minutes on controls, patient, STAT1^{KO} and IL-10RB^{KO} SV-40 fibroblasts stably transduced with IL-10RA-V5 when indicated on representative immunoblot image showing the analysis of STAT1 (pSTAT1) and STAT3 (pSTAT3) phosphorylation. STAT1, STAT3, GAPDH and V5 (IL-10RA) levels were also assessed.*** *D.* ***Western blot depicting phospho-STAT1 (pSTAT1) and phospho-STAT3 (pSTAT3) on reconstituted with WT IL-10RB or EV: patient, IL-10RB^{KO} and control SV-40 fibroblasts, with IL-10 stimulation of 40ng/ml for 30 minutes. GAPDH was used as a loading control, STAT1, STAT3 and V5 (IL-10RA1) levels were also depicted.*** *E.* ***Western blot of control, patient, STAT1^{KO}, TYK2^{KO} and IL-10RB^{KO} SV40-fibroblasts stably transduced with WT IL-22RA1-His when indicated, after treatment with IL-22 at the indicated concentrations for 30 minutes. The membrane has been probed with an antibody specific for phosphorylated STAT1 (pSTAT1) and phosphorylated STAT3 (pSTAT3). An antibody against GAPDH was used as a loading control; STAT1, STAT3, His (IL-22RA1) and IL-10RB levels were also shown.*** *F.* ***Western blot showing the detection of phospho-STAT1 (pSTAT1) and phospho-STAT3 (pSTAT3) in response to IL-22 10ng/ml 30 minute stimulation on controls, patient and IL-10RB^{KO} SV-40 fibroblasts stably transduced with IL-22RA1-His and transiently transfected with WT IL-10RB or EV. GAPDH was used as loading control. We show also***

STAT1, STAT3, IL-10RB and His (IL-22RA1). *G.* ***Response to IL-26 stimulation at the indicated concentrations for 45 minutes on controls, patient and IL-10RB^{KO} SV-40 fibroblasts stably transduced with IL-20RA-DDK when indicated on representative immunoblot image showing the analysis STAT3 (pSTAT3) phosphorylation. STAT3, GAPDH and DDK (IL-20RA) levels were also assessed.*** *H.* ***Western blot of control, patient, STAT1^{KO}, TYK2^{KO} and IL-10RB^{KO} SV40-fibroblasts stably transduced with WT IFNLR1-DDK when indicated, after treatment with IFN-λ1 at the indicated concentrations for 30 minutes. The membrane has been probed with an antibody specific for phosphorylated STAT1 (pSTAT1). An antibody against GAPDH was used as a loading control; STAT1, IFNLR1 and IL-10RB levels were also shown*** *I.* ***Western blot showing the detection of phospho-STAT1 (pSTAT1) in response to IFN-λ1 100ng/ml 30 minute stimulation on controls, patient and IL-10RB^{KO} SV-40 fibroblasts stably transduced with IL-22RA1-His and transiently transfected with WT IL-10RB or EV. GAPDH was used as loading control. We show also STAT1, IL-10RB and DDK (IFNLR1). All western blots performed at least three times, with the exception of G performed twice. C = control, Pt = patient, WT = wild type, SEM = standard error of the mean, NT = non transfected, EV = empty vector and p = phosphorylated.***

Figure 3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SV40-Fibroblasts
IL10RB (fold 2-ΔCT)
C1
C2
Pt
IL-10RBKO
B
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C
SV40-Fibroblasts
IL-10RA-V5
EV
IL-10 (ng/ul)
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STAT1
pSTAT3
STAT3
V5 (IL-10RA)
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V5
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IL-22 (ng/ul)
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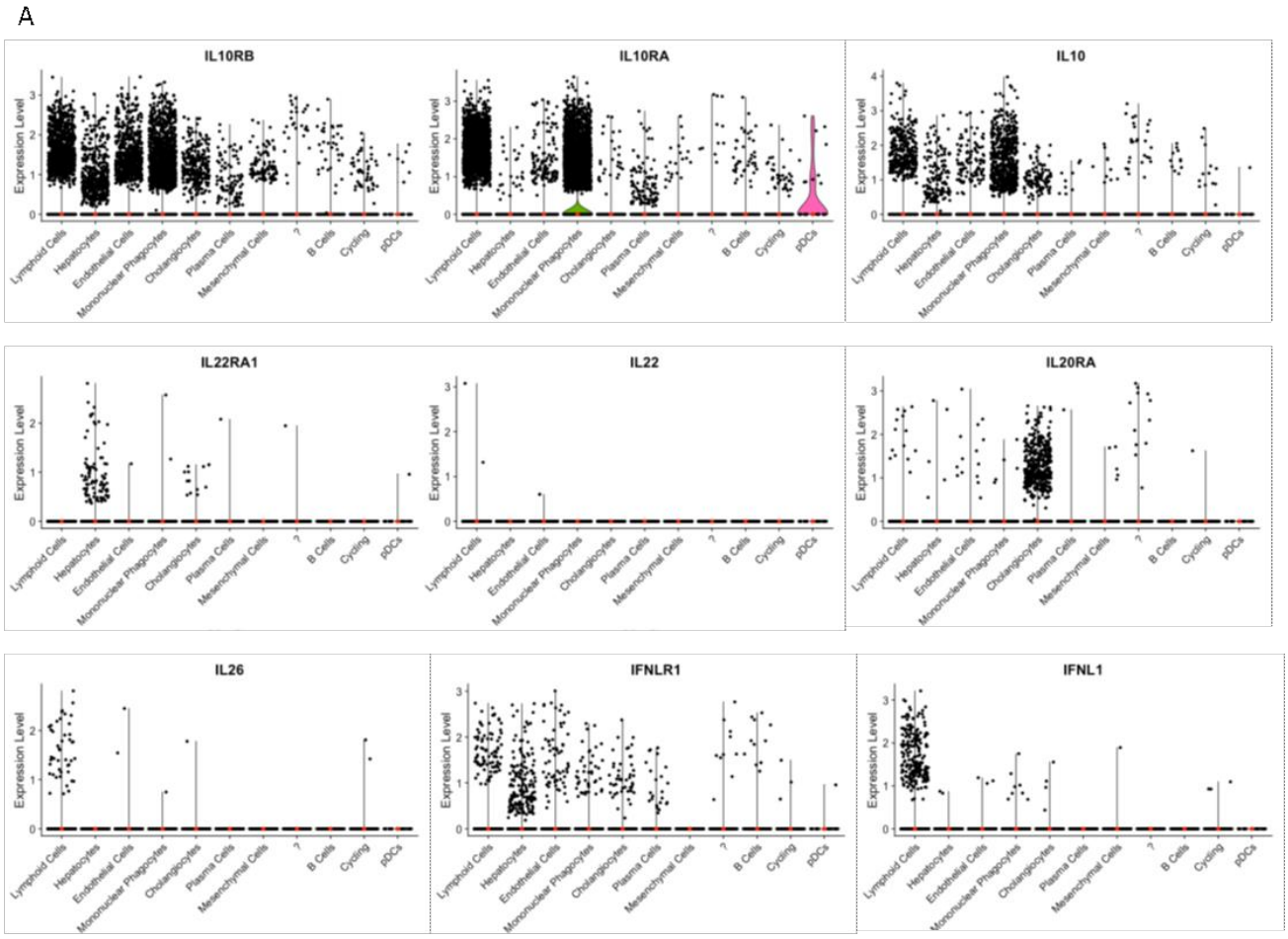
SV40-Fibroblasts IL-22RA1 transfected
IL-22
IL-22RA1
G
SV40-Fibroblasts
IL-20RA-DDK
IL-26(ng/ul)
DDK (IL-20RA)
H
SV40-Fibroblasts
IFNLR1-DDK
IFN-λ1 (ng/ul)
IFNLR1
I
SV40-Fibroblasts IFNLR1 transfected
IFN-λ1
DDK (IFNLR1)

Figure 4. *Human liver expression of IL-10RB axis components. A.* ***Single cell RNA-sequencing data showing differential expression of Il-10RB, its co-receptors and agonists in different liver cell types.*** *B.* ***Functional validation of the presence or absence of IL-10RB and its co-receptors in human hepatocytes by stimulation with IL-10 (40ng/ml), IL-22, IL-26, and IFN-λ1 (all 100ng/ml) and posterior phosphorylation of STAT1 and STAT3, representative western blot of n=2. GAPDH was use as a housekeeping control.***

Figure 4



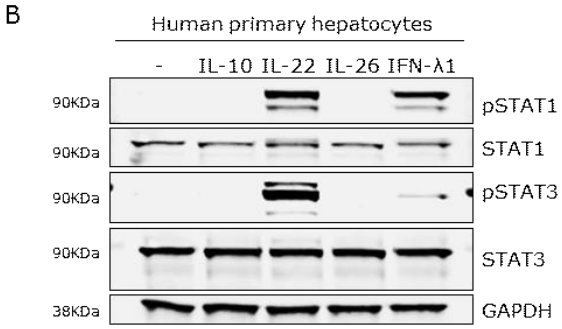
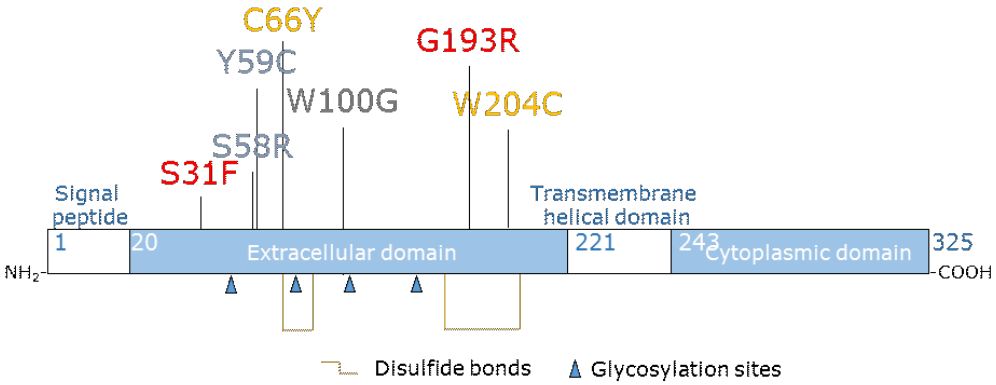


Figure 5. *Missense mutations in IL10RB show different cytokine-dependent function patterns.*

Figure 5



- Alleles not responding to IL-10, IL-22, IL-26 and IFN-λ1
- Alleles hypomorphic to IL-10, IL-22, IL-26 and IFN-λ1
- Alleles not responding to IL-10, and responding to IL-22, IL-26 and IFN-λ1
- Allele hypomorphic to IL-10, and responding to IL-22, IL-26 and IFN-λ1

Table 1: IL10RB patients carrying at least one missense mutation

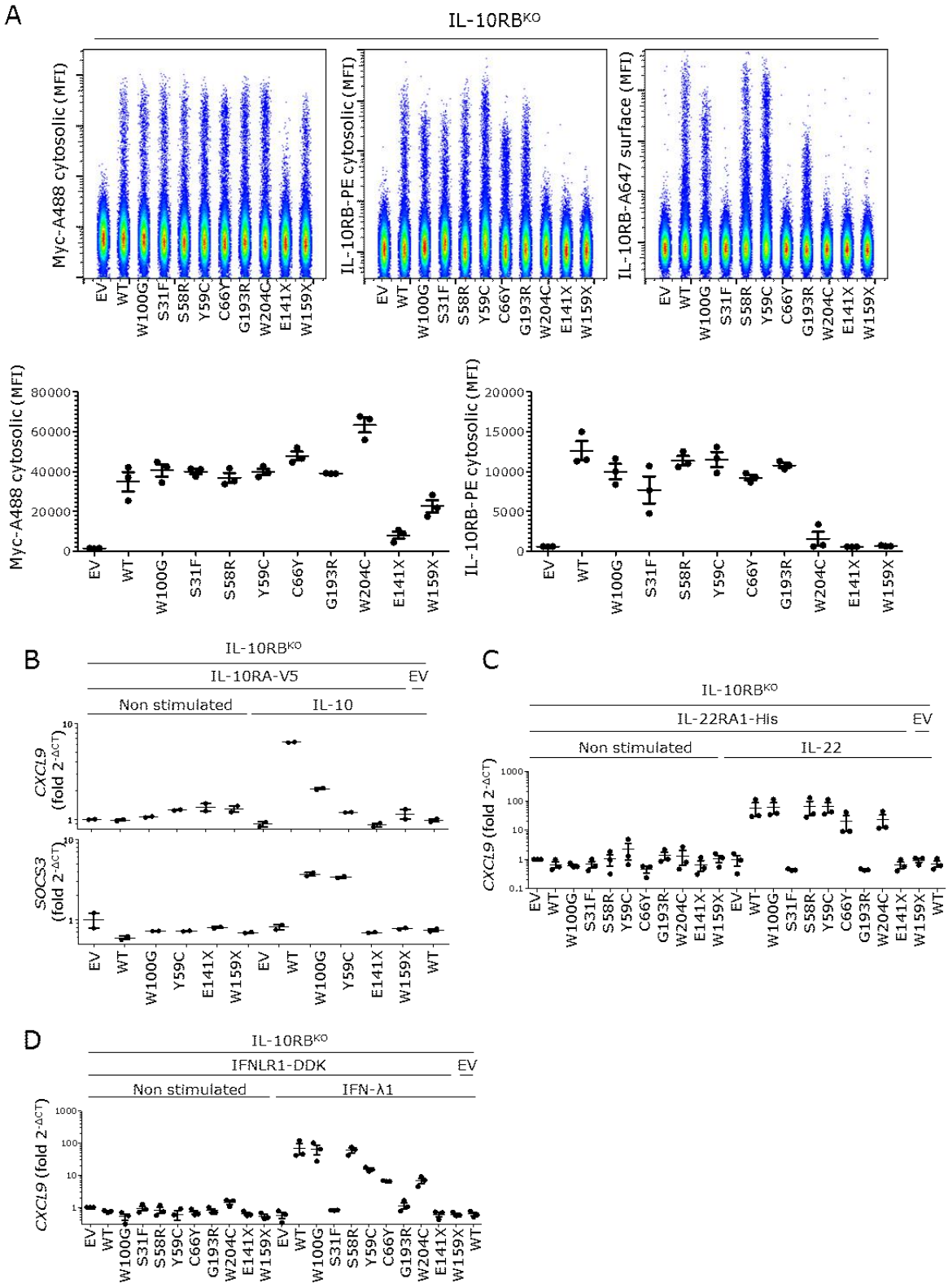
Mutation	Number of patients	Molecular mechanism	Patients' country of origin	Onset of disease (days)	References
S31F	1	Homozygous	Iran	60	Yazdani 2019
S58R	1	Compound heterozygous	USA	<30	Shouval 2014-2016-2017
Y59C	1	Homozygous	Italy	14	Neven 2013/Pigneur 2013
C66Y	1	Homozygous	Bangladesh	45	Kotlartz 2012
W100G	2	Homozygous	France/Saudi Arabia	84/?	Pigneur 2013/This article
G193R	1	Homozygous	Turkey	10	Engelhardt 2013
W204C	2	Compound heterozygous/Homozygous	France/China	14/?	Neven 2013/Pigneur 2013/Gong 2019

Table 2: Summary of results on the overexpression system

Stimulation	Read out	IL10RB overexpression system									
		WT	W100G	S31F	S58R	Y59C	C66Y	S193R	W204C	E141X	W159X
IL-10	WB-pSTAT1	+++	+	-	-	-	+	-	+	-	-
	WB-pSTAT3	+++	++	-	-	-	++	-	++	-	-
	qPCR-CXCL9	+++	-	ND	ND	-	ND	ND	ND	-	-
	qPCR-SOCS3	+++	+++	ND	ND	-	ND	ND	ND	-	-
IL-22	WB-pSTAT1	+++	+++	-	+++	+++	+	-	+	-	-
	WB-pSTAT3	+++	+++	-	+++	+++	+	-	+	-	-
	qPCR-CXCL9	+++	+++	-	+++	+++	+	-	+	-	-
IL-26	WB-pSTAT3	+++	+++	-	+++	+++	+++	-	+++	-	-
IFN-λ1	WB-pSTAT1	+++	+++	-	+++	++	+	-	+	-	-
	qPCR-CXCL9	+++	+++	-	+++	+	+	-	+	-	-

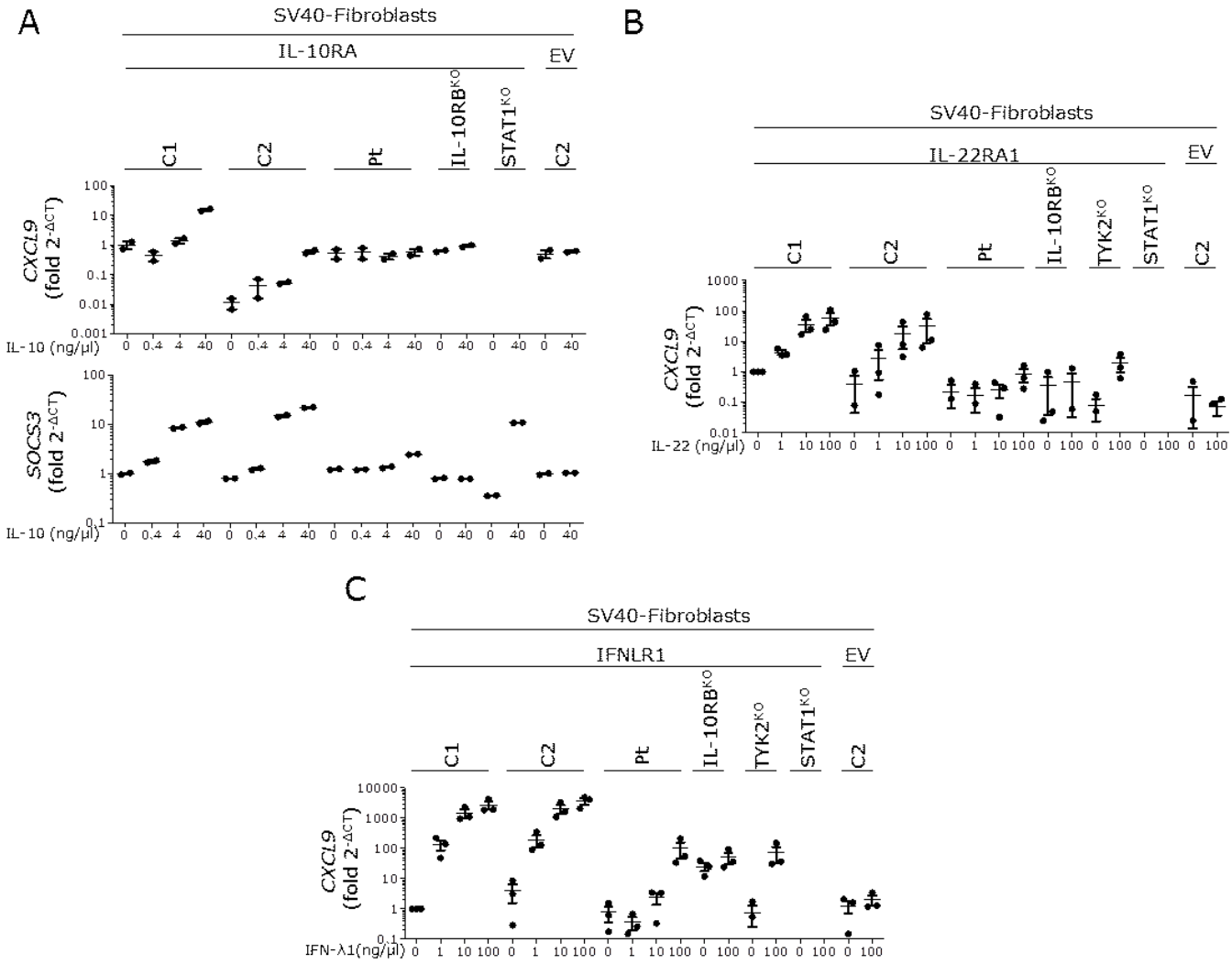
Supplementary figure 1. *W100G IL-10RB allele protein expression and functional assessment to IL-10, IL-22 and IFN-λ1 stimulation.* ***A. Flow cytometry of the cytosolic tag Myc; and surface and cytosolic IL-10RB on transfected IL-10RB^{KO} SV-40 fibroblasts 24 hours with indicated IL-10RB-Myc alleles tagged on the cytosolic c-terminal. We show dot-plot of one representative experiment of each staining and the accumulated three independent experiments by mean fluorescent intensity (MFI) on IL-10RB+ gate ± SEM for cytosolic IL-10RB and Myc.*** *B.* ***Response to IL-10 stimulation 40ng/ml for 2 hours on IL-10RB^{KO} SV-40 fibroblasts transfected with indicated IL10RB alleles and IL10RA-V5 on RT-qPCR showing the mRNA levels of CXCL9 and SOCS3. mRNA expression is shown corrected by the endogenous expression of HPRT1 mRNA for CXCL9 and GUSB mRNA for SOCS3. Results are normalized to the first column set as 1. Values shown are one representative experiment of three independent experiments performed in duplicate ± SEM.*** *C.* ***RT-qPCR depicting CXCL9 mRNA levels on IL-10RB^{KO} SV-40 fibroblasts transfected with indicated IL-10RB alleles and IL-22RA1-His with IL-22 stimulation 100ng/ml for 2 hours. Graph shows the copy numbers of CXCL9 mRNA corrected by the endogenous expression of HPRT1 mRNA and relative to the mean copy number of the first control set as 1 ± SEM. Values shown are the means of three independent experiments performed in duplicate.*** *D.* ***Induction of CXCL9 relative to HPRT1, as assessed by RT-qPCR on mRNA extracted from IL-10RB^{KO} SV40-fibroblasts transfected with the indicated IL-10RB alleles and WT IFNLR1-DDK, without or with stimulation for 2 hours with 100ng/ml IFN-λ1. CXCL9 levels are shown corrected by the endogenous expression of HPRT1 mRNA and normalized to the first column set as 1. Values shown are the means of three independent experiments performed in duplicate ± SEM. EV = empty vector, WT = wild type and SEM = standard error of the mean.***

Supplementary figure 1



Supplementary figure 2. *Patient cells functional assessment to IL-10, IL-22 and IFN-λ1 stimulation by RT-qPCR.* ***A. Response to IL-10 stimulation at the indicated concentrations for 6 hours on controls, patient, STAT1^{KO} and IL-10RB^{KO} SV-40 fibroblasts stably transduced with IL-10RA-V5 when indicated, showing the analysis of CXCL9 and SOCS3 mRNA levels were also assessed. HPRT1 and GUSB mRNA levels were used as endogenous loading controls for CXCL9 and SOCS3 respectively.*** *B.* ***RT-qPCR of control, patient, STAT1^{KO}, TYK2^{KO} and IL-10RB^{KO} SV40-fibroblasts stably transduced with WT IL-22RA1-His when indicated, after treatment with IL-22 at the indicated concentrations for 2 hours. We used probes specific to CXCL9 mRNA and HPRT1 mRNA, the last one used as the endogenous control.*** *C.* ***RT-qPCR of CXCL9 mRNA levels from control, patient, STAT1^{KO}, TYK2^{KO} and IL-10RB^{KO} SV40-fibroblasts stably transduced with WT IFNLR1-DDK when indicated, after treatment with IL-29 at the indicated concentrations for 6 hours. CXCL9 mRNA levels were corrected by HPRT1 mRNA levels. All RT-qPCR performed at least three times. Results are normalized to the first column set as 1. Values shown are the means of three independent experiments performed in duplicate ± SEM, with the exception of panel A showing one representative experiment out of two. C = control, Pt = patient, WT = wild type, SEM = standard error of the mean, NT = non transfected and EV = empty vector***

Supplementary figure 2



Supplementary table 1: Homozygous mutations present on patient 2

Chr	Pos	Ref	Alt	Function	Gene	AAChange	Sift_Pred	Polyphen2HVAR_Pred	CADD_Phred	MSC	GDI	Known PID?
21	34649025	T	G	missense	*IL10RB*	p.Trp100Gly	D,D	D,D,D,D	29.8	23.8	10.34	Yes
2	208725979	T	C	missense	*PLEKHM3*	p.Glu653Gly	D,D	P	29.7	2.31	3.26	No
16	88926306	C	G	missense	*TRAPPC2L*	p.Phe100Leu	D	P,P	25.9	23.84	5.19	No
6	41303876	C	T	missense	*NCR2*	p.Thr35Met	D,D,D	D,D,D	23.3	2.31	1.78	No
20	47269910	C	T	missense	*PREX1*	p.Glu779Lys	T,T	B,B	20.9	2.31	13.43	No
7	15725797	ATGG	A	indel-inframe	*MEOX2*	p.His77del			20.5	4.78	5.44	No
4	8307852	G	T	missense	*HTRA3*	p.Val451Leu	T	B	15.35	2.31	2.85	No
2	238737852	T	A	splicing	*RBM44*				10.96	2.31	4.67	No
2	238737860	A	G	splicing	*RBM44*	p.Arg868Arg			10.79	2.31	4.67	No
13	38320594	TA	T	splicing	*TRPC4*				9.94	2.31	1.08	No
2	238737854	T	G	splicing	*RBM44*				8.267	2.31	4.67	No
16	12009530	C	CCCG	indel-inframe	*GSPT1*	p.Gly16dup			7.76	2.31	10.44	No
7	131241029	G	GGGCGAC	indel-inframe	*PODXL*	p.Ser29_Pro30dup			5.726	2.31	3.35	No
20	32664864	C	CCAG	indel-inframe	*RALY*	p.Ala230_Gly231insSer			4.967	2.31	5.17	No
19	49657889	T	TTCC	indel-inframe	*HRC*	p.Glu202dup			4.004	2.31	10.86	No
13	25355965	GT	G	splicing	*RNF17*				3.637	2.31	9.75	No
3	158537415	CTTTTT	C	splicing	*MFSD1*				2.493	2.31	5.61	No
17	78081526	A	AGCAGCGG	splicing	*GAA*				0.341	0.01	7.92	No

Supplementary table 2: IL-10RB mutations published with CADD 1.3 score when applicable

Mutation	Allele frequency	CADD Score	Publication
p.W3X	Private	27.6	Shouval 2014-2017
c.50-2A>T	Private	16.92	Shouval 2016-2017
p.W18fsX12	0.00001988	24.7	Engelhardt 2013/Shouval 2017
p.S31F	Private	33	Yazdani 2019
c.174-3300_5483del	Private	-	Neven 2013/Pigneur 2013
p.S58R	Private	22.6	Shouval 2014-2016-2017
p.Y59C	Private	23.3	Neven 2013/Pigneur 2013
p.Y59_D110del	Private	-	Shouval 2014-2017
p.Y59_D110del_insCT	Private	-	Charbit-Henrion 2018
p.C66Y	Private	24.4	Kotlartz 2012
p.W100G	Private	29.8	Pigneur 2013/This article
c.331+1G>C	Private	25.2	Engelhardt 2013/Shouval 2017/Mukhopadhyay 2020
c.331+907_574del	Private	-	Kotlartz 2012
c.332-1356_5148del	Private	-	Shouval 2014-2017
p.E141X	Private	36	Begue 2011/Neven 2013/Pigneur 2013
p.W159X	0.000003977	43	Glocker 2009/Kotlartz 2012/Shouval 2014-2017/Beser 2015/Karaca 2016
p.G193R	Private	32	Engelhardt 2013
p.W202X	Private	-	Shouval 2014-2016-2017
p.W204X	0.000007953	43	Kotlartz 2012
p.W204C	0.00002386	31	Neven 2013/Pigneur 2013/Gong 2019
p.C209X	Private	36	Petersen 2017
c.646+2T>C	Private	22.9	Petersen 2017
p.S230X	Private	35	Kotlartz 2012
p.W246X	0.000003976	36	Huang 2017/Zheng 2019
p.F269fsX275	Private	26.3	Neven 2013/Pigneur 2013
c.*52C>T	Private	7.461	Kotlartz 2012/Shouval 2014-2016-2017

Annexe 3

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Annexe 4

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