Analysis of transcription mechanisms that limit type I interferon responses

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Science without imagination is just the mere reality,

only who wonders the imposible can reach what remains unseen.

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SUMMARY

This Thesis describes a key mechanism that prevents exacerbated activation of the interferon type I (IFN-I) pathway. This mechanism controls the magnitude of the response against pathogens such as virus and also safeguards the quiescence of hematopoietic stem cells (HSC). We identified the transcription factor NFAT5 as an inhibitor of IFN-I production.

The immune system has evolved to trigger specific and controlled responses to fight against the amazing spectrum of different pathogens that we can encounter. Based on the recognition of pathogen associated molecular patterns (PAMPs), innate immune cells activate specific signaling pathways depending on the type of pathogen recognized. These "patterns" are specific features of microbes not present in host cells such as cell wall components of bacteria and fungi, which are used to discriminate them from our own cells. Instead, viruses, the most abundant pathogens on earth, essentially are recognized by their nucleic acids, which are types of molecules also present in host cells. Once viruses are recognized, production of proinflammatory cytokines and IFN-I are fundamental to combat this slippery enemy. Activation of IFN-I production has pleiotropic effects that ensure the clearance of the pathogen. The first and best-characterized effect is the promotion of an antiviral state in infected and non-infected cells characterized by the expression of interferon stimulated genes (ISGs) that can target every step of the viral cycle to limit the infection. However, as a counterpart of its ability to recognize viruses, the IFN-I pathway can also be activated when our own DNA or RNA act as damage-associated molecular patterns (DAMPs). This signaling is a

hallmark process in the tumor environment or in autoimmune disorders. IFN-I has been described to influence multiple biological scenarios, such as its effect on HSCs. Upon IFN-I stimulation, quiescent HSCs enter into cell cycle and differentiate to produce blood cells in order to improve the capacity of the immune system to clear pathogens. Considering its broad range of effects, the expression of IFN-I must be tightly controlled. And seeing how uncontrolled IFN-I production could cause severe pathologies, a better understanding of mechanisms that limit its expression could lead to improved strategies in order to modulate IFN-I production in specific clinical settings.

In this Thesis, we characterize NFAT5 as a negative regulator of the expression of IFN-I genes. We show that NFAT5-deficient macrophages or dendritic cells respond to TLR3 stimulation or viral infection with enhanced production of IFN-I and higher ISG expression. NFAT5-deficient mouse models responded to in vivo viral infection with enhanced activation of the IFN-I pathway and improved control of viral load. They also responded to systemic TLR activation with exacerbated HSC exit from quiescence, making them vulnerable to exhaustion. These results highlight the complex balance between beneficial and detrimental effects of increasing IFN-I response.

This work defines for the first time a precise molecular mechanism that inhibits the expression of *IFNB1*. We show that NFAT5 binds the *IFNB1* promoter. We identified a binding site for NFAT5 at the *IFNB1* promoter that overlaps with a binding site for IRF3, the

master regulator of IFNβ. This overlapping causes a competition between IRF3 and NFAT5 that limits IFNβ production. We propose that this inhibitory mechanism is conserved across evolution from the appearance of interferons in vertebrates and could be extended to other genes regulated by IRF3 such as interferon alpha genes or other ISGs.

In a supplementary project also included in this Thesis, we defined NFAT5 as a positive regulator of basal *Ciita* expression in macrophages. Overall, work presented here in accordance with previous work from our group, poses NFAT5 as a transcription factor able to induce or repress the expression of specific target genes in response to pathogen recognition and also in the absence of stimuli. This Thesis confirms NFAT5 as a pleiotropic regulator of innate immune responses.

Esta tesis describe un mecanismo clave para evitar una excesiva activación de la vía del interferón tipo I (IFN-I) y así controlar la magnitud de la respuesta ante patógenos como virus y también salvaguardar la quiescencia de las células madre hematopoyéticas (HSC). Identificamos el factor de transcripción NFAT5 como un inhibidor de la producción de IFN-I.

El sistema inmune ha evolucionado para desencadenar respuestas específicas y controladas para luchar contra el increíble espectro de diferentes patógenos que podemos encontrar. Basándose en el reconocimiento de patrones moleculares asociados a patógenos (PAMP), las células de la inmunidad innata activan vías de señalización específicas dependiendo del tipo de patógeno reconocido. Estos "patrones" son características específicas de los microbios que no están presentes en células del huésped, como por ejemplo ciertos componentes de la pared celular de las bacterias y hongos, así que se utilizan para discriminar estos patógenos de nuestras propias células. Sin embargo, los virus, los patógenos más abundantes de la naturaleza, son reconocidos esencialmente por sus ácidos nucleídos, que son tipos de moléculas también presentes en las células del huésped. Una vez que se reconocen los virus, la producción de citoquinas pro-inflamatorias e IFN-I es fundamental para combatir a este enemigo tan evasivo. La activación de la producción de IFN-I tiene efectos pleiotrópicos que aseguran la eliminación del patógeno. El efecto mejor caracterizado es la promoción de un estado antiviral en células infectadas y no infectadas por medio de la expresión de genes estimulados por interferón (ISG) que pueden interferir en cada paso del ciclo viral

para limitar la infección. Sin embargo, como contrapartida a su capacidad para reconocer virus, la vía del IFN-I también puede activarse cuando nuestro propio ADN o ARN actúan como patrones moleculares asociados al daño (DAMP). Esta señalización es un proceso distintivo en el entorno del tumor o en trastornos autoinmunes. Más aún, se ha descrito que IFN-I influye en múltiples escenarios biológicos, como su efecto en las HSC. Tras la estimulación con IFN-I, las HSC inactivas entran en ciclo celular y se diferencian para producir células sanguíneas con el fin de mejorar la capacidad del sistema inmune para eliminar patógenos. Teniendo en cuenta su amplio rango de efectos, la expresión de IFN-I debe estar estrictamente controlada. Asimismo, considerando que la producción incontrolada de IFN-I puede causar patologías graves, una mejor comprensión de los mecanismos que limitan su expresión podría conducir a mejores estrategias con el fin de modular la producción de IFN-I en entornos clínicos específicos.

En esta tesis, caracterizamos a NFAT5 como un regulador negativo de la expresión de los genes IFN-I. Se demuestra que los macrófagos o células dendríticas deficientes para NFAT5 responden a la estimulación del TLR3 o ante una infección viral con una mayor producción de IFN-I y una mayor expresión de ISG. Los modelos de ratón que carecen de NFAT5 respondieron a la infección viral in vivo con una subida en la activación de la vía de IFN-I y un mejor control de la carga viral. Pero también respondieron a la activación sistémica de TLR3 con un incremento en la salida de quiescencia por parte de las HSC, hecho que provocó una mayor susceptibilidad a ser extenuadas. Estos resultados

resaltan el complejo equilibrio entre los efectos beneficiosos y perjudiciales del aumento en la producción de IFN-I.

Este trabajo define por primera vez un mecanismo molecular preciso que inhibe la expresión de *IFNB1*. Mostramos que NFAT5 se une al promotor de *IFNB1*. Identificamos un sitio de unión para NFAT5 en el promotor de *IFNB1* que se solapa con el sitio de unión para IRF3, el regulador principal de IFNβ. Esta superposición provoca una competición entre IRF3 y NFAT5 que limita la producción de IFNβ. Proponemos que este mecanismo inhibidor se podría haber conservado a lo largo de la evolución desde la aparición de los interferones en vertebrados y podría extenderse a otros genes regulados por IRF3, como los genes de interferón alfa u otros ISG.

En un proyecto adicional también incluido en esta tesis, definimos NFAT5 como un regulador positivo de la expresión basal de *Ciita* en macrófagos. En resumen, el trabajo presentado aquí, junto con el trabajo previo de nuestro grupo, sitúa NFAT5 como un factor de transcripción capaz de inducir o reprimir la expresión de genes específicos en respuesta al reconocimiento de patógenos y también en ausencia de estímulos. Esta tesis confirma NFAT5 como un regulador pleiotrópico de respuestas inmunitarias innatas.

PREFACE

The moment has arrived. I am ready to write my Thesis and I don't have anything but questions. I read the recommendations that the Department gives to the PhD students that start writing their Thesis, and I read that I should start by writing a section known as Preface, which (luckily for me) is accompanied by a short description to help the excited but naive writer: "Introduction to the Thesis that usually describes merit, value, or even place it within a certain context and circumstances".

So here am I, procrastinating the writing of the scary Introduction section and trying to start by an "easier" section with this Preface. I ask myself what are the merits and values of the Thesis? The answer to this question is challenging and it must be faced sincerely. Maybe this is a question that scientists never stop asking to themselves about their research. And so I decide that I want to try to answer this question, or at least contribute with my idea to what I believe that a PhD should imply. Because I think it is now, when I am about to become a PhD, when I see its real purpose clearer.

To talk about the merits and values of this Thesis, probably I should start writing about the novelty of the work presented in this Thesis, the important contribution to the field and how it could eventually imply many benefits for human health. But, above all this, what will most probably count as the merit of the Thesis is going to be whether its main work is being published in a high impact factor journal. Indeed, during these years I realized that everything is valued according to where one publishes it, and the value given to your work will be as high as the impact factor is, independently of

other aspects. However, I don’t think that most of my PhD student colleagues really consider that this should determine the value of their theses. It is just the consequence of the path that scientific community has taken in recent years, and that we should consider if it does good or bad to science itself. If the PI or the university only uses that scale of measurement to assess the work of a PhD student, and today there is a lot of pressure to do so, it is very likely many PhDs will be unfaithfully condemned. I say unfaithfully because now I see, behind the veil of being published, all that really means or should mean to do a PhD for students, PIs and the university. I consider that there are two things that really matter when doing a PhD: the curiosity and the sharing. Fortunately, during these years I met a lot of scientists that support this idea.

I think curiosity is inherent in most, if not all, young researchers who decide to start a PhD. We all started with many and stimulating questions and we want to know and explore the field we have chosen. Obviously, as time passes, one has to focus on a specific question, and that is also very important to learn. It is key to identify and target how to ask the specific questions in each of the cases. However, I think there should always be some space for the student to freely explore, even though most of the times nothing "valid" will result from it, but it will add more than many other positive results and will be very important and "valuable" for the PhD training.

Looking back, I think I explored my curiosity during the PhD, which is the first of the merits and values that I believe a PhD

should have. I have had the opportunity to develop this project, not only through the experiments from which I was predisposed by the previous knowledge of the laboratory, but through diverse approaches and experimental designs that I contributed to establish in the lab. I would like to highlight here the experiments performed by flow cytometry regarding the activation of hematopoietic stem cells, the site-directed mutation experiments on the *IFNB1* promoter, and also the analysis of the evolutionary conservation of the *IFNB1* promoter.

The second value that I think one should achieve during the PhD is the sharing. Science, and I thing that even more important in public-funded science, should be based in sharing knowledge and sharing questions. If we finish the PhD without having learned to share, we will lack a fundamental value. It is usual nowadays to find some reluctance or limitations to share the knowledge we acquire, either between different research groups or even within the same group. Especially true when there are economic interests involved or when there is a fear of losing recognition for the work done. Therefore, I feel satisfied to see that, on the one hand, in my lab I learned to put aside my ego and share the projects I have done during my PhD and, on the other hand, I had the opportunity to collaborate in other projects of the laboratory and also to collaborate and learn from other research groups.

In this sense, the merits during my Thesis have also been varied. In the first year of my PhD I collaborated with two researchers of the IMIM, Dr. Cristos Gekas from Dr. Anna Bigas laboratory and Dr.

Jordi Farrés from Dr. Jose Yelamos laboratory, who taught me the basics of flow cytometry analysis and bone marrow transplantation. During my second year I was fortunate to establish collaboration with the Neuropharmacology laboratory of the DCEXS-UPF. In my third year, I started to be part of the project of MHCII regulation by NFAT5 and we also started a very productive collaboration with the Infection Biology laboratory of Dr. Andreas Meyerhans of the DCEXS-UPF. During the fourth year of my doctorate I had the great opportunity to co-tutorize a master thesis, which allowed me to learn how to guide and have under my responsibility another student. In my last year, I also collaborated with the group of Dr. Andres Hidalgo of the CNIC in Madrid and probably one of the things I'm most proud of is that I organized the first PhD student symposium of the DCEXS-UPF.

All these experiences have helped me learn something that I completely ignored and that I could not have even imagined. In the end I understood that sharing knowledge with others is not only the true purpose of science, but instead, it is what makes it more fun. I have really enjoyed my PhD, I feel satisfied and fulfilled, and it is largely thanks to sharing this time and knowledge with different people.

At the end, I think that this Thesis reflects the main values of "explore following curiosity" and "share for fun" that I think should be learned and put in value during the PhD. I hope, with humility, to be an example for future colleagues, and inspire them to do their theses beyond the conventional guidelines.

INTRODUCTION

1. Concerning Immune System

"Whoever fights with monsters should look to it that he himself does not become a monster"

It is intriguing to consider that there exist a parallelism between Nietzsche's paradigm and the primitive immune system that appeared in nature. Probably, prokaryotic bacteria came to the same conclusion when they had to face pathogens. The most primitive strategy to fight against pathogens has been recently reported in bacteria and it is no other than to kind of become a pathogen by incorporating fragments of the pathogen DNA into the host DNA (Barrangou et al., 2007). This proto-immune system known as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) nowadays has become fashionable in the laboratories around the world due to its derived applications in the field of genetic editing (Doudna and Charpentier, 2014). Effectively, the CRISPR is considered to be the first immune system used by bacteria as an antiviral strategy. Since the first protective system originated in bacteria, it is fascinating to observe how the immune system has evolved exponentially. In eukaryotes, arguably the acquisition of surface receptors to discriminate between food and other homologue cell to make contact is the base to develop multicellular organisms. The capacity to recognize self from nonself is the basic function of innate immune system, and has been perfected throughout evolution. In complex invertebrates such as *D.melanogaster* or *C.elegans* we found a developed innate immune system, including the expression of pattern recognition receptors

(PRRs) specialized in recognize pathogens. Interestingly, these organisms already acquired genes that encode for proteins of intracellular signaling pathways that connect the surface receptors with the activation of the transcription factor NF-κB, which will be conserved in evolution. Furthermore, the immune system development reaches astonishing levels of complexity in vertebrates. This complexity is mainly due to the coexistence of two unique features in jawed vertebrates: the acquisition of interferons (IFNs), and the development of sophisticated immune system comprised by diverse specialized immune cells, that can be constantly generated and renewed by hematopoietic stem cells (HSCs), resulting in the classification of innate and adaptive immune system. These two characteristics that appeared and evolved together through evolution (there cannot be found separately in any living organisms) will be explained in the first two chapters of the Introduction.

In the first chapter of the Introduction we will review the general characteristics of the immune system. First, how is formed and developed in vertebrates (1.1), explaining the main source of the cells of the immune system, which is the hematopoietic stem cell (HSC) (although, as we will see, the origin of some differentiated immune cells is independent of HSC). Second, how it is activated when recognizes a potentially damaging signal (1.2), explaining how the cells recognize the pathogens and the main molecular signaling pathways that lead to the activation of the immune system.

In the second chapter of the Introduction we will describe in detail the interferon response, which as commented previously is also responsible for the great complexity of the immune system in vertebrates.

Finally, in the third chapter of the Introduction, we will review the most recent findings about the transcription factor NFAT5, which place it as a new regulator of immune responses in vertebrates.

1.1 A brief overview from embryo to adult homeostatic hematopoiesis

Starting from the beginning, which is the origin of immune cells? Or, maybe we should say, which are the origins of immune cells? There are two different origins for the cells of the immune system, the hematopoietic stem cells (HSCs) and the alternative embryonic hematopoietic progenitors. It is well characterized that HSCs arise from the Aorta-Gonad-Mesonephros (AGM) around embryonic day 11.5 (E11.5) (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Müller et al., 1994a). HSCs then originate the adult hematopoietic system. Furthermore, during the adult life of an organism, HSCs maintain and regenerate blood cells, either during homeostatic renewal or upon emergency. However, not all immune cells originate from HSCs. During early embryo development, important immune functions must be conducted, which cannot wait for the appearance of the HSCs and rely on the alternative embryonic progenitors.

To solve this timing issue it has been postulated that immune cells originate in 3 consecutive waves (Clements and Traver, 2013;

Dzierzak and Bigas, 2018). The first wave of hematopoietic cells production occurs around E7.5 embryo stage. At this stage, the size of the embryo poses the need for oxygen transport and tissue remodelling, which is mediated respectively by erythrocytes and macrophages derived directly from the yolk sac (Palis et al., 1999; Tober et al., 2007). The second wave is initiated at E8.25 when hematopoietic progenitors that display multipotent capacity but still lack self-renewal capacity are simultaneously generated from several origins such as the yolk sac, the allantois and the paraaortic splanchnopleura. It continues with the liver colonization of the transient erythromyeloid progenitors (EMP) by E9.5 to E10.5, where they further mature to release definitive erythrocytes to the circulation at E11.5 (McGrath et al., 2011). Importantly, EMPs will also originate the life-long tissue resident macrophages that help to form and to remodel the tissues/organs of the embryo (Gomez Perdiguero et al., 2015; Hashimoto et al., 2013). Moreover, in some cases, these tissue resident macrophages will remain in the adult and will be one of the first immune components to sense and activate the response against pathogens. Of note, it has been reported that also T cells and some subsets of B cells can be found at this stage of the embryo development (Cumano et al., 1993; Yokota et al., 2006), suggesting that not only myeloid but also lymphoid lineages can be generated previous to the HSCs onset. Finally, the third wave occurs at E11.5 by a well-reported process called endothelial-to-hematopoietic transition (EHT) that generates HSCs derived from endothelial cells (Jaffredo et al. 1998), being the AGM the main site of production. Moreover, it is reported that

HSCs can be found in other sites, especially in the placenta (Gekas et al., 2005; Lee et al., 2010; Ottersbach and Dzierzak, 2005), the embryonic head (Li et al., 2012) and the yolk sac (Yoder et al., 1997). The AGM-HSCs already possess long-term multi-lineage engraftment and repopulation capacity and can be found in the fetal liver, thymus and spleen (Müller et al., 1994a; Ivanovs et al., 2011). The fetal liver is the main hematopoietic organ during embryo development, which serves as a site for massive HSC expansion and differentiation until the skeletal system formation and its vascularization create a niche for adult HSCs (Ema and Nakauchi, 2000; Morrison et al., 1995).

The maintenance and generation of immune cells in adult homeostasis or in response to pathogen infections rely on HSCs. Myeloid cells such as granulocytes, monocytes, and dendritic cells are very short lived and peripheral T and B lymphocytes although are relatively long lived and may be sustained in part by homeostatic proliferation, they require the input of new cells to maintain a diverse polyclonal repertoire of antigen specificities (Geering et al., 2013; Geissmann et al., 2010). Therefore, correct haematopoiesis guarantees the functioning of the adult immune system. For this, to study and understand immune responses, it is crucial to clearly set how to define HSCs in terms of experimental methodology and understand the signaling pathways that can modulate their activity.

1.1.1 Strategies to define and characterize HSCs

There are two main strategies to define and study HSCs in the laboratory. The first strategy characterizes HSCs by the specific pattern of surface markers while the second strategy characterizes HSCs based on their functional capacity to restore the hematopoietic system after different stress situations.

When analysing the cellular surface markers, HSCs do not express any of the markers that determine the different mature hematopoietic lineages such as B220, TER119, GR1, CD11b or CD3, and therefore they are considered as lineage negative (Lin^-). Also, HSCs express the surface markers Sca1 and c-Kit, which are associated with stem cell capacity. Altogether, HSCs are enriched in Lin^-, and Sca1 and c-Kit positive populations, known as LSK. However, inside the LSK populations there is an heterogeneous mixture of HSCs and other multipotent progenitor (MPPs), which lack long-term reconstitution capacity and could be lineage-committed. In turn, the MPPs can be subdivided in four main groups respectively named MPP1 or short-term HSC (ST-HSC), MPP2, MPP3 and MPP4 (Figure 1.1.1).

Extensive research in the past years has aimed to improve marker strategies to differentiate the subpopulations inside the LSK. Although there are different markers combinations, one of the most accepted and the one used in this Thesis, is the marker strategy proposed by the Morrison group, which is based on the use of signaling lymphocytic activation molecule (SLAM) protein family (Bryder et al., 2006; Iida et al., 2014; Kiel et al., 2005). In this

strategy, HSCs are enriched inside LSK negative for CD48 and positive for CD150 markers. Therefore, HSC are defined by being Lin^-, $Sca1^+$, $cKit^+$, $CD48^-$ and $CD150^+$, but still further research has extended our ability to increase the HSC purity based on other surface markers. For example, the use of the CD34 marker specifically allows distinguishing between long term HSC (LT-HSC) that are $CD34^-$ and characterized to be in a deeply quiescent state with the highest reconstitution potential and short-term HSC (ST-HSC) or more committed progenitors that are $CD34^+$ and in a more active cycling state (Engelhardt et al., 2002; Osawa et al., 1996).

Moreover, SLAM markers are used to distinguish among the different MPP subpopulations. In this regard, a new surface marker strategy, which combines SLAM markers with the use of CD135 (Flk2) have been recently proposed to define 4 types of MPPs (Cabezas-Wallscheid et al., 2014). In this study, MPP1 is defined as LSK $CD150^+CD48^-CD34^+CD135^-$. Of note, this subset coincides with which is also named as ST-HSC by other groups (Busch et al., 2015; Pietras et al., 2015) as this cell possesses self-renewal and multilineage potential. Despite the lack of clear and uniform criteria to distinguish between purely HSC and very first committed progenitors (MPP1), there is more consensus in the definition of subsequent MPP subsets. The use of CD135 became particularly useful to distinguish between MPP3 (LSK $CD150^-$ $CD48^+CD34^+CD135^-$) and MPP4 (LSK $CD150^-$ $CD48^+CD34^+CD135^+$). Also this study revealed, by reconstitution assays, that MPP2 cells (LSK $CD150^+CD48^+CD34^+CD135^-$) are

multipotent (but with less reconstitution capacity compared with HSC and MPP1) whereas MPP3 and MPP4 populations show a direct differentiation bias towards myeloid and lymphoid cell types, respectively (Cabezas-Wallscheid et al., 2014).

The classical linearity conception of hematopoietic hierarchy has been redefined by the idea of lineage biased multipotent progenitors MPP2-4 found below the ST-HSC/MPP1 (Figure 1.1.1). Despite all MPPs potentially may give rise to two oligopotent progenitors, the common myeloid and common lymphoid progenitors (CMP and CLP), this new step in the differentiation process involves multipotency loss towards definitive specific lineage capacity (Akashi et al., 2000; Kondo et al., 1997). On one branch, CMPs, defined as $Lin^{-}Sca1^{-}cKit^{+}CD34^{+}$ $CD16/32^{-}$, give rise to megakaryocyteerythrocyte progenitors (MEPs), defined as $Lin^{-}Sca1^{-}cKit^{+}CD34^{-}CD16/32^{-}$, and granulocyte-macrophage progenitors (GMPs) defined as $Lin^{-}Sca1^{-}$ $cKit^{+}CD34^{+}CD16/32^{+}$. MEPs and GMPs will finally generate differentiated immune components such as erythrocytes and platelets or granulocytes, macrophages and conventional dendritic cell types, respectively. On the other branch, CLPs, defined as Lin^{-} $Sca1^{+}cKit^{lo}CD34^{+}$ $IL7R^{+}$, will give rise to B cells, T cells, NK cells and plasmocytoid dendritic cells (pDCs), a cell type intended to produce high amounts of interferon type I (Manz et al., 2001; Rodrigues et al., 2018). In summary, the use of surface markers to define HSC is subject to constant changes and it has been currently updated due to the appearance of new markers that have increased our ability to differentiate among the subtypes and the

purity of HSC and MPPs populations. Moreover, ongoing works based on single cell analysis will contribute to a new level of refinement in the definition of hematopoietic stem and progenitor cells (HSPCs).

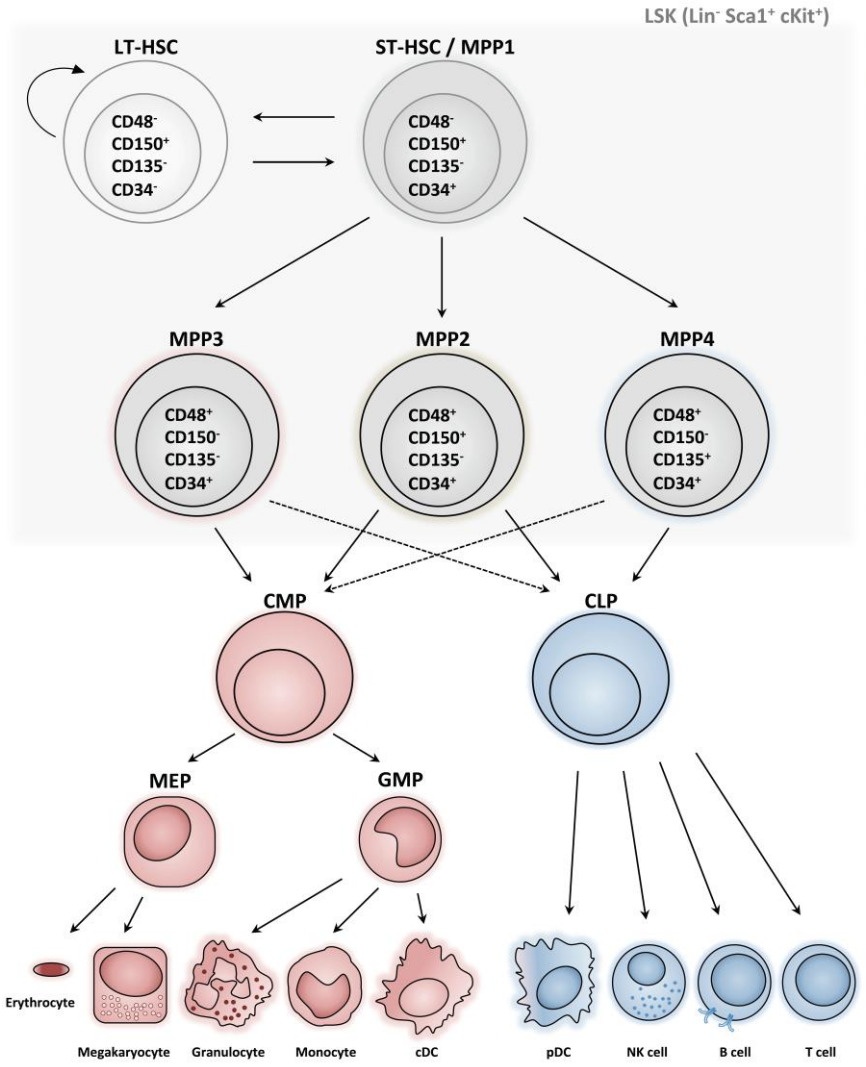

Figure 1.1.1 Hierarchy of the hematopoietic system. Scheme shows the surface markers used to distinguish hematopoietic stem and progenitor cells (HSPCs) within the LSK population (grey background) and its derived lineage committed progenitors (CMP, CLP) and the terminate differentiated cells (bottom cells). Red and blue colours represent myeloid and lymphoid lineages respectively. Dashed arrows indicate a minor contribution to the specific lineage.

As previously commented, independently of their surface markers, another way to characterize and analyze the HSCs is by the use of functional assays. As we highlighted, HSCs are defined by their self-renewal and multipotency capability, as well by their capacity to restore the hematopoietic system after different stress situations. Therefore, these characteristics can be experimentally assessed in functional assays such as bone marrow transplantation experiments in lethally irradiated host or chemotherapy ablation by 5-Fluorouracil (5-FU) administration.

The gold standard to evaluate the functionality of HSCs is the transplantation assays where bone marrow from donor mice is transplanted into irradiated mice, in order to deplete the host bone marrow. In that manner, the ability of donor bone marrow to replenish and restore the hematopoietic system is evaluated. However, there are some considerations to take into account to evaluate the results. For instance, the impact of other cells that indirectly influence HSC functionality like the mesenchymal stromal cells (MSCs) or the inflammation caused by lethal irradiation in order to empty the HSC niche are two variables that could significantly influence the experiment (Abbuehl et al., 2017; Cao et al., 2011; Morikawa et al., 2009). Another issue to evaluate HSC functionality by bone marrow transplantation is the necessity to perform serial transplantations as only HSC, and not more committed HSPCs as ST-HSC or MPP1, are able to serially reconstitute the entire blood system. Multipotent progenitors can reconstitute the blood system of lethally irradiated recipients up to several months, but they fail to do so in a secondary or tertiary

transplant. Therefore, serial rounds of transplantation are needed to discriminate between multipotent progenitor cells and HSCs functionality (Lemischka et al., 1986).

An alternative or complementary experimental approach broadly used to measure HSC function is the ablation of the hematopoietic system by administration of 5-Fluorouracil (5-FU) (Randall and Weissman, 1997; Venezia et al., 2004). Compared to transplantation, this technique is faster and reduces variables such as inflammation-derived-irradiation. In vivo, 5-FU is converted into two different isoforms that will interfere with the incorporation of the endogenous uracil or thymidine nucleotides. Thus 5-FU inhibits RNA and DNA synthesis during the S phase of the cell cycle (Focaccetti et al., 2015). For this, 5-FU reduces cell viability mainly of cycling cells. There are two different experimental strategies usually followed when using 5-FU, which provide different information regarding HSCs (Randall and Weissman, 1997). When 5-FU administration is done after HSC activation, 5-FU gets incorporated in activated-cycling HSC and directly reduces their cell viability. The outcomes derived from decreased viable HSCs serves as readout of the level of HSC activation caused by the initial stimulus. This strategy is used to compare which condition implies more HSC activation as higher activation can cause a reduction in viability (Pietras et al., 2014). The other strategy is done by injecting 5-FU without previous HSC activation. This experimental model assesses the intrinsic capacity of HSCs to enter cell cycle in order to replenish more committed progenitors and differentiated cells that are sensible to 5-FU, as they are homeostatically in active

cell cycle (Kleppe et al., 2017). With this strategy, a single injection of 5- FU causes an increase in cycling HSC of up to 20% around 4 days after the administration (Venezia et al., 2004). Therefore, serial injections of 5-FU, even in the absence of previous HSC activation, will cause a reduction of organism viability. One thing that has in common both strategies is that the system is compromised and HSCs are forced to enter into cell cycle leading to an eventual exhaustion of the hematopoietic system.

1.1.2 Signaling pathways that regulate HSC activity

To approach the study of the immune response against pathogens, it is crucial to define HSCs in terms of experimental methodology and also to understand the signaling pathways that modulate their activity. Regarding the second question, we can consider that the pathways that trigger HSC activity and development will be crucial to achieve a proper adult immune system. Indeed, only a reduced group of signaling pathways has been reported to regulate HSCs and consequently the development of the immune system, and those include the following pathways: NOTCH, FGF, EGF, WNT, HEDGEHOG, TFGβ, HIPPO, JAK/STAT, TNF/IFN/NF-κB, JNK and RAR (Dzierzak and Bigas, 2018).

Of especial relevance for the work in this Thesis are the pathways that involve JAK/STAT and IFN/NF-κB axis. These pathways not only regulate HSC development during embryogenesis but also in adult homeostasis and upon pathogen infection (Essers et al., 2009; Gough et al., 2012; Josefsdottir et al., 2017; Kim et al., 2016; Stein and Baldwin, 2013). In fact, in the last decade, many studies have

started to analyze and define how HSCs are affected upon infection (Baldridge et al., 2010; Hirche et al., 2017). The current vision, as Margaret Goodell proposes, is "viewing the HSC as a foundation for the immune response" (King and Goodell, 2011), as the way the immune system works and responds to infections will largely depend on the activity of HSCs and the generation of immune cells to control the pathogen.

During homeostasis, adult HSCs are mostly quiescent, with approximately 90% of them being in the G_0 phase of the cell cycle. Instead, the MPPs are actively cycling and are responsible for the majority of the cell expansion for the homeostatic daily production of billions of blood cells (Busch et al., 2015; Sawai et al., 2016). The discovery that HSCs become activated and proliferate in response to systemic infection, opens a range of questions about how HSCs are regulated by the classic inflammatory signaling molecules that are produced in this situation. The activation of dormant HSCs is and must be reversible in order to avoid exhaustion or neoplasia of the system (Wilson et al., 2009). Therefore, activation and cycling behaviour of HSCs must be under a strict-balanced control to achieve the exact measure of activation and cell production. The sensing of inflammatory signals by HSCs has effects beyond the activation of cell cycle and has been reported to also modulate HSC differentiation and self-renewal capacity. For instance, interleukin 1 (IL-1), tumor necrosis factor α (TNF-α) and interferons (IFNs) have been described to induce HSC proliferation, trigger myeloid or megakaryopoietic programs and adversely impact on LT-HSC

self-renewal capacity (Essers et al., 2009; Kleppe et al., 2017; Matatall et al., 2014, 2016, Pietras et al., 2014, 2016). Although the complete molecular mechanism is still unknown, several works report that the effect of these inflammatory molecules on HSC signature depends on the activation of the NF-κB transcription factor and on the signaling mediated through the JAK/STAT pathway (Baldridge et al., 2010; Grossmann et al., 1999; Kleppe et al., 2017; Pietras et al., 2016). Therefore, IFNs emerge as key molecules to modulate HSCs. The effects of IFNs on HSCs will be further described below (section 2.3.2).

Despite current knowledge about how HSCs become activated and respond to inflammatory molecules produced during infection, it remains unknown whether HSCs can directly sense and respond to pathogens via their own TLRs (Baldridge et al., 2011; Takizawa et al., 2017). Indeed, a recent work from the Rice laboratory shows that stem cells as HSC intrinsically activate antipathogen transcriptional programs to be refractory to possible infections (Wu et al., 2018). In fact, pathogen recognition and production of inflammatory molecules are functions classically associated to the "first responders" immune cells, macrophages and dendritic cells (DCs). After reviewing the formation of the immune system taking the HSCs as a reference, we will now review how the immune system senses and triggers the response against pathogens, with macrophages and DCs as the reference cell types.

1.2 Response to pathogens

The innate immune system is the first line of defence against pathogens. Overall, macrophages and DCs are specialized in discriminating not only the "non self" products but also potential harmful insults that can be derived from the host. To accomplish this function, macrophages and DCs express a huge variety of receptors to ensure recognition of different types of potential-dangerous molecules. These receptors can be classified into two main superfamilies: the scavenger receptors (SRs) and the pattern recognition receptors (PRRs). The SRs were initially described to bind and internalize modified low-density lipoprotein (LDL) and recognize a wide variety of ligands to mediate the clearance of apoptotic bodies by promoting phagocytosis (Goldstein et al., 1979; Zani et al., 2015). The PRRs are specialized in the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). It is when macrophages sense pathogen infection (PAMPs) or tissue damage (DAMPs) that they activate the inflammatory response. Macrophages and DCs have different types of PRRs, which are specialized in the recognition of different molecules. From their discovery by Jules Hoffmann and Bruce Beutler (Lemaitre et al., 1996), many types of PRRs have been identified, allowing a comprehensive understanding of the signaling pathways triggered by innate immune cells and how adaptive immunity is primed.

1.2.1 Pattern Recognition Receptors Pathway

PRRs are mostly expressed in antigen-presenting cells such as macrophages and DCs, but they can also be found in other immune and non-immune cells. Cells possess different types of PRRs placed at strategic locations in the cell to ensure pathogen recognition, including plasma membrane, endosomes and cytoplasm. PRRs generally follow a common activation mode, where each PRR recognizes a specific feature of the pathogen and leads to the transduction of a signaling cascade via the assistance of an adaptor molecule. Hereunder there is a table of the main PRRs with the corresponding molecule that recognize and the adaptor molecule that each of them uses to transduce the signal (Table 1.2.1).

Despite the variety of receptors that exist to trigger different signaling cascades, it is remarkable to point that all of them converge into at least one of these three families of signaling molecules: mitogen activated protein kinases (MAPKs), nuclear factor-κB (NF-κB) and interferon regulatory factors (IRFs). Then, the specificity in the gene pattern activation induced in response to each of the stimulus is achieved by the specific combination of transcription factors activated by these pathways. And for instance, the relative activation of MAPK-NF-κB-IRF pathways could favor proinflammatory response (MAPK-NF-κB axis) or interferon type I response (NF-κB-IRF axis).

Pathogen recognition receptors (PRRs)			Localization	Ligand	Origin	Adaptor	IFN-I
MEMBRANE RECEPTORS	TLRs	TLR1	Plasma membrane	Triacyl lipopeptides	Bacteria	MyD88	PC
		TLR2	Plasma membrane	Peptidoglycans / Lipopeptides	Bacteria / Fungi / Virus	MyD88	PC
		TLR3	Endosomes	dsRNA	Virus	TRIF	IFN-I
		TLR4	Plasma membrane (and endosomes)	LPS / Envelope proteins	Bacteria / Virus	MyD88 / TRIF	Both
		TLR5	Plasma membrane	Flagelin	Bacteria	MyD88	PC
		TLR6	Plasma membrane	Diacyl lipopeptides	Bacteria / Fungi	MyD88	PC
		TLR7 (mouse)	Endosomes	ssRNA	Virus / Bacteria	MyD88	IFN-I
		TLR8 (human)	Endosomes	ssRNA	Virus / Bacteria	MyD88	IFN-I
		TLR9	Endosomes	dsDNA (CpG motif)	Virus / Bacteria	MyD88	IFN-I
	CLRs	Dectin-1	Plasma membrane	β-Glucan	Fungi	FcRγ	PC
		Dectin-2	Plasma membrane	α-Mannans	Fungi / Bacteria	FcRγ	PC
CYTOSOLIC RECEPTORS	NLRs	NLRP / NLRC	Cytosol	Peptidoglycans	Bacteria	Caspase 1	PC
		NOD2	Cytosol	ssRNA	Bacteria	MAVS	PC
	RLRs	RIG1	Cytosol	dsRNA / ssRNA	Virus	MAVS	IFN-I
		MDA5	Cytosol	long dsRNA	Virus	MAVS	IFN-I
	DNA sensors	cGAS	Cytosol	dsDNA	Virus	STING	IFN-I
		IFI16	Cytosol	dsDNA	Virus	STING	IFN-I
		DDX41	Cytosol	dsDNA	Virus	STING	IFN-I

Table 1.2.1 Types and characteristics of the different PRRs. Table describes, for each PRR, the cellular localization, main type of ligand and organism that triggers their activation and the downstream adaptor. All PRRs can induce the production of proinflammatory cytokines (PC) but only some of them are specialized towards the production of IFN-I. Last column shows the tendency of each PRR to induce the production of IFN-I vs proinflammatory cytokines.

Toll-like receptors (TLRs) are the largest and most-characterized group of PRRs. When pathogen recognition is achieved via TLRs, the balance of MAPK-NF-κB-IRF depends on the adaptor molecule that is used, which can be either MyD88 (Myeloid differentiation primary response 88) or TRIF (TIR-domain-containing adapter-inducing interferon-β). The MyD88-dependent pathway is activated upon recruitment of MyD88 to the TIR domain of all TLRs except TLR3. This promotes the sequential activation of the IL-1 receptor-associated kinases (IRAKs) that then interact with the adaptor TRAF6, which in turn activates TAB2/TAB3, the regulatory components of the kinase TAK1 (Akira et al., 2006). At this point, TAK1 can either phosphorylate to activate the MAPKs branch leading to the activation of transcription factors that trigger the expression of inflammatory cytokines, or it can instead phosphorylate the IKKβ (Inhibitor of NF-κB Kinase). Phosphorylation of IKKβ promotes the phosphorylation and degradation of the cytoplasmic repressor IκB, allowing translocation of NF-κB into the nucleus where it can activate the expression of multiple targets such as interferon type I genes and inflammatory cytokines (Kawai and Akira, 2006). The TRIF-dependent pathway is activated upon TLR4 and particularly TLR3 activation. TRIF is recruited to the TIR domain of TLR3 or TLR4. Then, TRAF6 is recruited by TRIF, resulting in the activation of the NF-κB branch. Importantly, the use of the adaptor molecule TRIF also leads to the activation of the IRFs branch via the recruitment of TRAF3, which activates the TBK1 (TANK binding kinase) and IKKε (Inhibitor of Nuclear Factor Kappa-ε Kinase) that catalyse the

activatory phosphorylation of interferon regulatory factor 3 (IRF3) and can also promote the degradation of the cytoplasmic NF-κB-repressor IκB. Phosphorylated IRF3 then dimerizes and translocates into the nucleus where, in cooperation with NF-κB (detailed in 2.2.2), promotes the transcription of IFN-I and the activation of IFN-I responses (Kawai and Akira, 2010).

Pathogen recognition can also be achieved via the cytosolic receptors, highlighting the key role in virus recognition. For example, the helicases RIG-I and MDA5, known as RIG-I-like receptors (RLRs), recognize viral RNA through their RNA-helicase domain. Signal transduction by these receptors is performed via their two N-terminal caspase-recruitment and activation domains (CARDs) and uses the adaptor molecule MAVS (mitochondrial antiviral-signaling protein) to activate kinases TAK1 and TBK1, which will activate NF-κB and IRFs. For this, cytosolic receptor pathways are known to preferentially activate the interferon signature. Of note, RIG-I and MDA5 receptors have been described essential to induce TLR-independent type I IFN genes (Yoneyama et al., 2004, 2005). Another relevant example is the receptor cGAS that recognize cytosolic DNA. When cGAS recognizes DNA is actived and produces the cyclic dinucleotide molecule cGAMP(2′-5′), that bind to and activates the ER-resident receptor STING. This cGAS-STING pathway links DNA detection with activation of TBK1/IRF3 and IFN-I production (Cavlar et al., 2012). Interestingly, the cGAS-STING pathway can also be triggered by host DNA, which makes it especially relevant for connecting DNA

damage with IFN-I production and understanding the cause of several interferonopathies (Crowl et al., 2017).

Although signaling through the PRRs is essential to protect against infection, excessive PRR responses lead to immune disorders such as acute and chronic inflammation, autoimmune diseases and, unexpectedly, have even been found to favour pathogen spreading (Kawai and Akira, 2010; Teijaro et al., 2013; Wilson et al., 2013). For this reason, PRR signaling needs a strictly controlled negative regulation to avoid excessive detrimental activation. Negative regulation of PRRs is achieved by different mechanisms that are reviewed in Kondo et al., 2012. Briefly, the negative mechanisms can be classified in three groups: dissociation of adaptor complexes, degradation of signal proteins and transcriptional regulation. As good example of the dissociation of adaptor complexes is the respective binding of the proteins sterile alpha-and armadillo-motif-containing (SARM) and IRF4 to the adaptors TRIF and MyD88 to block their signal (Carty et al., 2006; Negishi et al., 2005). Next, the classical example of negative regulation of the PRRs by degradation of signal proteins is the function of the suppressor of cytokine signaling (SOCS) proteins that promote degradation of TRAF (Yoshimura et al., 2007). Finally, there are fewer examples of mechanisms that negatively regulate transcription responses downstream PRRs. However, those examples are more restricted to transcriptional regulation of proinflamatory cytokines and less is known about negative transcriptional regulation of type I interferon genes. For instance, IκBδ is a TLR-inducible gene found to block the expression of IL-6 and IL-12p40 cytokines (Kuwata et al.,

2006). Also, cyclic AMP-dependent Transcription Factor (ATF3) has been shown to recruit histone deacetylase 1 (HDAC1) to the promoter region of proinflamatory cytokines to limit the access of activator transcription factors (Whitmore et al., 2007).

Taking into account all the background presented here, it is clear that the knowledge on multiple signaling pathways and their interrelations will be key to understand the immune system response as a whole. Of note, IFN-I response emerges as a signaling pathway of great interest from different points of view. First, IFN-I is one of the two main signatures activated in response to pathogens, especially in macrophages and DCs. Second, beyond its classical antipathogenic functions in macrophages and DCs, it has been reported to possess other immunoregulatory activities depending on the cell type that receives IFN-I. Two outstanding examples with potential therapeutic implications are the effects of IFN-I on tumour progression or HSC activation. Third, the wide spectrum of IFN-I effects tend to be as a double-edge sword, uncontrolled IFN-I production could lead to aberrant viral spreading, exhaustion of the hematopoietic system or the development of autoinflammatory diseases known as interferonopathies (Pietras et al., 2014; Sato et al., 2009; Siednienko et al., 2012; Teijaro et al., 2013; Wang et al., 2017b; You et al., 2013). Finally, the IFN-I pathway can be activated upon endogenous self-molecules very abundant in our cells, DNA and RNA. Taking all of these in consideration, it is of great importance to understand the mechanisms that fine-tune its expression and activity.

2. Interferon Type I (IFN-I)

Interferon was mention for first time back in 1804, when Edward Janner described a process that caused (or a process known since then as) “viral interference” when he observed that an infection with Herpes virus prevented a subsequent infection with Vaccinia (Jenner E. Letter to the Editor. Med. Phys. J. 1804;12:97–102). However, it was not until 150 years later that this "viral interference" was proven to be caused by a singular factor, secreted by the host cells (ISAACS and LINDENMANN, 1957). This singular factor was named interferon (IFN) and it was characterized to be produced in response to viral infections to promote host resistance to such infections (Isaacs et al., 1957; Lindenmann et al., 1957). Since then, more than 100,000 works about the IFNs have been published (Borden et al., 2007) and contributed much knowledge about the nature of IFN. Initially, research about IFN focused on defining the antiviral function of this molecule. Further research in the 80s and 90s, contributed mostly to the definition of several molecular mechanisms that regulate IFN production. For instance, these studies led to the description of the IFN signaling pathway, the enhanceosome, and the discovery and description of the functions of IFN-I-Stimulated Genes (ISGs). This mechanistic information paved the way to describe the signaling that controls IFN-I production downstream PRRs. Finally, more recently, research has pointed out the impact of IFN responses on several other biological scenarios beyond the canonical antiviral response, highlighting the pleiotropic role of IFN as an antitumoral element, its role in chronic inflammatory disease and its ability to activate

HSCs (Bonifazi et al., 2001; Essers et al., 2009; Gutterman, 1994; Pietras et al., 2014; Rohatiner et al., 2005).

Our knowledge on the IFN-I response has increased exponentially and has made us realize that its diverse effects could be interconnected. For this, trying to describe the individual function of interferon in one specific process is complicated, and it is more accurate to study its effects considering the multiple molecules and cell types that take part in IFN-I response. Therefore, in this chapter, we will first review the IFN pathway and second we will describe its diverse cellular effects. In more detail, we will first briefly introduce the types of interferon that exist and their appearance and conservation throughout evolution (chapter 2.1). After that, we will dissect the IFN pathway (2.2) in three steps. Conceptually this subdivision of the IFN pathway may help to better describe how IFN production is activated (2.2.1), which are the molecular mechanisms that lead to IFN production (2.2.2) and finally, once IFN is produced, to describe its molecular (2.2.3) and cellular or physiological effects (2.3).

2.1 IFNs classification and evolutionary conservation

IFNs are a family of cytokines that includes three different types of molecules, named type I, type II and type III or IFN-like group. This classification is determined by the three main types of receptor that specifically recognize each type of IFN (I, II and III) (González-Navajas et al., 2012). The IFN secreted can act in a paracrine or autocrine manner through its specific receptor to exert its effects. All three types of IFNs can be induced by PRR

activation, activate similar gene programs and exert antipathogenic effects. However, the three types of IFNs are recognized by different receptors and trigger different signaling pathways.

For the purpose of this Thesis, we will focus mainly in the type I Interferons (IFN-Is). Within the IFN-I family, up to seven different subfamilies have been described (with some of them only found in specific species): IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNδ, and IFNτ (Pestka et al., 2004). All IFN-Is are recognized by the same receptor known as IFNAR, which is comprised by two transmembrane subunits, INFAR1 and IFNAR2. Evolutionarily, IFN-I appeared at the onset of jawed vertebrates and although the number of IFN-I genes varies among species, all species within this group have one gene that encodes for IFNβ and at least two that encode for IFNα (Roberts et al., 1998). Phylogenetic analysis concluded that the three main subfamilies of IFN-I (IFNβ, IFN-α and IFNω) diverged after the division of birds and mammals. In turn, the expansion of the IFNα subtype occurred concomitant with the radiation of the major mammalian orders (Hughes, 1995). Particularly, in humans, there is only 1 type of IFNβ, IFNε, IFNω and IFNκ, and 12 subtypes of IFNα (derived from 14 genes from which one is a pseudo gene and two of them, IFNA13 and IFNA1, produce the same protein), and all of them are located in chromosome 9 (Pestka et al., 2004). Similarly, in mice, there is 1 type of IFNβ, IFNε, and IFNκ, and 14 subtypes of IFNα, and their genes are clustered in chromosome 4 (van Pesch et al., 2004).

There is an intriguing feature of the vertebrate immune system that is worth considering when talking about IFN evolution. Indeed, the

appearance and preservation of IFN-Is throughout the vertebrate evolution could drive us to insightful relationships between IFN-I and different immune functions. Interestingly, IFNs appeared and expanded in the vertebrate genomes together with the appearance of B, T and NK immune cells, which intriguinly have not been described to exist in any organism that lacks IFN, and vice versa. Moreover, it is worth noting that vertebrates are also the only organisms that use apoptosis as a defense strategy (Stetson and Medzhitov, 2006). The proper elimination of infected cells by apoptosis is a key step during the immune response against pathogens. Apoptosis then leads to a reduction in the number of cells that must be counteracted by their renewal. As we will see, there are known roles for IFNs in both promoting apoptosis in macrophages and also in promoting entry into cell cycle of the HSCs to ensure the renewal of differentiate immune cells. Then, it is interesting to consider how different cells respond to IFN-I in a different way based on their renewal capacity. Therefore, seems that vertebrates took great profit of the IFN-I acquisition to build a whole new net of interrelations among the immune system that builds the modern and effective mammalian immune system.

2.2 The IFN-I pathway

From pathogen recognition to the immune response, the IFN-I pathway can be divided in three main steps. First, the signaling cascade upstream of IFN-I production, that comprises from the stimulation of the receptor to the activation of the transcription factors. Second, the transcription factors assemble a complex

known as the enhanceosome, which directly activates the expression of IFN-I. Third, once IFN-I has been produced, the signaling cascade downstream of IFNAR in the target cell (Figure 2.2).

There is an increasing interest in defining the mechanisms that negatively regulate the IFN-I pathway (Arimoto et al., 2018), as limiting the activity of INF-Is has been found key to ensure the balance between beneficial and detrimental effects of IFN-I that will be reviewed in chapter 2.3. Since examples of negative regulation that restrict general PRR signaling were highlighted in chapter 1.2.1, in the following sections, we will describe the best characterized negative regulatory mechanisms for each step of the IFN-I pathway.

2.2.1 Upstream of IFN-I

Almost all cells express some of the PRRs that trigger IFN-I production. As we reviewed in chapter 1.2, there are two distinct PRRs able to induce IFN-I, TLRs and cytosolic PRRs. These two types of PRRs are associated to different adaptor molecules to trigger the signaling pathways that independently lead to the production of IFN-I. On one hand, in the cytosol, we find the ubiquitously expressed cytosolic receptors that recognize RNA (mainly RIG-I and MDA5) or DNA (mainly cGAS, DDX sensors or IFI16). On the other hand, endosomes of specialized sentinel cells posses TLRs that recognize different pathogen products such as TLR3, TLR4, TLR7, TLR8 and TLR9 (Figure 2.2).

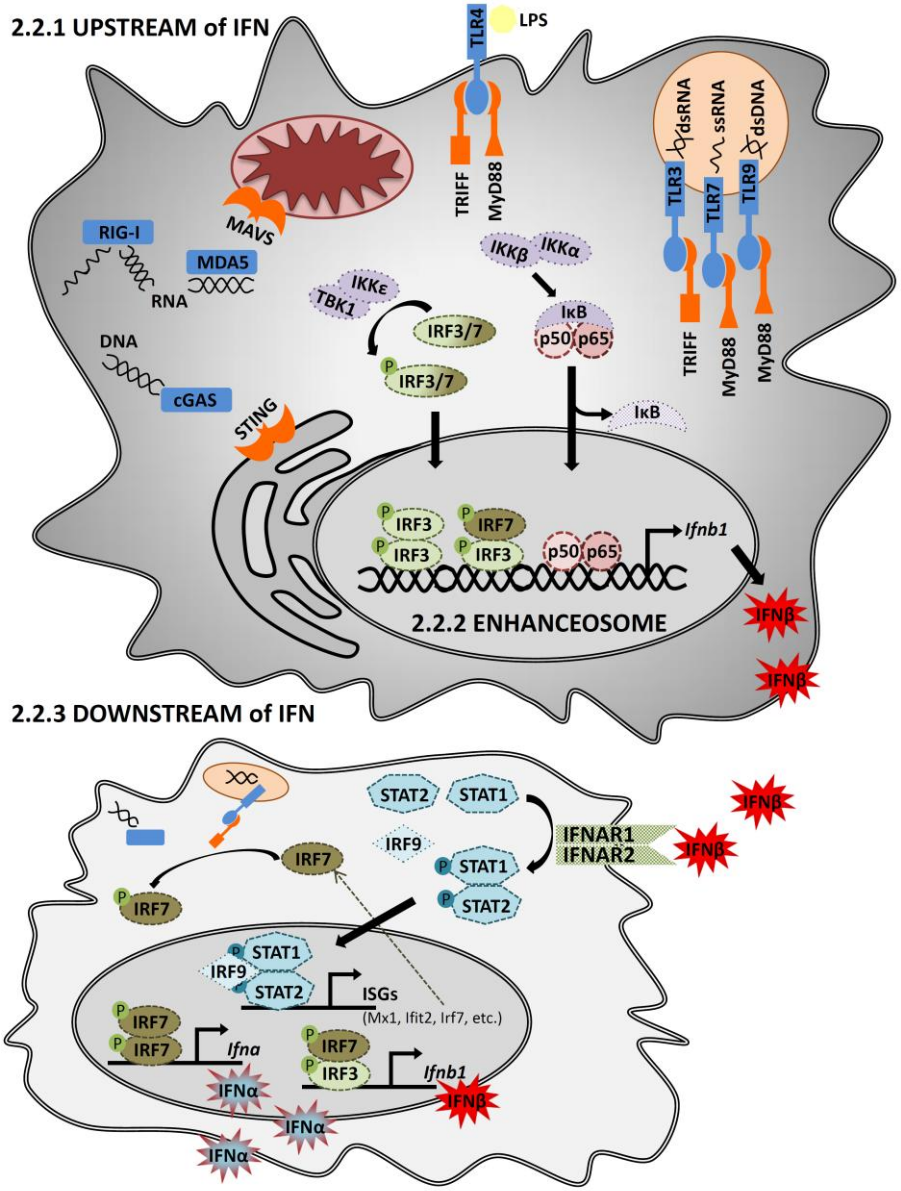

Figure 2.2 IFN-I pathway divided in 3 main steps. 2.2.1 Upstream of IFN-I. PRRs (in blue) use adaptor molecules (in orange) to transduce the signal and activate signaling kinases (in purple) that eventually activate IRFs or p65/NF-κB. **2.2.2 Enhanceosome** formation triggers *Ifnb1* expression. **2.2.3 Downstream of IFN-I.** IFNβ is recognized by IFNAR and activates the STATs, which in turn activate the transcription of several ISGs, including IRF7, facilitating the amplification of IFN-I response by IFNα production.

The recognition of a pathogen by different receptors leads to the activation of downstream signaling pathways via the action of different and specific adaptor molecules. For instance, the cytosolic receptors that sense RNA use the mitochondrial anti-viral signaling protein (MAVS) adaptor while the ones that sense DNA use the Stimulator of Interferon Genes (STING) adaptor to transduce the signal (Hornung, 2014). The endosomal receptor TLR3 and TLR4 induce IFN-I in a manner dependent on TRIF adaptor (Kawai and Akira, 2010) while the other endosomal receptors TLR7, TLR8 and TLR9 induce IFN in a manner dependent on MyD88 adaptor (Tabeta et al., 2004). Downstream of the adaptor molecules MAVS, TRIF and MyD88, there is a third step in the IFN-I signaling cascade, which is mediated by members of the TNF Receptor-Associated Factor (TRAF) family of adaptor molecules (Häcker et al., 2006; Liu et al., 2013; Oganesyan et al., 2006). At this point, the four signaling cascades (cytosolic RNA, cytosolic DNA, TLR-TRIF and TLR-MyD88) can activate NF-κB or IRFs. In the case of NF-κB, activation is promoted by the phosphorylations performed by the kinase IKKβ, which result in the degradation of the cytoplasmic NF-κB-repressor, IκB, and the consequent NF-κB activation and shuttling into the nucleus (Kawai and Akira, 2006). In the case of IRFs, activation is promoted by the activation of the kinases TBK1 and IKKε, which catalyse the phosphorylation of IRF3 and its posterior dimerization and translocation into the nucleus. Finally, inside the nucleus, NF-κB and IRF3 cooperate and ensemble in a multiprotein complex called the enhanceosome to activate the transcription of IFN-I genes (Figure 2.2).

These differentiated receptors are not functionally redundant and they can be activated alone or simultaneously, according to the type of stimulus and cell type, activating the production of IFN-I independently. For example, upon infection by the vesicular stomatits virus (VSV), fibroblasts and conventional dendritic cells (cDC) deficient for RIG-I are unable to produce proper amounts of IFN-I (Kato et al., 2005). Alternatively, in the case of plasmacytoid dendritic cells (pDCs), the production of IFN-I depends mainly on the MyD88-dependent pathway but not in the MAVS and STING-dependent cytosolic receptors (Kato et al., 2005, 2006). However, these two pathways are necessary as MAVS-deficient mice infected with VSV show a strong systemic IFN-I production mediated by pDCs via the TLR receptors but are unable to control the infection (Sun et al., 2006).

Despite differences at the top of the signaling cascades, it is worth to remark that all these pathways converge in the activation of three protein kinases, which in turn activate three specific transcription factors for IFN-I expression; IRF3, IRF7 and NF-κB. Thus, all the signaling cascades lead to the activation of the IκB kinase-ε (IKKε) and TANK-binding kinase 1 (TBK1) kinases, responsible for the phosphorylation of IRF3 and IRF7, and the activation of the IκB kinase (IKK) kinase complex responsible for the activation of NF-κB (Fitzgerald et al., 2003; Karin, 1999) (Figure 2.2).

When we focus on transcription factors, the central dogma of IFN-I production is that phosphorylated IRF3 forms homo or heterodimers with IRF7 that translocate to the nucleus and, together with NF-κB, bind to the *IFNB1* promoter in a well-defined

mechanism referred as enhanceosome assembly. Interestingly, it has been shown that IRF3 activation, which is expressed in homeostatic conditions, induces its homodimerization and leads to an initial IRF3-dependent IFNβ burst that triggers the transcription of IRF7 among many other ISGs. This generates a positive feedback loop in which IRF3 can then form heterodimers with IRF7 and lead to the induction of a second wave of gene transcription that includes the expression of IFNα genes. The expression of IFNα genes only rely on the binding of IRF3 and IRF7, and not on other factors such as NF-κB, to the viral-responsive elements (VREs) located in the IFN alpha promoter regions (Honda et al., 2005a; Marie et al., 1998; van Pesch et al., 2004). Notably, this positive feed-back regulation of type I IFN signaling is also reinforced by the induction of some components of the pathway such as TLR3, RIG-I or MDA5 by IFN-I production that amplify the IFN-I response (Kang et al., 2002; Ma et al., 2015; Sadler and Williams, 2008).

A remarkable exception of this mechanism is observed in pDCs. In vivo, pDCs are responsible for the production of large amounts of IFN-I upon viral infection (Siegal et al., 1999). pDCs have the capacity to produce IFN-I rapidly and efficiently because they constitutively express IRF7. Therefore, once they recognize the virus through TLR7, TLR9 or cytosolic receptors (pDCs do not express TLR3), the signaling cascade will activate IRF3 and IRF7 to immediately produce IFNβ and specially IFNα (Colonna et al., 2004).

There are two known inhibitory strategies described to negatively regulate this first part of the pathway. First, cells can target the PRRs or their adaptor molecules to be degraded by the proteosome. For instance, in a manner dependent on IFN, the E3 ubiquitin ligase ring finger protein 125 (RNF125) ubiquitinates RIG-I, MDA5 and MAVS (Arimoto et al., 2007) and the IFN-induced protein 35 (IFI35) mediates RIG-I proteasomal degradation (Das et al., 2014). Also, Pellino3 is an ubiquitin ligase induced by TLR3 signaling that targets TRAF6 for degradation, impairing IRFs activation and establishing a negative feed-back loop to control IFN-I expression (Siednienko et al., 2012). Another representative example of negative feed-back regulation is the IFN-stimualted gene 15 (ISG15). Interestingly, ISG15 is a ubiquitin-like protein that can bind, in a process known as ISGylation, to other proteins of the IFN signaling cascade targeting them to degradation limiting the activation of the pathway (Jeon et al., 2010). Other strategies able to inhibit downstream signaling molecules include the NLRC5-mediated inhibition of NF-KB responses by binding to IKKα and IKKβ (Cui et al., 2010). Accordingly, NLRC5-deficient mice showed enhanced IFN-I expression in response to TLR stimulation (Tong et al., 2012). More classic examples are represented by SOCS1, IFIT1 (Isg56) and YAP. Those proteins have been found to respectively block IRAK1, STING and the dimerization of IRF3 (Li et al., 2009; Nakagawa et al., 2002; Wang et al., 2017b).

2.2.2 Enhanceosome assembly

To talk about the transcriptional regulation of IFN-I is to talk about the work of Maniatis and Thanos. Their studies constitute the foundation of the molecular mechanism by which cells express the *IFNB1* gene and allowed them to define the enhanceosome (Thanos and Maniatis, 1995a).

Until the end of the 20th century, it was difficult to understand how the same transcription factor could be responsible to activate different gene programs upon different types of stimuli. The classical example to state this controversy is NF-κB. NF-κB was known to target a different subset of genes depending on which specific stimuli induce its activation (UV irradiation, cytokines, viruses or bacterial products). However, there was not a mechanistic explanation about how this specificity could be achieved. Now we know, partly thanks to the work on the *IFNB1* enhanceosome, that two main features account for this specificity of NF-κB. First, the coordinate activation and assembly of NF-κB with other transcription factors and, second, there is a variable number and combination of binding sites for the transcription factors in the enhancer/promoter region of target genes.

The enchanceosome is a multiprotein complex formed by the assembly of multiple transcription factors that act cooperatively to activate transcription. *IFNB1* enhanceosome is the first and best characterized example of how the assembly and cooperation of different transcription factors ensures specificity in the activation of specific genes in response to a given stimulus. Now we are going to

review the main works that have collaborated in our detailed understanding of this transcriptional regulation mechanism of the *IFNB1* gene. Both the regulatory sequences and the factors involved were characterized in parallel to establish the final model we know today (Figure 2.2.2).

The first evidences of the existence of a regulatory region upstream to the *IFNB1* gene were reported in the 80's. The team of Maniatis described the existence of two different regulatory regions upstream of the *IFNB1* gene. They showed that deletion of the more proximal region (-77 bp to -19 bp) caused a reduction in the induction of *IFNB1* gene while the deletion of the more distal region (-210 to -107) causes an increase in the constitutive expression of *IFNB1* gene without affecting its inducibility (Zinn et al., 1983). Two years later, the more proximal region was restrained from -77 to -37 and was described as an inducible enhancer element necessary for the *IFNB1* activation upon polyIC treatment (Goodbourn et al., 1985). For this, they called this particular sequence the Interferon gene Regulatory Element (IRE) and demonstrated that this sequence confers polyIC inducibility to genes, independently of its location and orientation. Since then, multiple works were focused on defining the specific sequence and regulatory proteins associated to this regulatory region. Nowadays, the sequence of 70bp comprised between -110 and -37 from the transcription start site (TSS) of *IFNB1* is known as the enhanceosome sequence, which in turn is composed of four positive regulatory domains (PRDs) (Maniatis et al., 1998). From 5' to 3', PRDs are named as PRDIV-PRDIII-PRDI-PRDII.

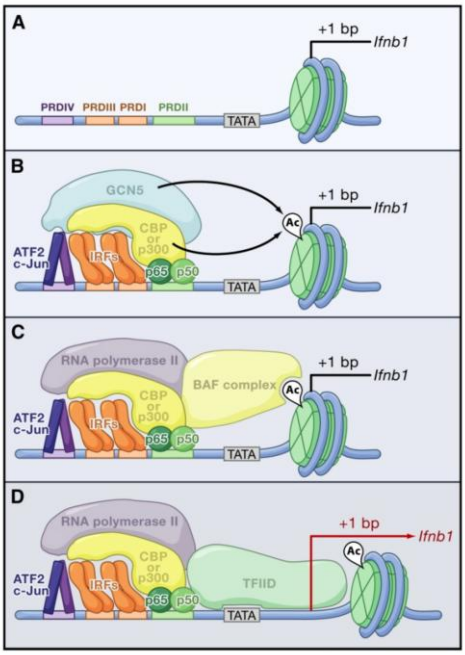

Figure 2.2.2. Assembling of IFNβ enhanceosome and *Ifnb1* gene transcription. (**A**) In unstimulated conditions the *Ifnb1* promoter is free of transcription factors at the PRDs. (**B**) Upon PRR activation, AP-1, IRFs, and NF-κB translocate to the nucleus and bind their respective PRDs, enabling the recruitment of HATs such as GCN5 and CBP/p300. (**C**) Histone modifications facilitate the recruitment of other chromatin remodeling factors that eventually expose the transcription start site (TSS). (**D**) The transcription factor TFIID associated to the RNA pol II can then bind the TATA box and transcription of *Ifnb1* is initiated. Image from *Honda et al., 2006*.

The PRDs include the specific binding sites for the transcription factors necessary to form the enhanceosome and induce *IFNB1* expression. PRDIV contains the sequence that partly represents the ATF2/cJun binding motif (-TGAG/CTCA-). PRDIV is not essential for the induction of *IFNB1*, as ATF2 knock-out mice are still able to induce proper levels of IFNβ upon viral infection (Reimold et al., 1996). However, PRDIV is described to be necessary for a maximal enhancer activity of *IFNB1* (Du et al., 1993; Kim and Maniatis, 1997). PRDIII and PRDI are the key regions to ensure enhancer activity as they contain the interferon regulatory factors (IRFs) binding sequence (-GAAANNGAAA-). PRDIII-PRDI bound by IRF3 homodimers or IRF3/IRF7 heterodimers are major drivers of *IFNB1* expression (Lin et al., 1998; Schafer et al., 1998). In fact, PRDIII-PRDI can function even as a single-virus inducible

enhancer (Wathelet et al., 1998). Both IRF3 and IRF7 play essential but distinct roles to activate the expression of IFN-I genes and while IRF3 or IRF7 single knock-out mice produce much less IFN-I and are more susceptible to viral infections it is only the double knock-out that is completely unable to induce IFN-I (Honda et al., 2005b; Sato et al., 2000). While the relative importance of IRF3 or IRF7 will depend on the type of virus/stimulus and the cell type that produces interferon (as commented in 2.2.1), their activities are regulated by the binding to PRDIII-PRDI elements (Andrilenas et al., 2018). Finally, PRDII contains a well-conserved binding site for NF-KB (-GGGRNA/TYYCC-). Several works have characterized how the binding of the heterodimer p65/p50 (NF-KB) to PRDII is an essential step in the viral induction of *IFNB1* (Chen and Ghosh; Thanos and Maniatis, 1995b; Wan and Lenardo, 2009). Complementary, in the case of PRDII, and also in PRDIV, the high mobility group protein (HMG-I) has been reported to play an architectural role that facilitates the binding of NF-κB and ATF2 to PRDII and PRDIV respectively.

Multiple in silico experiments have allowed scientists to elaborate a nice and detailed model about how each individual protein component binds a specific DNA sequence to assemble the IFNβ enhanceosome in a highly structured and sequential manner (Figure 2.2.2). The first and essential step to form the enhanceosome is the binding of IRF dimers to PRDIII and PRDI (Escalante et al., 2007; Panne et al., 2007). This key step is followed by the binding of the other two main components, ATF2/cJun and NF-κB (Dragan et al., 2008; Panne et al., 2007). According to crystal structures, IRFs

dimers are not in direct contact with ATF2/cJun but instead cooperate and interact with NF-κB. In fact, IRF3 and NF-κB extensively collaborate to recruit Pol II and regulate the transcription of diverse antiviral genes (Freaney et al., 2013). Besides these three components and the architectural protein HMG-I, there are other members of the enhanceosome such as the transcriptional coactivator and histone aceytiltransferase (HAT) p300 and CBP. IRF3/IRF7, ATF2/cJun and NF-κB form an interaction surface where the complex p300/CBP can bind. Interestingly, p300/CBP binding is stronger when it interacts with IRF3/IRF7 heterodimer (Vo and Goodman, 2001; Wathelet et al., 1998). The binding of p300/CBP and other HATs like GCN5 induces chromatin remodeling to expose the TSS and eventually facilitate *de novo* recruitment of RNA polymerase Pol II and its associated transcriptional machinery, ultimately initiating *IFNB1* transcription (Agalioti et al., 2000; Lomvardas and Thanos, 2001).

It is important to state that the enhanceosome assembly and the consequent expression of IFNβ follows an stochastic pattern, which means that only a specific percentage of infected cells will be IFN-producers and the rest of infected cells will not produce IFN (Zawatzky et al., 1985). It is reported that the stochastic expression of IFNβ is due to cell-to-cell differences in the levels of limiting components of the pathway that triggers IRF activation and does not dependent on NF-κB components. This is in accordance with the fact that IFNα genes, which are also expressed stochastically, are independent of the NF-κB pathway (Zhao et al., 2012). For example, experiments overexpressing cytosolic receptors RIG-I or

MDA5, the adaptor molecule MAVS, TBK1, IRF3 or IRF7 result in increased percentage of infected cells that produce IFN-I. The stochastic expression of IFN-I is also increased by pre-stimulation with IFNβ, suggesting that the limiting factors contributing to this pattern can be increased by initial burst of IFNβ production. This last observation links to the fact that IFN-I expression also changes from initial monoallelic expression to later biallelic expression once the availability of the limiting factors is increased (Zhao et al., 2012).

As commented previously, it is important to activate but also to limit or inhibit the IFN-I response. This stochastic expression provides an additional mechanism of regulation to control an exacerbated IFN-I production that could be very harmful.

Strictly referred to mechanisms that can directly repress type I interferon expression, although initial works point to the existence of a negative regulatory control at the level of the enhanceosome, the current knowledge is very limited. The 5'UTR of *IFNB1* contains two negative regulatory domains (NRDs) known as NRDI and NRDII. Both NRDs participate in the establishment of the silent state of *IFNB1* promoter in unstimulated conditions. NRDI overlaps PRDII and NRDII is located upstream of PRDIV (Goodbourn et al., 1986; Klar and Bode, 2005; Lopez et al., 1997; Weill et al., 2003). Regarding *IFNB1* inducibility, it was proposed that a virus-inducible repressor could lead to a post-induction turnoff of the IFNβ gene expression (Whittemore and Maniatis, 1990a). Further work postulated that this repressor should either do so by binding directly to the PRDs of the promoter or should somehow inactivate

the factors bound to PRDI and PRDII (Whittemore and Maniatis, 1990b). This was further demonstrated *in vitro* as there was a factor, BF1, described to exert as a repressor by binding PRDI (Keller and Maniatis, 1991). Unfortunately, BF1 factor was not further characterized in other works nor its functions were validated *in vivo*. More recently, other mechanism have been reported to inhibit *IFNB1* expression through a direct interaction between IRF3 with SMAD proteins (Sugiyama et al., 2012) or Rubicon (Kim et al., 2017) but regarding the promoter region, there is only evidence for the antagonic role of IRF2 in regulating *IFNB1* expression (Matsuyama et al., 1993). Another repressor reported recently is ATF3, but in this case the binding is located to a distal site of the *IFNB1* promoter (Labzin et al., 2015). Overall there is still not a complete understanding of the negative regulators of the enhanceosome activity.

2.2.3 Downstream of IFN-I

Once IFNβ is produced, it can act in an autocrine or paracrine manner since virtually all cells express the interferon type I receptor (IFNAR). As commented, IFNAR is an heterodimeric transmembrane receptor of two subunits, IFNAR1 and IFNAR2. All subtypes of IFN-I are able to recognize this receptor, as all IFN-Is contain the common residues that serve as anchoring point to ensure the binding to IFNAR (Piehler et al., 2012). Besides these common residues, there are specific residues that confer different affinity rates for the receptor among the different subtypes of IFN-I. Thus, the different signaling cascades and biological outcomes originated

by the different IFN-I subtypes could be influenced by their relative amounts and by their affinity for the IFNAR receptor (Ng et al., 2015). Interestingly, among the IFN-I family, the IFNβ subtype has the highest affinity for IFNAR (Jaks et al., 2007; Lavoie et al., 2011).

Albeit binding of IFNβ to IFNAR can trigger different signaling cascades, the common and first step in these signaling cascades is the activation of the receptor-associated protein tyrosine kinases Janus Kinase 1 (JAK1) and Tyrosine Kinase 2 (TYK2). Once JAK1 and TYK2 kinases are activated, they can promote the canonical or the non-canonical pathways, which lead to the activation of different signal transducer and activator of transcription (STAT) proteins and the subsequent activation of specific gene programs.

In the canonical pathway, JAK1 and TYK2 phosphorylate STAT1 and STAT2 molecules, leading to their dimerization and nuclear translocation. The heterodimer STAT1-STAT2 binds to IRF9 to form the ISG factor 3 (ISGF3) complex. The ISGF3 trimeric complex then recognizes the IFN-stimulated response elements (ISRE) in the promoters of interferon stimulated genes (ISGs) (Ivashkiv and Donlin, 2014; McNab et al., 2015). IRF7, one of the ISGs that gets induced, promotes a positive feedback loop to reinforce IFNAR signaling (Marie et al., 1998; Ning et al., 2011) (Figure 2.2). Moreover, other IFN-I signaling molecules are also ISGs, such as RIG-I, MDA5, TLR3 or STAT1, contributing to this positive feedback loop (Table 1.2.1). At this point, an interesting observation arises that connects with the negative regulation of this part of the pathway. Also other ISGs create a negative feedback

loop opposing the positive loop. For example, the SOCS proteins such as SOCS1 and SOCS3 inhibit IFNAR signaling through association with the kinases TYK2 and JAK1, respectively (Piganis et al., 2011; Sarasin-Filipowicz et al., 2009).

In the non-canonical pathway, the activation of JAK1 and TYK2 does not lead to the heterodimerization of STAT1-STAT2 but, instead, it signals to promote STAT1 homodimers and other STAT combinations. STAT1 homodimers are more commonly associated with the IFNγ pathway, which explains the partial overlap that exists in the gene programs activated by IFN-I and IFN-II. Both type I and type II IFNs can induce the activation of IFNγ-activated site (GAS) elements through STAT1 homodimers. However, in contrast to type I IFNs, IFNγ cannot induce the formation of ISGF3 complexes and therefore is not able to promote the engagement of ISRE sites to activate those genes that have only ISREs in their promoters (van Boxel-Dezaire et al., 2006; Decker et al., 2002). IFN-I can also activate signaling cascades through STATs that are usually associated with other cytokine-mediated responses, including STAT3, STAT4, STAT5A and STAT5B (Farrar et al., 2000; Torpey et al., 2004). In fact, it has been suggested that the balance of the different STATs activated downstream of IFNAR determines the relative activation between antiviral, proinflammatory, suppressive and anti-proliferative gene programs (Gil et al., 2012; Nguyen et al., 2002; Wang et al., 2011a). Also, binding of IFNβ to IFNAR could activate the phosphoinositide 3 kinase (PI3K)–mammalian target of rapamycin (mTOR) pathway and multiple mitogen-activated protein kinase (MAPK) pathway

(Lekmine et al., 2003; Platanias, 2005). This diversity of signaling pathways may in part explain the diverse IFN-I effects. IFNAR signaling leads to the transcription of a broad range of genes in addition to those dedicated to viral restriction, including genes that encode cytokines and chemokines, antibacterial effectors, pro-apoptotic and anti-apoptotic molecules, cell cycle regulators and molecules involved in metabolic processes (McNab et al., 2015; Rauch et al., 2013). In consequence, there are broad effects of IFN-I signaling that, as we have proposed, must be strictly controlled to prevent cell damage and disease. In the following section we will review the most outstanding effects of IFN-I identified in the recent years.

2.3 IFN-I effects

Type I interferons are secreted by infected or damaged cells and exert their effects in all cells (infected/damaged and healthy ones). During the 21st century, multiple and diverse interferon effects have been discovered. In this chapter, we will review in detail those that have been better characterized and also attracted more interest: the antiviral response, the influence on tumor growth and anticancer therapy, and finally the regulation of the hematopoiesis and the impact on HSCs.

2.3.1 Antiviral response

The antiviral effect is the best-characterized function of interferons. In this section, we will divide the antiviral effects of IFN in cell intrinsic or cell extrinsic. One that is designated to induce the

expression of ISGs that directly limits the spread of the virus (cell intrinsic) and another that functions to globally modulate the immune system and better resolve the infection (cell extrinsic). At the end of the section, we will discuss the recently described role of IFN-I as a double-edge sword regarding viral infections.

The cell intrinsic effects are referred to the expression of ISGs that directly limit the spread of the virus in the infected cells or specially in neighboring non-infected cells. Despite the function of many ISGs is still unknown, it is safe to say that the function of most ISGs is to repress every stage of the viral life cycle (Borden et al., 2007). To exemplify this fact we can consider the antiviral mechanism of three representative families of ISGs: oligoadenylate synthetases (OAS), the IFN-induced proteins with tetratricopeptide repeats (IFIT) and the Mixovirus resitance proteins (Mx) families. The OASs family of ISGs promotes the degradation of viral RNA transcripts (as well as host RNAs) by the activation of the ribonuclease L (RNASEL) (Silverman, 2007). The IFIT family of ISGs is directed to block the translation of viral components by inhibiting the activity of the translation initiation factor (EIF3) (Fensterl and Sen, 2015). The Mx family of ISGs is formed by GTPases designated to bind viral nucleocapsids and interfere with intracellular trafficking and viral assembly (Haller et al., 2007).

ISGs are key to promote the antiviral response, and their importance in combating viral infections stands out by the fact that most viruses developed mechanisms that limit IFN-I production and/or its signaling to prevent ISGs induction (Devasthanam, 2014). For example, the nucleoprotein (NP) characteristic from arenavirus such

as the lymphocytic choriomeningitis virus (LCMV) was reported to interfere with the activation of IRF3 and prevent IFN-I expression (Martínez-Sobrido et al., 2006). As a reply to this viral strategy, some ISGs can be directly induced by IRFs in response to PRR activation, independently of the IFN-I production (Schoggins and Rice, 2011). However, ISG expression solely is not sufficient to stop the viral infection.

Beyond the induction of ISGs, IFN-I is able to globally modulate the immune system to ensure a coordinated and transversal antiviral response. In this regard, type I interferons modulate different immune processes that affect several cell types. Besides macrophages and DCs, NKs and cytotoxic T cells (CTLs) are key actors in the clearance of infected cells and their cytotoxic activity is regulated by IFN-I in different ways. For example, IFN-I induces the production of chemokines such as CXCL9 to promote the recruitment and accumulation of effector cells (Antonelli et al., 2010; Proost et al., 2006). Also, type I IFNs potentiate hematopoietic differentiation of bone marrow progenitors into DCs, activates immature DCs and promotes conversion of pDCs into myeloid-derived DCs (Sevilla et al., 2004; Zuniga et al., 2004). Then, activated DCs produce IL-12 that is crucial for driving T helper 1 (Th1) responses during some bacterial and viral infections, and important for IFNγ production by T cells and NK cells (Gautier et al., 2005). IFN-I also induces the secretion of other cytokines such as IL-15 that play a positive role in the proliferation and maintenance of NKs and memory CD8 T cells (Yamaji et al., 2006; Zhang et al., 1998). Furthermore, IFN-I-induced upregulation of

MHCI and CD80 and CD86 facilitates presentation of viral antigens to CD8 T cells and the subsequent elimination of infected cells (Le Bon et al., 2003). IFN-I regulates directly the expansion and functions of NKs and CD8 T cells. During viral infections, IFN-I promote survival and clonal expansion of the CD8 T cell pool but it is also reported that promote growth-inhibitory signals in these cells (Aichele et al., 2006; Marrack et al., 1999; Marshall and Swain, 2011). The different ratio of STAT signaling downstream of IFNAR could be, again, behind these diverse roles of IFN-I as STAT1-deficient T cells mostly drive pro-survival/expansion of CD8 T cells probably through STAT3/STAT5 signaling, rather than antiproliferative effects through STAT1 (Gimeno et al., 2005; Tanabe et al., 2005). Similarly, IFN-I promotes the survival and clonal expansion of the NK cell pool and it is reported that NKs lacking the IFNAR receptor show impairment in their functionality (Kolumam et al., 2005; Lee et al., 2000).

Despite extensive research in the field, the lack of systems that can detect low IFN-I levels strongly hinders the studies that aim to understand how slight changes in IFN-I produce profound biological effects. For instance, there are at least three relevant scenarios in which low amounts of IFN-I are produced but cannot be directly detected, such as the expression in homeostatic conditions, in the late phase of infection and in patients with type I interferonopathies (Gough et al., 2012). These measurement problems are one of the reasons that explain the lack of direct evidence to relate these interferonopathies with a direct increase in the production of interferon. In these scenarios, the most common

strategy to indirectly assess the levels of IFN-I is to measure the mRNA of ISGs or the presence of immuno-suppressors such as IL10 and PD1L1 (Baechler et al., 2003; Bennett et al., 2003).

The importance of strictly measuring and controlling the levels of IFN-I is exemplified by the existence of interferonopathies that account for a series of diseases that have their origin in the production or rather, the overproduction of interferon. The most common and known interferonopathies are the Aicardi-Goutieres syndrome (AGS), the Singleton-Merten syndrome (SMS) and the systemic lupus erythematosus (SLE). Interferonopathies are defined as monogenic disorders that, due to the mutation in some factors of the IFN-I pathway, produce a signaling increase. For instance, patients with AGS and SMS have gain-of-function mutations in the cytosolic nucleic acid receptors IFIH1 (MDA5) and RIG1 genes or in the signal transducer STING gene leading to higher constitutive activation of IFN-I (Rodero and Crow, 2016). Also loss-of-function mutations in negative IFN-I regulators such as ISG15 or in proteins that prevent cytosolic nucleic acid accumulation like TREX are reported in patients diagnosed with AGS (Crowl et al., 2017; Zhang et al., 2015). However, due to our inability to properly measure IFN-I levels, not much evidence exists to link these pathologies with an increase in IFN-I. In support of this link, it has been documented that there is a phenotypic overlap between AGS, SLE and certain congenital viral infections, suggesting that the aberrant up-regulation of IFN-I signaling in all these cases could be their common driving cause (Crow, 2010; Rice et al., 2013). Moreover, although still without any direct evidence, some authors have

proposed an association between the abnormal increase of IFN-I and the appearance of autoinflammatory symptoms that can lead to autoimmune diseases (Cattalini et al., 2016; Cuadrado et al., 2015). For instance, it has been shown that exacerbated IFN-I signaling can promote antigen presentation and increased effector T and B cell responses in a way that generates autoantibodies (Ivashkiv and Donlin, 2014). Exacerbated IFN-I also potentiates inflammatory signals, as myeloid cells would express increased levels of STAT1 triggering responses downstream IFNAR and IFNGR in a positive feedback loop (Kalliolias and Ivashkiv, 2010).

IFN-I has been shown to exert as a double-edge sword in the responses against pathogens. Recent works have shown that deletion of the IFNAR receptor or alternative impairment of IFN-I signaling upon infection can lead to opposite outcomes, indicating a dual role for IFN-I in the responses against pathogens. Many variables have been proposed to account for this dual role of IFN-I, for instance, the exact amount of IFN-I that is produced and the timing, strength and type of virus that infects the cells. Thus, the type of virus and its specific infection kinetics will determine the balance between the positive and the detrimental effects of IFN-I, and will determine the suitability of blocking or exacerbating IFN-I production as a treatment. For example, AP-1 and NF-κB, transcription factors that promote IFN-I production, are also reported to favor, directly or indirectly, the replication of viruses such as cytomegalovirus (CMV) (Gustems et al., 2006; Isern et al., 2011). We will next review the viruses mostly used for the analysis

of IFN-I responses (Table 2.3.1) and describe what some authors call the interferon paradox (Odorizzi and Wherry, 2013).

Viral genome		Example	IFN-I related observations	References
DNA	dsDNA	CMV	Monocytes trigger cGAS-STING pathway and pDCs trigger TLR9 pathway CMV transcription is facilitated by AP-1 and NF-κB	DeFilipis et al., 2010; Paijo et al., 2006 Gustems et al. 2006; Isern et al. 2011
	ssDNA	rare examples	Parvovirus is related with the appearence of arthitritis, but not with autoinflammatory SLE or reumathoid arthitris	Moore, 2000
RNA	dsRNA	VSV	Detection through TLR3 and RIG-I Highly sensitive to IFN-induced antiviral responses IFNAR deletion causes high increase in mortality	Trottier et al., 2005; Muller et al., 1994; Lang et al., 2007
	ss(+)RNA	West Nile	RIG-I dependency 100% mortality in IFNAR deficient mice	Samuel and Diamond, 2005
	ss(-)RNA	LCMV	Docile low dose = acute self-resolving infection (IFN-I is necessary to clear the virus) Docile high dose = chronic infection (IFN-I, paradoxically, promotes viral spreading)	Wilson et al., 2013; Teijaro et al., 2013

Table 2.3.1 Classification of virus types. Table shows examples for each type of virus and observations connecting each virus with IFN-I response. Highlighted in green, viruses used in the main article of this Thesis.

IFN-I activates its prototypical antiviral effects upon acute infections performed using VSV, *Armstrong* or *Docile* (low dose) strains of LCMV or Vaccinia viruses (VV). In these situations, the deletion of IFNAR causes an increase in viral load together with a reduction in antiviral T cell function (Samuel and Diamond, 2005;

Sheehan et al., 2015; Zhou et al., 2012). Increased IFN-I signaling in these situations helps to better control the infection and to reduce the viral load by promoting the antiviral activity of DCs, NKs and CTLs. Conversely, upon chronic infections caused by *Clone13* strain or high dose of *Docile* strain of LCMV, IFN-I is associated to detrimental roles either by inducing immunosuppressive effects that cause uncontrolled viral replication, or by triggering inflammation and tissue damage. In this scenario IFNAR deletion albeit causes an initial increase in the viral loads, paradoxically, leads to a substantial reduction in viral titers at later time points after the infection. Also blockade of IFN-I signaling after the establishment of the chronic infection, enhances viral control, reduces the presence of immunosuppressor molecules such as IL10 and PD1L, and restores tissue organization (Teijaro et al., 2013; Wilson et al., 2013). Interestingly, it was also reported that IFNβ more than IFNα is the responsible of LCMV persistence, as treatment of infected mice with antibodies against IFNβ improves T cell responses and accelerates viral clearance compared to blocking IFNα treatment (Ng et al., 2015). Mice infected with Influenza Virus also show reduced immunopathology, morbidity and mortality when IFNAR is deleted or pDCs are depleted (Davidson et al., 2014). IFN-I can have pathogenic effects upon the infection with non-viral pathogens. For instance, infections with Mycobacterium tuberculosis (M. tuberculosis) promote the IFN-I-dependent production of IL10, which antagonizes with IFNγ and inhibits IL-1 production, both important factors of the antimicrobial program (Mayer-Barber et al., 2011; McNab et al., 2013). The existence of

pathological effects caused by IFN-I gives more hints to eventually answer an old and intriguing question of the field, which is why interferon treatments are so ineffective against important chronic infections such as those by HIV or HCV. In conclusion, the functions of type I IFNs can switch from being primarily antimicrobial and immune-stimulatory during acute infection to being predominantly immunosuppressive at later, chronic stages of infection. However, there are some discrepancies for this general view as, for example, the treatment with IFN-I during early stages of persistent LCMV infection promotes a rapid viral control (Wang et al., 2012). The reason for these discrepancies might be the type of virus studied, the genetic susceptibility of individuals, the house-keeping or homeostatic conditions used in the studies with animal models and the subtype and timing of IFN-I species produced, so more research will be needed to fully understand both sides of IFN-I and how to control them.

The main conclusion we can draw from this section is that the fine-tuning of the IFN-I production is key to acquire the exact level of immune response activation and that as important to induce the IFN-I response, is to be able to inhibit it. The correct measurement and strength in the production of interferons will determine their beneficial effect (stop the infection) versus the deleterious one (immunosuppressive and interferonopathies). The immediate future in the field is full of interesting challenges. We need to improve our understanding in the specific effects that the IFN-I has in each stage of the infection or the disease, which will allow us to design better strategies to block or activate the IFN-I pathway.

2.3.2 Hematopoiesis and HSC activation

Among all the effects of IFN-I, the last to be discovered less than ten years ago is the effect that it exerts on HSCs. Since Essers and Sato first proposed this role for IFN-I, it lead to some controversy fuelled by the lack of a complete understanding of its mechanism of action on HSCs. So far it has been proposed that interferon influences the proliferation, apoptosis and differentiation of HSCs. Again, as seen previously, it has been shown that the effects of IFN-I on the HSCs can be exerted directly via IFNAR signaling in the HSCs or by indirect effects of IFN-I on other immune cells that in turn regulate HSCs such as macrophages, T cells or stromal cells. Interestingly, regarding possible IFN-I effects, stem cells in general and HSCs in particular, are recently reported to be quite refractory to induce ISG expression in response to IFNAR signaling (Wu et al., 2018). HSCs intrinsically express some subsets of ISGs, including IFITM gene family, to be protected against viral infection.

IFN-I can directly regulate proliferation of HSCs. As seen previously, IFN-I is associated with anti-proliferative properties on many cell types but, albeit the first *in vitro* observations proposed an anti-proliferative role for IFN-I also in HSCs (Verma et al., 2002), further works challenged this idea and demonstrated that, instead, IFN-I stimulates the proliferation of HSC *in vivo* (Essers et al., 2009; Sato et al., 2009). Therefore, *in vivo*, HSCs will proliferate in response to systemic increases in IFN-I. Indeed, it has been shown that upon HSC activation via IFN-I, HSCs decrease the expression of cyclin-dependent kinase inhibitors and of the

prototypical quiescent transcriptional programs (Pietras et al., 2014). Albeit the proliferation and differentiation of HSCs upon IFN-I production could help in the regulation of the immune system to promote viral clearance, it can also be deleterious for the HSC pool. During homeostasis, quiescent HSCs are protected from damaging processes such as replication stress, DNA damage and genomic instability. It is well established that experimentally polyIC induced production of IFN-I leads to HSC activation, which causes rapid IFNAR-dependent HSC entry into cell cycle (Essers et al., 2009; Pietras et al., 2014). Albeit the precise order of the events is still unknown, cycling HSCs have been shown to contain higher levels of reactive oxygen species (ROS) and to accumulate DNA double-strand breaks that lead to the activation of the DNA damage response (DDR) pathway (Walter et al., 2015). Consequently, an aberrant/harmful positive feed-back loop is produced as this DNA damage induces endogenous IFN-I production via DAMPs recognition through ATM-IKKα/β-IRF3 pathway (Yu et al., 2015). Therefore, as proposed recently, as IFN-I stimulation of HSCs increase their ability to enter cell cycle also increase their risk of experiencing replication stress. Replication stress would eventually generate DNA damage and produce ROS, which would in turn amplify the signal by inducing the production of more INF-I via DAMP generation (Tasdogan et al., 2016). Collectively, these effects eventually lead to the activation of the pro-apoptotic and tumor suppressor transcription factor p53, which can promote HSC apoptosis (Takaoka et al., 2003).

Beyond proliferation and apoptosis, IFN-I promotes differentiation of HSCs. First, upon IFN-I exposure, there is an increase in the HSC compartment of the bone marrow (BM). Also, it has been observed that IFN-I promotes cell intrinsic alterations that prime HSC towards myeloid lineage differentiation and diminish their lymphoid potential (Rossi et al., 2005). Finally, in the same line of observations, IFN-I promotes the differentiation of HSCs to the MPP2 precursors. Although some reports propose as a driving cause of this differentiation bias the upregulation of the oncogenic transcription factor c-Myc or of the megakaryocyte lineage factors, little is known about the mechanisms underlying this process (Ehninger et al., 2014; Haas et al., 2015).

Beyond its direct impact on HSCs, IFN-I also regulates indirectly the functions of HSCs through different niche cells such as macrophages, T cells or stromal cells (Smith et al., 2016). Macrophages reinforce the initial IFN-I signaling by a positive feedback loop and by the production of TNFα, which crosstalks with the IFN-I pathway and exacerbates the above-mentioned effects (Legarda et al., 2016). Also, IFN-I can cause IFNγ production by T cells and NK cells. This IFNγ also activates HSCs upon infection (Baldridge et al., 2010). The impact of IFN-I on stromal cells that surround the HSCs can also influence their activation. A currently topic of investigation is to explore the contribution of surrounding stromal cells on HSC functionality. Stromal cells play a key role providing signals that support HSC properties, including self-renewal capacity and long-term multilineage repopulation capacity (Anthony and Link, 2014).

There are two differentiated zones in the HSC niche in the BM, the endosteal zone and the perivascular zone. While in a quiescence state, HSCs remain in contact with stromal cells in the endosteal zone, upon different signals, including IFN-I signal, HSC-niche interaction is lost and HSC move towards the perivascular zone, where they encounter higher ROS levels that promote HSC activation and cycling (Kunisaki et al., 2013; Ugarte and Forsberg, 2013).

The link between IFN-I and HSC functionality, which promote both exit from quiescence and DNA-damage exacerbation is one of the major causes of HSC senescence and aging (Walter et al., 2015; Yu et al., 2015). Thus, it would be of great applicability the discovery of a mechanism dedicated to limit exacerbated IFN-I responses, which lead to HSC senescence and hematopoietic failure and exhaustion of the system (Figure 2.3.2). In this regard, different experiments using BM transplantation or 5-FU challenge reported how HSCs are less able to reconstitute the immune system after the administration of polyIC, indicating a negative role for IFN-I in the HSC functionality (Essers et al., 2009; Pietras et al., 2014; Walter et al., 2015). Accordingly, two outstanding works defined how ADAR1 and IRF2, two negative regulators of IFN-I, are essential to maintain HSC capacity and preserve them from exhaustion (Hartner et al., 2009; Sato et al., 2009).

The fine tune production of IFN-I is crucial to preserve untimely HSC aging. Despite the transitory effect of IFN-I and the capacity of HSC to re-enter quiescence, the functional impact of IFN-I lasts long time after the stimuli. HSC “remember” each stress episode

that pushes them to be activated and proliferate. The signature created through lifespan could determine HSC loss of function and aging (Bernitz et al., 2016; Pietras et al., 2014). Then, understanding how the environmental stress response (ESR) regulates cell cycle and alters cell function is a fundamental puzzle for which we still only have some pieces (Canal et al., 2018). From all the stress signals that triggers the ESR, the IFN type I response emerge due to their impact on transcriptional antipathogen-program and at the same time potent cell cycle activation on stem cells. This dual capacity could be the source of many mutations through transcription replication conflicts (TRCs) that occur when transcription and replication coincide. Nonetheless, recent works highlighted two important features regarding the possible sources of genomic instability in activated HSC. First, stem cells and particularly HSCs are reported to be refractory to IFN-I in terms of activating the transcriptional antipathogen-program (Wu et al., 2018), which will protect HSC from TRCs derived from IFN-I. Second, in response to DNA damage, quiescent HSC use preferentially the nonhomologous end joining (NHEJ)-mediated DNA repair mechanism rather than the homologous recombination (HR) (Beerman et al., 2014; Mohrin et al., 2010). As NHEJ is associated with errors in DNA repair and the acquisition of genomic rearrangements and mutations, the activation of HSCs could protect them from the accumulation of mutations that could persist in the hematopoietic progeny. Understanding the specific mechanisms that use IFN-I to activate quiescent stem cells and impair its self-renewal and multipotent capacity would provide insights into the

molecular basis of many disorders such as leukaemias, aplasias or premature senescence.

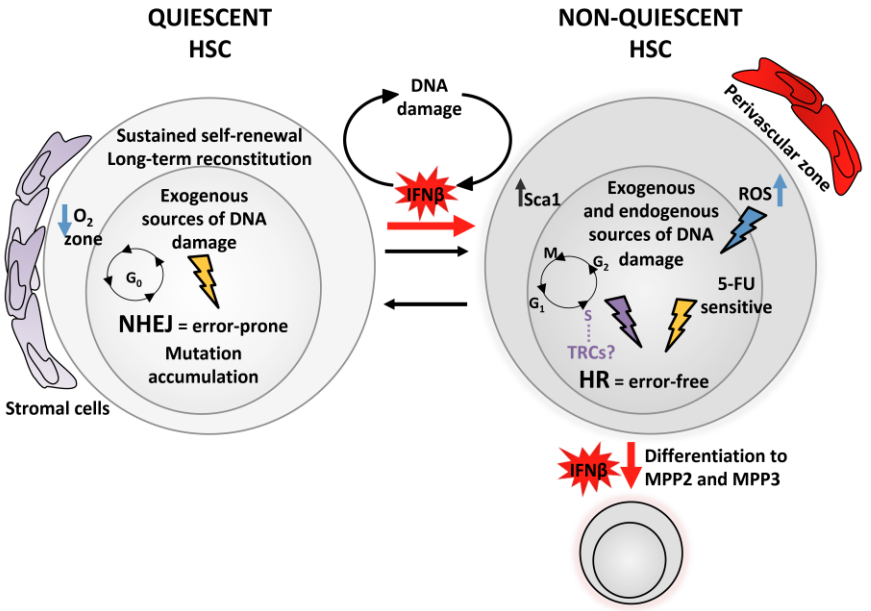

Figure 2.3.2 IFN-I activates HSC. The image shows differential characteristics between quiescent and non-quiescent HSCs. Quiescent HSCs are less exposed to DNA damaging sources that are mainly exogenous but repair DNA damage by error-prone NHEJ. Non-quiescent HSCs are more exposed to endogenous and exogenous DNA damaging sources but repair DNA damage by error-free HR. IFN-I has multiple effects as it promotes upregulation of Sca1, exit from quiescence, accumulation of ROS and differentiation to MPPs. Notice the positive feedback loop between IFN-I signaling and DNA damage.

2.3.3 Antitumoral

Seeing the multiple cellular effects of IFN-I, and how these effects diverse depending on the cell type it is difficult to imagine a biological process in which interferons would not play a role. One good example of this is the link between IFN-I and cancer. On the one hand, IFN-I has the ability to activate the innate and adaptive immunity, and therefore it has the potential to play an antitumor

role. On the other hand, as we reviewed previously, IFN-I links DNA damage with senescence in stem cells so is worth to analyze if IFN-I has the same effects on tumor cells, as DNA damage is a hallmark of cancer. Indeed, IFN-I has been already used with some success for the treatment of several types of cancer such as lymphoma, melanoma and renal carcinoma (Ferrantini et al., 2007; Moschos and Kirkwood, 2007).

Studying the effect of IFN-I in tumor growth has an additional level of complexity as first, one should understand how the immune system affects cancer development and progression and second, how IFN affects the immune system. Things get complicated because the immune system plays a dual role in cancer, and then interferon will act concordantly. The immune system is expected to eliminate malignant cells, however, it has been described that instead it has a dual opposite role as host-protective and tumor-promoting in a process known as cancer immunoediting (Schreiber et al., 2011). Cancer immunoediting can be divided in three phases. The first phase (elimination phase) is when the immune system efficiently eliminates malignant cells. The second phase (equilibrium phase) is when the immune system not eliminate some genetically unstable malignant cells, which "selects" or "immunoedits" cancer cells. Finally, the third phase (escape phase) occurs when neoplastic cell variants escape to immune detection and form clinically manifest neoplasms (Dunn et al., 2004a, 2004b). IFN-Is can play roles in the three phases because of the broad spectrum effects that promote in different cell types.

The shift from the first to the second phase in the process of cancer immunoediting relies mostly on the efficient recognition and antigen presentation strategies to target and eliminate malignant cells. Therefore, IFN-I signaling is crucial for target malignant cells through its roles in regulating DAMPs signaling and antigen presentation. DAMPs signaling can be activated in tumor-infiltrated APCs, when these cells phagocyte death cells, or in tumor cells, as these cells are characterized for accumulate DNA damage. In both cases, IFN-I is produced as a consequence of the presence of cytosolic DNA, which is recognized by cGAS (Mackenzie et al., 2017). In APCs, IFN-I signaling is necessary to activate and initiate an effective tumor-antigen presentation to CD8 T cells. As reviewed previously, IFN-I promotes IFNγ production by T cells, which in turn upregulates the antigen presentation by MHCII (Steimle et al., 1994). For example, it was reported that mice lacking IFNAR in CD8α+ DCs are unable to recognize and cross-present immunogenic malignant cells or mice deficient for cGAS show lower rates of tumor rejection (Diamond et al., 2011; Wang et al., 2017a). In tumor cells, the activation of cGAS-STING pathway can promote both antitumor and protumor effects. The antitumor effects rely on IFN-I that, similarly as previously mentioned for HSC, in tumor cells sustain DNA damage responses and promote senescence. Indeed, cGAS-STING mediated production of IFN-I is reported to be key in the antitumor strategies based on induce DNA damage in tumor cells (radiation) and prevent malignancy (Harding et al., 2017). This is further support by the observation that the downregulation of STING in cancer cells promotes tumorigenesis

(Xia et al., 2016a, 2016b). Instead, the protumor effects rely on the inflammation-driven carcionegenesis that result from the inflammatory cytokines, such as TNF, that are produced in tumor cells also through cGAS-STING activation (Balkwill, 2009; Li and Chen, 2018).

IFN-I also has effects regarding neoplastic transformation. For example, the deletion of *IFNB1* or IFNAR1 genes in mouse fibroblast is associated with higher rates of cellular transformation and the deletion of IFNAR1 from intestinal epithelial cells increases tumor growth in a model of colitis (Chen et al., 2009; Tschurtschenthaler et al., 2014). Also, using a model of breast cancer, the metastatic dissemination to the bones was accelerated in IFNAR deficient mice and, interestingly, administration of IFNα inhibited bone metastases in this cancer model (Bidwell et al., 2012). Therefore, IFN-I has antitumoral effects by its ability to globally stimulate the immune system. However, IFN-I antitumoral effects are not restricted to controlling the immune system, but instead, many works described the anticancer effects of directly activating IFNAR signaling in malignant cells. For example, it has been shown that IFNAR signaling induces apoptosis in B cell lymphoma (Yang et al., 2012) and inhibits cell cycle progression in hepatocarcinoma cell lines (Maeda et al., 2014).

Hence, the reported antitumoral effect of IFN-I administration could be because of their impact in the activation of the immune cells or by direct IFNAR signaling in malignant cells (Badgwell et al., 2004; Sistigu et al., 2014; Yang et al., 2014). Therefore, an interesting panorama opens up to improve antitumor strategies by

directing the increase of IFN signaling to a specific compartment and cell type of the tumor context (Zitvogel et al., 2015).

3. Nuclear Factor of Activated T cells 5 (NFAT5)

Nuclear factor of activated T cells 5 (NFAT5), also known as tonicity-responsive enhancer-binding protein (TonEBP), is a transcription factor (TF) that was first cloned and identified in 1999 by two independent groups (Lopez-Rodríguez et al., 1999; Miyakawa et al., 1999). These groups followed different strategies leading to the identification of this new transcription factor, which therefore was baptized using two different names.

López-Rodriguez and colleagues from Rao laboratory described an homology of about 43% and 16% between the new transcription factor and NFAT1-4 (NFATc) and p50 subunit of NF-κB respectively. Therefore, they decided to name the new factor as NFAT5 and, as we shall see in the next section, it was classified as a member of the Rel family.

Miyakawa from Moo Kwon's group identified the new transcription factors by using a yeast one-hybrid strategy, which assesses protein-DNA interactions. Specifically, they described the new factor is able to bind the tonicity-responsive enhancer (TonE) elements present in genes involved in osmostress responses, and therefore called it TonE-binding protein (TonEBP). Also, this work and others led to the characterization of the first known role of the TonEBP (NFAT5 from now on) in the response to hypertonic stress. NFAT5 is activated in response to hypertonic stress to induce

an osmoprotective gene expression program (Ko et al., 2000; Miyakawa et al., 1999). Moreover, it is described that NFAT5 null mice show severe renal abnormalities associated with impaired activation of osmoprotective genes (López-Rodríguez et al., 2004).

However, the role of NFAT5 goes beyond hypertonic stress. Roles for NFAT5 have been described in embryonic development (Go et al., 2004; Mak et al., 2011; Maouyo et al., 2002), cell migration (Jauliac et al., 2002) and muscle differentiation (O'Connor et al., 2007). Also, in analogy with other members of the Rel family (NFATc and NF-κB), there are increasing evidences that postulate NFAT5 as a regulator of the immune response. In the next section, we will contextualize NFAT5 in relation to the other Rel family members to better understand the spectrum of functions of NFAT5 in the immune system.

3.1 NFAT5 as a Rel family member

NFAT5 belongs to the Rel family of transcription factors, which also comprises NF-κB and NFATc proteins. NFAT5 was the last transcription factor to be added to the Rel family. For this reason, NFAT5 was initially described based on comparisons to what was already known for the other two well-characterized members of the family. However, in the last years, NFAT5 has been shown to be a fully independent member of the Rel family. Our laboratory and others have characterised NFAT5 roles, which place it as a master regulator of the immune system at the same level as NF-κB or NFATc. To this end, we will see first its similarities with NFATc or

NF-κB and second the particularities that differentiate NFAT5 as a separate member of the family.

The three types of the Rel family members are present and well conserved in mammals (Figure Ya). To study their origin and evolution across species, researchers have usually used as a reference the Rel-like Homology Region (RHR) sequence conservation. Studies in *Drosophila melanogaster* have described that in this organism there is only an NF-κB protein and a single NFAT-like factor, called dNFAT. Remarkably, the dNFAT of *D. melanogaster* has been shown to share higher homology with the mammalian NFAT5 than with the mammalian NFATc, as it shares 51% aminoacid identity with NFAT5, it conserves its dimerization residues, and it is related to salt stress tolerance, which indicates that NFAT5 appeared earlier than NFAT1-4 proteins (Aramburu et al., 2006; Keyser et al., 2007). This indication is further supported by studies reporting that sequences with high alignment with the mammalian NFATc proteins appear for the first time in vertebrate genomes and not before, which further confirms NF-κB and NFAT5 appearing earlier than NFATc as members of the Rel family (Graef et al., 2001; Hogan et al., 2003).

As commented, NFAT5 is defined as a member of the Rel family because it shares the RHR with the other two types of the Rel family members. The DNA-Binding Domain (DBD) and the Dimerization Domain (DD) are located inside the RHR. The DBD is located in the amino-terminal region of the RHR (RHR-N) and the DD is located in the carboxy-terminal region of the RHR (RHR-C). Same as NF-κB transcription fators, but unlike the NFATc,

NFAT5 is constitutively dimeric (homodimeric in this case), and its dimerization is essential for DNA binding and transcriptional activity (Hayden and Ghosh, 2004; López-Rodríguez et al., 2001). On the other hand, the DNA binding site selected by the homodimeric NFAT5 closely resembles the one selected by the monomeric NFATc (Lopez-Rodríguez et al., 1999; Miyakawa et al., 1999; Rao et al., 1997). As a consequence of this mixed homology, it is usual to define NFAT5 as a hybrid between NF-κB and NFATc regarding its structure (similar to NF-κB) and sequence to bind DNA (similar to NFATc) (Figure 3.1).

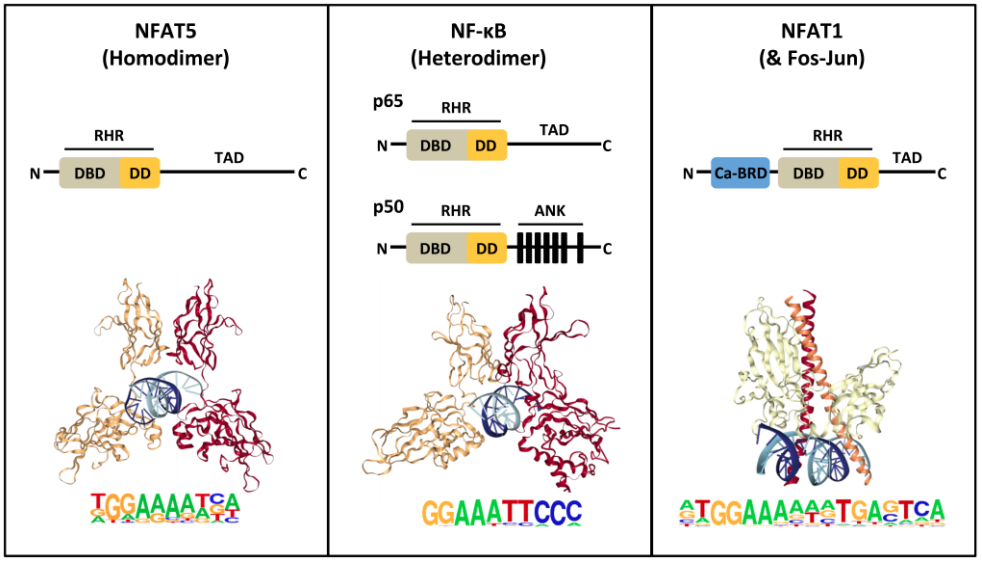

Figure 3.1 Schematic representation of the Rel family members. The image shows the protein sequence domains, structure of the Rel homology region (RHR) bound to DNA, and the DNA consensus site for NFAT5, NF- κB and NFAT1. Crystal structures reveal a high homology between NFAT5 and NF-κB RHRs. Both NFAT5 and NF-κB form dimers (orange subunit with red subunit) to bind DNA, while NFATc proteins (white subunit) are structurally different, contain a Calcineurin-binding regulatory domain (Ca-BRD) and cooperate with AP-1 to bind DNA.

Beyond the homology in the Rel domain, there is no recognizable similarity between the other regions of NFAT5 and NF-κB or NFATc proteins. For example, NFAT5 has a C-terminal domain containing a characteristic long TAD that gets phosphorylated and activated in response to hypertonicity (Lee et al., 2003; López-Rodríguez et al., 2001). This domain of more than 900 amino acids makes NFAT5 the longest protein among the Rel family with almost 1500 amino acids, and it is only found in vertebrates, but not in the insect protein dNFAT. In the N-terminal region of NFAT5 there is no similarity with either NF-κB or NFATc. In this regard, NFAT5 lacks the conserved calcineurin docking sites and residues targeted for phosphorylation that are present in the N-terminal regulatory region of NFATc proteins.

There are also major differences in the mechanisms that activate the different members of the Rel family, which also reflect their diverse functions. Up to this date, it has not been fully described which is the exact mechanism that activates NFAT5, whereas the mechanism of activation of NF-κB and NFATc is well defined. NFATc is activated by Ca^{2+} and the Ca^{2+}/calmodulin-dependent serine phosphatase calcineurin. In resting cells, NFATc proteins are phosphorylated and reside in the cytoplasm. Upon Ca^{2+}-dependent stimulation, NFATc proteins become dephosphorylated by calcineurin, translocate to the nucleus, and become transcriptionally active (Flanagan et al., 1991; Rao et al., 1997; Shaw et al., 1995). Also, the mechanism of activation of the conventional NF-κB pathway is well defined (see 2.2.1). Briefly, NF-κB dimers are retained in the cytoplasm of resting cells by the inhibitor IκB. Then,

upon stimulation, activation of the inhibitor of κB kinase β (IKKβ) phosphorylates and promotes the degradation of IκB, releasing NF-κB to translocate to the nucleus and activate transcription (Hayden and Ghosh, 2004; Karin, 1999).

All the Rel members are able to bind DNA, however, the affinity towards DNA and the specific mechanism of binding differs among them. As commented, NF-κB and NFAT5 bind DNA as hetero and homodimers respectively. Moreover, in the case of NFAT5, this binding is asymmetric and the affinity of NFAT5 for DNA is much lower than that of the other Rel members but its dissociation rate is lower too (Stroud et al., 2002). Also, while the DBD domain of NFATc contains residues that contact with cFos and cJun and therefore cooperates with AP1 to bind DNA, the DBD domain of NFAT5 lacks those residues and its binding to DNA is independent of AP1 (Lopez-Rodríguez et al., 1999). This independence from AP1 could be interesting in the context of the IFN-I response, where, as we saw in the previous chapter, AP1 (ATF2/cJun) plays a role in activating the transcription of the *IFNB1* gene.

The functions of Rel family members are mainly determined by the functions of the two well-known factors: NF-κB and NFATc. Both NF-κB and NFATc are factors known to carry out their functions in the context of the regulation of the immune system. We will brifely review the well-known functions of NF-κB and NFATc and then, we will compare these factors with NFAT5 to propose a certain homology also at the functional level among the members of the Rel family.

From its discovery during the study of immunoglobulin expression in B lymphocytes, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) was described to have a central role in the innate and adaptive-immune response, where it was found to activate different transcriptional signatures in different immune scenarios (Sen and Baltimore, 1986). During 30 years of research, NF-κB has reveal to possess an incredible broad role in activating gene expression upon many cellular responses beyond the activation and development of B cells (Zhang et al., 2017). Because of this wide spectrum of influence, we will cite those functions more connected with the work presented here. First, NF-κB is involved in the activation of the IFN-I response, acting downstream of the PRRs (see 2.2.1). Nevertheless, the best-recognized function of NF-κB is the regulation of inflammatory responses. NF-κB induces a pro-inflamatory profile in response to some PRR agonist, TNF, IL-1, and C-type lectin receptors (Ghosh and Karin, 2002; Medzhitov et al., 1997; Tak and Firestein, 2001; among others). In addition to mediating induction of various pro-inflammatory genes in innate immune cells, NF-κB regulates the activation, differentiation and effector function of inflammatory T cells as it mediates the TCR signaling (Liu et al., 2017; Oh and Ghosh, 2013). As a result, NF-κB promotes Th1 differentiation and therefore induces the secretion of IFN-γ, which in turn promotes conventional pro-inflammatory polarization (M1) of macrophages and reinforces the inflammatory response.

NFAT stands for nuclear factor of activated T cells but despite the name, NFATs expression is not restricted to T cells, but instead

they are also expressed in many other immune cell types. NFAT1-4 (NFATc) proteins, as we mentioned above, are activated by calcineurin upon intracelular increases in calcium. The mobilization and increase in calcium occurs as a consequence of the stimulation of different receptors. Examples of such receptors are the antigen receptors on T and B cells (Clipstone and Crabtree, 1992; Northrop et al., 1994), the Fcγ receptors on macrophages and NKs (Aramburu et al., 1995) and the C-type lectin and CD14 receptors in DCs, macrophages and neutrophils (Goodridge et al., 2007; Zanoni et al., 2009). NFATc triggers the activation of many immunomodularoty citokines of the adaptive response by T cells such as IL-1, IL-2, IL-6, IL-10, GM-CSF or IFN-γ (Crabtree, 1989; Lawrence et al.; Luo et al., 1996; Sica et al., 1997; among others).

3.2 Immune functions of NFAT5

If NFATc and NF-κB Rel family members are reported to have major roles in regulating the innate and adaptive immune responses, we will see now how NFAT5, the other Rel family member, is not far behind.

3.2.1 Functions in adaptive immune cells

Recently, it has been reported that NFAT5 regulates T cell functionality. First, Berga-Bolaños and colleagues defined NFAT5 as an important factor in thymocyte survival and correct development in the thymus (Berga-Bolaños et al., 2013). They proposed that NFAT5 positively regulates the transition of thymocytes from the β-selection checkpoint to the double-positive

stage by modulating the outcome of the signaling that occurs downstream of the pre-TCR. Specifically, they showed that despite normal pre-TCR signaling NFAT5-deficient thymocytes express lower pro-survival factors (A1 and Bcl2) and higher pro-apoptotic markers (p53 and Noxa). Also, NFAT5, related with its initial reported role as osmoprotective factor, is necessary for the correct expansion of T cell in response to osmostress, which could explain the immunodeficiency observed in NFAT5 null mice (Berga-Bolaños et al., 2010). In this regard, a patient was diagnosed with primary immunodeficiency associated with organ-specific autoimmunity, but normal diagnostic tests excluded the common disorders associated with these symptoms. Further analyses demonstrated that the patient had an hemizygous loss of NFAT5, suggesting that NFAT5 may play an important role in T cell immune responses and that NFAT5 deficiency may be linked to human autoimmunity (Boland et al., 2015).

Finally, further pointing out the role of NFAT5 in regulating the adaptive immune system, work from our group described a new relationship between NFAT5 and the T lymphocyte polarization. In a model of activation of T cells in vivo, NFAT5-deficient CD4 T cells exhibited a modest pattern of enhanced IFNγ and IL-17A expression relative to FOXP3 and CTLA4 leading to a more pronounced Th1 and Th17 response (Alberdi et al., 2017). Therefore, similarly to the other members of the Rel faminy, NF-κB and NFATc, NFAT5 can also regulate adaptive immune processes.

3.2.2 Functions in innate immune cells

Compared to our knowledge about the role of NFAT5 in adaptive immune cells, there are more works about its roles in innate immune cells, particularly in macrophages.

Macrophages are a good model to study the role of NFAT5 as, for example, they constitutively express moderate levels of NFAT5 and signaling through TLRs further induces its expression (Buxadé et al., 2012). Interestingly, it has been found that TLR-dependent induction of NFAT5 expression can be mediated by NF-κB and opposed by p38. NF-κB was reported to be necessary to upregulate NFAT5 upon TLR4 activation as inhibiting IKKβ prevented the induction of NFAT5. Also supports this idea the observation that NF-κB is bound to the promoter region of NFAT5 upon LPS stimulation (Buxadé et al., 2012). Regarding the p38 MAPK link, contradictory, it was also shown that its activation led to an induced expression of NFAT5 (Kim et al., 2014).

NFAT5 positively regulates transcription of multiple genes in macrophages. The molecular mechanism by which NFAT5 regulates gene transcription is not well understood. Fundamentally, NFAT5 binds directly to promoter regions that contain its consensus site (-GGAAA-) to positively regulate gene expression (Lopez-Rodríguez et al., 1999). However, some data proposes that NFAT5 could interact with NF-κB to regulate transcription. It was described that NFAT5 and NF-κB would participate in the formation of what was called as the NF-κB enhanceosome to activate the transcription of pro-inflammatory cytokines.

Intriguingly, the function of NFAT5 in the so-called NF-κB enhanceosome would not require its binding to DNA but, instead, NFAT5 would interact with the p300 co-activator and be a bridge between NF-κB and several lineage-specific transcription factors such as Sp1 at the promoter of pro-inflammatory genes (Lee et al., 2016).

The main function of NFAT5 described in macrophages is to induce the transcription of pro-inflammatory cytokines and promote M1 polarization (Buxadé et al., 2012; Kim et al., 2014; Tellechea et al., 2018). NFAT5 is constitutively bound to the promoters of some primary response genes such as *Tnf*, *Il1* and *Ccl2* and, also, under low doses of LPS, NFAT5 binds to the promoter region of secondary response genes such as *Il6*, *Il12b* and *Nos2* (Buxadé et al., 2012). Moreover, an NFAT5-dependent pro-inflammatory shift has been reported in both M1 and alternative polarized (M2) macrophages, as NFAT5 upregulates pro-Th1 factors produced in both M1 macrophages (IL-12) and M2 macrophages (Relma and Arg1) (Tellechea et al., 2018). Also, another work proposed that NFAT5 promotes the M1 polarization of macrophages by interfering with the binding of the Sp1 transcription factor and therefore reducing the expression of several M2-promoting factors (Choi et al., 2016).

Regarding the biological outcomes of NFAT5 in innate immune responses, there is some contradictory or opposite results among the works reported. For instance, NFAT5 helps to promote the immune clearance of the *Leishmania* parasite by facilitating the expression of iNOS (Buxadé et al., 2012). This observation was further

validated when it was reported that NFAT5-defficient classically or alternatively-polarised macrophages show less bactericidal capacity (Tellechea et al., 2018). However, opposed to this protective role of NFAT5 in front of bacterial infections, NFAT5 was found to facilitate the replication of the HIV virus in macrophages by binding an enhancer region conserved in the genome of the HIV-1 subtypes and promote viral replication (Ranjbar et al., 2006). Moreover, co-infection of HIV with *Mycobacterium tuberculosis* (MTb) further enhances HIV replication via the same NFAT5-dependent mechanism. Briefly, MTb infection would be recognised by TLRs that would eventually induce the expression of NFAT5 in a positive feedback loop that would further promote HIV replication (Ranjbar et al., 2012). Beyond data about pathogen infection, a recent work suggested that NFAT5-deficient macrophages may have impaired antitumor capacity, when co-injected with tumour cells, due to their reduced ability to activate a pro-inflammatory state and promote Th1 responses (Tellechea et al., 2018). Finally, NFAT5 has also been associated with some immune disorders such as rheumatoid arthritis. In this scenario, NFAT5 could act as a pro-survival factor for macrophages by mediating CCL2 production, which would cause macrophage accumulation and exacerbation of the arthritis at the affected area (Choi et al., 2017; Kim et al., 2014; Lee et al., 2018; Yoon et al., 2011). Therefore, NFAT5 could take part in different physiological scenarios that range from the inflammatory response of macrophages and T cells to the outcome of infectious diseases.

3.2.2 Upcoming functions

In this Thesis, we will demonstrate that the parallelism among the Rel members goes far beyond what is already known and we will show definitive evidences supporting that NFAT5 states as a bona fide immune regulator and provides a fine-tune control of the expression of diverse genes, as previously known for the other two Rel members. This functional similarity, reflected in the existence of non-redundant functions in the regulation of inflammatory responses and the control of pathogens, serve us as a guideline to open new lines of research and finally reveal two new roles for NFAT5.

Included as a supplementary article in this Thesis, we have characterized the role of NFAT5 in sustaining MHCII expression in macrophages. This work describes a genomic enhancer controlled by NFAT5 that regulates the activity of the myeloid promoter of *Ciita* specifically in macrophages.

IFN-I response is extensively connected with NF-κB (chapter 2.2 of the Introduction) and has also been connected with NFATc (Bao et al., 2016). As detailed in the Results section of this Thesis, our data indicates that NFAT5 is able to negatively regulate the expression of IFN-I to prevent a systemic exacerbation of the IFN-I response that, as reviewed in the Introduction, can have harmful deleterious consequences.

OBJECTIVES

Our group is interested in understanding the role of NFAT5 in the immune system. As a member of the Rel family of transcription factors, our starting hypothesis is that NFAT5 will share functional characteristics with the other Rel members, NF-κB and NFATc. NF-κB and NFATc proteins are essential players in the response to pathogens as key transcription factors activated by pattern recognition receptors (PRRs). Indeed, previous results from our group and others define NFAT5 as a positive regulator of proinflammatory response upon TLR4 activation in macrophages. Further analysis of the TLR4-dependent gene transcription suggested a potential role of NFAT5 in regulating the expression of Interferon Stimulated Genes (ISGs). Therefore, the central objective of this Thesis is to study the potential implication of NFAT5 in the type I Interferon response.

Specifically, the main objectives of this PhD were:

1- To study the role of NFAT5 in type I interferon (IFN-I) responses, dissecting its specific function in different types of cells and upon different PRR activation.

2- To assess the physiological impact of NFAT5 in the regulation of IFN-I responses upon viral infection.

3- To analyze whether NFAT5-regualted IFN-I production can control hematopoietic stem cell activation.

4- To characterize the molecular mechanism by which NFAT5 inhibits the expression of IFN-I genes.

RESULTS: MAIN ARTICLE

The transcription factor NFAT5 limits infection-induced type I interferon responses

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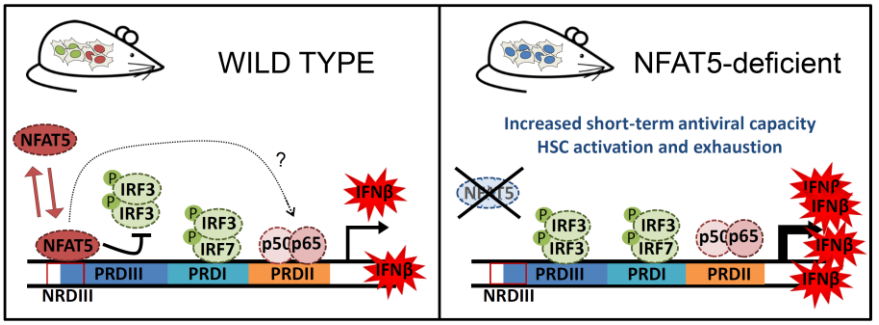
DISCUSSION

Is IFN-I omnipresent? Indeed, publications from the last 10 years demonstrate how difficult it is to try delimiting when and where IFN-I is biologically relevant. From its discovery as an antiviral factor, unstopping growing evidence has demonstrated a profound influence of IFN-I in multiple biological scenarios. As seen in the Introduction, some of these pleiotropic functions include the influence of IFN-I in the adaptive immune response, HSC activation and tumor progression. Also IFN-I impacts on cellular metabolism, circadian rhythms or the development of different autoimmune and proinflammatory diseases (Ohdo et al., 2001; Rodero and Crow, 2016; Wu et al., 2016).

To this day, the updated understanding of the effects of IFN-I is based on two characteristics. The first one, as we highlighted, its capacity to influence different processes seems endless, there are no limits to what interferon can or cannot modulate. The second one, that emerge in the last years, is that IFN-I production has been found to promote double-edged or opposed responses depending on time, strength or type of the cell where the signaling take place. For example, these variables of IFN-I determine the difference between apoptosis and cell division or between stemness and senescence. Therefore, understanding how this balance works and learn how to tightly control and limit IFN-I levels in order to modulate its functions will undoubtedly be a key field of research in the upcoming years.

In this Thesis we characterize a novel mechanism mediated by the transcription factor NFAT5 that limits the IFN-I response. We prove that NFAT5 binds the IFNβ promoter and impairs the binding

of IRF3, the master positive regulator of IFN-I. Accordingly, we show that knocking out NFAT5 or preventing its binding to the IFNβ promoter results in increased IFN-I induction in response to PRR activation. We find increased IFN-I response in NFAT5-deficient BMDM, BMDC, and tissue isolated macrophages, cDCs and pDCs upon stimulation with polyIC or viral infection with RNA (VSV and LCMV) and DNA virus (mCMV). Also, we demonstrate that NFAT5 limits IFN-I production with significant biological outcomes. We prove *in vivo* that NFAT5 restrains the antiviral functions of IFN-I upon acute viral infection and, interestingly, that NFAT5, by controlling systemic IFN-I production, protects HSCs quiescence (Discussion Figure).



Discussion Figure. This model represents the molecular mechanism by which NFAT5 limits IFN-I response. In wild type mice, NFAT5 can bind the *IFNB1* promoter. IRF3 binding leads to IFN-I production (green cells) but when NFAT5 binds NRDIII, binding of IRF3 is impaired and IFN-I production is limited (red cells) allowing a fine-tune control of IFN-I production in wild type mice. In NFAT5-deficient mice, although NF-κB binding is reduced, IRF3 binding is enhanced leading to higher systemic IFN-I production (blue cells) that can give rise to diverse physiological consequences that will be discussed in this section.

IFN-I pathway: a mechanistic and evolutionary update

We identified an unknown negative regulatory domain (NRD) at the *IFNB1* promoter that contains the consensus binding site for

NFAT5. Two NRDs (NRDI and NRDII) have been previously described at the *IFNB1* promoter (Goodbourn et al., 1986; Nourbakhsh et al., 1993; Weill et al., 2003). We confirmed that NFAT5 binds to this new NRD, which we propose to call NRDIII, to limit the expression of the *IFNB1* gene (Fig. 5a and Fig. 6d). We genetically separated the role of the different regulators of IFNβ such as IRFs, NF-κB and NFAT5 by performing point mutations at the enhanceosome region of the *IFNB1* promoter. We mutated this new described NFAT5 binding site and also the three previously known positive regulatory domains (PRDs) present at the *IFNB1* promoter to sequentially disrupt the binding of NFAT5, IRFs and NF-κB (Fig. 6c). As expected, mutations of the PRDs impaired the activation of the *IFNB1* promoter (especially upon the mutation of PRDIII) but, interestingly, the mutation of the NFAT5 binding site increased the activation of the *IFNB1* promoter upon polyIC treatment (Fig. 6d). Accordingly, overexpression of the isolated NFAT5 DNA-binding domain reduced the polyIC-induced activity of *IFNB1* promoter (Supplementary Fig. 8a). We propose that the absence of NFAT5 alters the stoichiometry of the factors IRF3/7 and NF-κB. Specifically, we propose that the overlap between the PRDIII and the newly identified NRD causes a direct competition between IRF3 and NFAT5, which serves to control IRF3 binding and to limit IFNβ induction. In support of this hypothesis, cells deficient for NFAT5 increased the recruitment of IRF3 and displayed net increase in promoter activity as indicated by increased acetylation of histone H4 (Fig. 5b,c). Accordingly, taking advantage of the observations from mutant combinations, we confirm that

PRDI and PRDII mutants still show an NFAT5-sensitivity, while PRDIII mutant showed no NFAT5-sensitivity. Mutation in the NFAT5 binding site caused an increase in polyIC-induced *IFNB1* promoter activity which was highly similar to the increase observed when we analyzed the double mutants PRDI-NFAT5 and PRDII-NFAT5 (Fig. 6e). These results strongly indicate that NFAT5 binding to the new identified consensus site impedes IRF3 binding to the PRDIII, bringing about the control of IFN-I induction (Fig. 6d).

The central mechanism that is reported to induce IFN-I responses is that PRDs are bound by IRFs and NF-κB to positively regulate *IFNB1* activation, which triggers the expression of ISGs. Unexpectedly, only the mutation of PRDIII and not that of PRDI or PRDII, abolished the *IFNB1* promoter activation, which suggests that among PRDs, PRDIII is capital for the expression of *IFNB1*. Since IRF3 binds to PRDIII and PRDI, and NF-κB (p65) binds to PRDII, results suggest that binding of IRF3 dominates over the binding of p65 to activate *IFNB1* transcription. In agreement with that, NFAT5-deficient cells induced higher IFNβ expression than wild type cells and also presented higher IRF3 binding but, surprisingly, less p65 binding to the *IFNB1* promoter (Fig. 5c). To confirm our hypothesis of the greater contribution of IRF3/7 versus NF-κB and the relevance of PRDIII versus other PRDs in the activation of *IFNB1* expression, we could perform additional experiments. One of these experiments would be ChIP analysis on promoter mutants, to confirm that the target mutations we designed effectively impaired the binding of the corresponding factors. Also

by ChIP analysis, it would be interesting to confirm that the changes in the stoichiometry of the enhanceosome factors observed in NFAT5 deficient cells are also observed when we just impede the binding of NFAT5 to the NRDIII region. Together with the results presented here, these experiments would help to complete our understanding in the mechanism that controls IFNβ production. For instance, understanding the precise level of each factor required to reach its maximum induction. In this sense, although IRF3/7 is dominant versus NF-κB for *IFNB1* transcription, our results also indicated that at least some binding of NF-κB is necessary to reach the maximum *IFNB1* transcriptional induction as we detected reduced but clear p65 recruitment in NFAT5 deficient cells and also the activity of the double mutant PRDII-NFAT5 is lower than that of the NFAT5 mutant (Fig. 5b and Fig. 6c right panel).

Also this new role characterized for NFAT5 could lead to a better understanding of the stochastic expression of IFN-I. We propose that NFAT5 could influence on this stochastic pattern in a manner that the NFAT5 deficiency will increase the percentage of activated/infected cells that produce IFN-I. The limiting factors that underlie the stochastic expression could be IRF3 or other components of the IFN-I pathway that are induced by IFNβ, such as RIG-I, MDA5 or IRF7 (Zhao et al., 2012). Therefore, it seems likely that NFAT5 controls the level of IFN-I expression by limiting the percentage of IFN-producer cells. With this view, some infected cells will ensemble the enhanceosome by proper IRF3/7 binding and produce IFN-I, while other infected cells will not produce IFN-I because NFAT5 limits IRF3/7 binding. In a scenario with no

NFAT5 most of, if not all, the infected cells would have the potential to express IFN-I without any impediment (Discussion Figure). To analyze whether NFAT5 competition with IRFs is what controls the stochastic expression of IFN-I seems one attractive approache to further characterize the IFN-I response.

The relevance of the repression mechanism imposed by NFAT5 is indicated by the high conservation of this regulatory region among vertebrates through evolution. All analyzed species that produce IFNβ contain in their promoter region the consensus site for NFAT5 overlapping with a binding site for IRF3. As we previously see, in the case of human or mouse this overlapping is produced between PRDIII and the adjacent NRDIII containing the NFAT5 binding site. Intriguingly, in the case of sheep or opossum, the NFAT5 binding site instead of overlapping with PRDIII, it does so with PRDI (Supplementary Fig. 7). Therefore, the conserved overlapping between IRF-regulated PRDs and the NFAT5 binding site suggests a relevant contribution for NFAT5 in the control of IFN-I production across the evolution of the vertebrates.

IRFs regulate the expression of other genes beyond *IFNB1*. As we described in the Introduction, interferon alpha genes (IFNAs) can be activated directly by IRFs. Also it is reported that IRF3 directly targets the expression of some ISGs (Honda et al., 2006), an antiviral strategy to avoid the IFN-dependency as some virus can block the production of IFN and cytokines such as IL-15 or IL-12 (Goriely et al., 2006). Therefore one question that emerges is whether NFAT5 could also directly repress the transcription of alpha interferons. One important fact about alpha interferons is that

they are mainly regulated as ISGs and therefore induced upon IFNβ activation of the IFNAR. In that case, differences in IFNα expression between wild type and NFAT5-deficient cells could be due to the higher production of IFNβ by knockout cells and not because of a direct effect of NFAT5 on IFNα promoters. Thus, two experimental strategies were proposed to address the effect of NFAT5 in the expression of interferons alphas. The first one was to analyze whether there were differences in the expression of IFNα between wild type and NFAT5 deficient BMDM in an IFNAR-deficient background. In the absence of IFNAR signaling, any expression of IFNα will be due to a direct regulation by NFAT5 and not influenced by IFNβ levels. Although IFNα expression levels are low due to its strong dependence on IFNAR, we could appreciate that NFAT5 deficiency leads to higher expression of IFNα early after infection (Supplementary Fig. 3b). The second strategy is based on analyzing the expression of IFNα in a cell type specialized in the production of IFN-I, the plasmacytoid dendritic cells (pDC). As we reviewed in the Introduction, these pDCs follow a different signaling pathway, based on constitutively expressing IRF7 and thus do not depend on the initial production of IFNβ to express IFNα. In the in vitro viral infection experiments we observed higher levels of IFNα in NFAT5-deficient pDCs compared to wild type pDCs (Fig. 3c). Despite these results, it would be fair to say that we cannot be completely sure that NFAT5 directly regulates the expression of IFNα; since in the first case, the expression of IFNα of IFNAR-deficient macrophages is poorly induced upon stimulation, and in the second case, the pDCs are generated in a

mixed culture where cDC are also produced, and therefore we cannot rule out that the differences in the pDC expression of IFNα are influenced by the higher production of IFNβ by NFAT5 deficient cDCs (Supplementary Fig. 3a). So, in order to further analyze whether NFAT5 directly regulates IFNα expression, there would be two strategies to follow based on isolating pDC from spleen of NFAT5 deficient mice and control littermates and then infect them in vitro. We could then analyze the RNA expression of IFNα in a homogeneous culture of pDC, and perhaps, we could detect NFAT5 and IRF7 binding in the promoter region of interferon alpha genes performing ChIP experiments, as done in BMDM. Nonetheless, regarding this possible ChIP analysis on IFNα promoters or the ChIP analysis on IFNβ promoter exposed in this Thesis, it is worth to keep in mind that the detection of transcription factors could be quite limited due to the stochastic expression of IFN-I within a cell population upon stimulation (Zhao et al., 2012). Despite the technical challenges that these experiments could present, the fact that we observe NFAT5 consensus sites proximal to IRFs sites in human and mouse promoters of different IFNα genes, reinforces the idea of a direct regulation of alpha interferons by NFAT5.

Besides direct regulation on IFNα, and similarly as IRFs, we also reported that NFAT5 directly bind to the promoter of some ISGs. Ultrasequencing of immunoprecipitated chromatin (ChIP-seq) analysis revealed several ISGs as potential direct targets of NFAT5 (Supplementary Fig. 6). Outstanding examples are *Ifi27* or *Ifitm* genes. In the first case, we validate by qChIP that NFAT5 is

strongly recruited to the promoter of *Ifi27* upon polyIC stimulation (Fig. 5a). Contradictory, in the case of *Ifitm* family members, we observe clear higher expression of some members (*Ifitm6* and *Ifitm1*) in wild type over NFAT5-deficient cells (Fig. 1b). As we previously propose that NFAT5 binds to overlapped IRFs regulatory regions of IFN-I, and this coexistence seems highly conserved through species since the appearance of interferons, these evidences open the possibility to study to what extent this mechanism occurs also in other genes beyond those encoding for type I interferon.

A final remark regarding the activation of IFN-I pathway could be considered. We reviewed in the Introduction that the relative activation of the signaling pathways that trigger IRFs or NF-κB determines the level of IFN-I response versus the conventional proinflammatory response. In this regard, the role of NFAT5 emerges as a possible judge in both contexts. On the one hand, NFAT5 is reported to favor proinflammatory cytokine expression (Buxadé et al., 2012) when triggering signaling pathways that favor NF-κB activation and proinflammatory responses (LPS-TLR4). On the other hand, in this Thesis we propose that NFAT5 limits the IFN-I response upon the activation of signaling cascades that promote IRFs and IFN-I pathway activation: TLR3 activated by synthetic dsRNA, TLR7 (or TLR8) activated by single strand RNA viruses and TLR9 and cytosolic receptors that recognize double strand DNA virus. Of note, these results also highlight that NFAT5 could regulate type I interferon production both in TRIF and MyD88-dependent signaling pathways.

The interferon paradox in antiviral response: NFAT5 as the last frontier?

In our search towards understanding the role of NFAT5 in the IFN-I response, we established its involvement on antiviral responses both at cellular (in vitro) and physiological level (in vivo). As we will discuss below, the experiments performed in this regard are concentrated in studying the response to an early/acute antiviral response.

NFAT5 deficient cells show higher expression of diverse set of interferon stimulated genes (ISGs) (Fig. 1a-c) as a consequence of their increased IFNβ production (Fig. 1f) and IFNAR signaling (Fig. 1g). This higher expression of ISGs in NFAT5-deficient cells was mainly due to the paracrine effect of IFN-I (Supplementary Fig. 3c). This observation indicates that cells deficient for NFAT5 express more ISGs mainly due to the initial higher production of IFN by neighbor cells that have been infected and not due to a bias downstream of IFNAR. One possible experiment to reinforce this idea would be the co-culture of wild type and NFAT5-deficient cells, for their subsequent infection and isolation to analyze the expression of IFN-I and ISGs in infected and non-infected NFAT5-deficient and control cells separately. We then expect to find higher levels of IFNβ in infected NFAT5-deficient cells than in its infected control counterparts, but no differences in the ISG expression when comparing both types of non-infected populations, except perhaps in ISGs that could be directly regulated by NFAT5 independently of IFN. At this point maybe one wonder about the possibility that perhaps NFAT5 deficient cells were more susceptible or able to be

activated, that is, expressing higher levels of PRRs. Nonetheless, we have discard this scenario as we performed a wide transcriptome analysis and then focused on the PRRs that can lead to IFN-I production to observe no differences between wild type and NFAT5-deficient cells (Supplementary Table 1), of course this result would be insured if we confirm the corresponding protein/receptor levels.

On the other hand, to analyze in vivo the antiviral response we infected *Nfat5*$^{fl/fl}$ Vav-Cre and control littermates with a low dose of LCMV Docile strain and we analyzed their response 2 days later. Since early after LCMV infection, the main IFN-I producer is the plasmacytoid dendritic cell (pDC) (Siegal et al., 1999), we use a NFAT5-deficient mouse model that lacks NFAT5 in all immune cells (*Nfat5*$^{fl/fl}$ Vav-Cre). Our expectations were far surpassed by observing that *Nfat5*$^{fl/fl}$ Vav-Cre mice nearly doubled the systemic production of IFN-I (Fig. 2a) and their viral load detected in the spleen was clearly reduced (Fig. 2b). Detailed mRNA analysis on pDC or peritoneal macrophages revealed that both types of cells express higher levels of *Ifnb1* but lower levels of viral RNA (Fig. 2c,d). These results, that support our hypothesis, are nonetheless also subject to certain limitations that we must also take into account when interpreting the data. For example, the drawback of working with this mouse model is that adaptive immune cells are also deficient for NFAT5, and resolution of LCMV infection is dependent on the CTL response (Moskophidis et al., 1993). Therefore, to better analyze whether this initial higher production of IFN-I and control of viral spreading have a physiological impact at

long time points and it really serves to better control the infection, we rather not use this mouse model since it could also have a bias on NFAT5-dependent T lymphocyte functions (Alberdi et al., 2017; Berga-Bolaños et al., 2013). For this reason, we also analyzed the antiviral response at a later time point in the LysM-Cre mouse model, which only lacks NFAT5 in myeloid cells. Interestingly, even without the increased production of IFN-I supplied by pDC, we observed that at day 7 after infection this NFAT5-deficient mouse also presented improved viral clearance (Fig. 2e). In light of these results, one can wonder about the possibility that this phenotype may be influenced by the fact that cells deficient for NFAT5 could be differentially susceptible to be infected. To rule out this possibility, we designed an experiment based on inactivating the virus prior the infection and then analyzed the viral load inside the cells early after the infection (3 hours). Since our results showed no differences between wild type and knock out cells in the viral load (data not shown) this suggested no bias in the viral entrance or capacity of infection. In summary, the biological scenario conferred by increased IFN-I responses in NFAT5-deficient mice provides an improved defense against viral infection.

Another layer of complexity is that we now know that the effects of IFN-I on antiviral response could vary during the course of the infection. The old-classical view of IFN-I antiviral function is presented during early stages of the infection or in acute infections, where the loss of IFN-I signaling results in uncontrolled viral replication (Müller et al., 1994b) and, as we also see in our work, the increase in IFN-I response has beneficial antiviral effects.

However, is still quite unclear why the sustained IFN-I signaling in chronic infections is unable to clear the virus. Indeed, recent works show that initial production of IFN-I upon viral infections could lead to several immune disorders and favor viral spreading at late time points after the infection (Teijaro et al., 2013; Wilson et al., 2013). The contradictory effects of IFN-I during viral infections are known as "the interferon paradox" (Odorizzi and Wherry, 2013). This paradox leads us to ask ourselves to what extent we can ensure that the lack of NFAT5 will always produce beneficial effects in the antiviral response, or whether NFAT5 role could be a safeguard mechanism to prevent exacerbated detrimental effects during chronic infections. Future experiments to try to answer these questions, analyzing the effects of NFAT5 deficiency in the LysM-Cre mouse model at late time points of the chronic infection could be of great interest. We could envision that the initial hyperproduction of IFN-I in NFAT5-deficient mice will exacerbate the suppressive effects on the adaptive immune system and cause diverse effects like T cell exhaustion, higher production of immunosuppressive molecules such as IL-10, PD-L1 or IDO1, tissular disruption of the spleen, or even higher viral spreading.

Besides the time and strength of IFN-I production during the viral infection, the effects of IFN-I on antiviral responses also depends on the cell type. Indeed, a recent report describes that stem cells are resistant to viral infection as a consequence of intrinsically expressing ISGs independently of IFN-I signaling (Wu et al., 2018). This antiviral capacity of stem cells is based on the intrinsic expression of a particular subset of ISGs that particularly include

IFITM expression. Since different *Ifitm* genes are NFAT5 targets in our work, it could be of particular interest to further explore whether NFAT5 influences *Ifitm* expression in stem cells to regulate their sensitivity to viral infection. The fact that IFITMs are part of the ISGs differentially regulated and expressed in homeostatic conditions in stem cells, could be linked to our observation that they are, contrary of majority of ISGs, positively regulated by NFAT5.

NFAT5 as the protector of the hematopoietic realm

The broad effects of IFN-I vary depending on the cell type that received IFN-I. For instance, it is known that IFN-I activates hematopoietic stem cells (HSC) to exit quiescence and enter the cell cycle (Essers et al., 2009). This is why as an in vivo model to address the relevance of NFAT5-controlled IFN-I production we explored HSC. As expected, mice deficient for NFAT5 in the whole hematopoietic system (Vav-Cre) or only in myeloid cells (LysM-Cre) showed higher polyIC-induced production of IFN-I compared to wild type littermates (Fig. 4a,b). Both mouse NFAT5-deficient model presented decreased HSC quiescence and increased cell cycle entry (Fig. 4d and Supplementary Fig. 4c). It is known that IFN-I signaling, besides activating HSC, also changes the proportions of different MPPs, being particularly clear the increase of MPP2 subset, which we defined here as LSK $CD150^{+}CD48^{+}$, and that is almost absent in homeostatic conditions (Pietras et al., 2015). Consequently, NFAT5 deficient mice show higher percentages of different precursors populations such as LSK $CD150^{+}CD48^{+}$ or LSK $CD150^{-}CD48^{+}$ in response to polyIC treatment (Fig. 4c). As expected, HSC from NFAT5 deficient mice show higher levels of

IFNAR signaling analyzed by three independent measurements: levels of phosphorylated STAT1, expression of the ISG protein Sca1 (Fig. 4e,f) and also the RNA expression of some ISGs (Supplementary Fig. 4d). Interestingly, we confirmed that the difference in polyIC-induced HSC activation between NFAT5-deficient mice and wild type mice was due to the higher production of IFN-I, as treatment of both mice with IFNα showed no differences (Supplementary Fig. 4e,f). Similarly, experiments in BMDM stimulated with IFNα also induced ISGs in an NFAT5-independent manner (Fig. 1e). In support of the IFN-I hyperproduction as the cause of HSC activation, we showed that NFAT5-deficient and wild type mice activated their HSCs similarly upon polyIC induction in IFNAR deficient mice, a finding that also excludes the possibility that other cytokines differentially produced between wild type and knockout mice could account for the observed HSC differences (Fig. 4g).

Since NFAT5-deficient mice produce higher IFN-I it could be beneficial to better prevent viral infections but also could cause deleterious enhanced HSC activation. Indeed, excessive IFN-I signaling or cell cycle activation on HSC causes impairment in their stemness function (Essers et al., 2009; Sato et al., 2009). Therefore, we asked whether IFN-I hyperproduction in NFAT5-deficient mice could lead to significant HSC exhaustion. Reaching our expectations, excessive activation of HSC in NFAT5-deficient mice impacted negatively in mice survival after challenge with the chemotherapeutic agent 5-Fluorouracil (Fig. 4h). This result strongly supports the notion that NFAT5-controlled production of

IFN-I is a relevant contributor to protect HSC stemness and functionality.

NFAT5 beyond IFN-I: a window to the future

During the realization of this project, different hypotheses about possible functions of NFAT5 have arisen. Some of these hypotheses were based on the relationship we have established with the IFN-I pathway and other ideas, as they usually do, arose as a consequence of some unexpected results. We have clearly stated that NFAT5 is a new regulator of the IFN-I response, which inevitably links NFAT5 with the several downstream functions of IFN-I. For instance, the IFN-I pathway is an important regulator of early embryonic hematopoiesis development (Kim et al., 2016) and, therefore, NFAT5 might also play important roles regulating it. Data obtained during the realization of our experiments reveal, in adult homeostasis, that no differences between NFAT5 deficient (Vav-Cre) and wild type mice were observed in total bone marrow cellularity, percentage of bone marrow HSPCs and differentiated peripheral blood cell populations (Supplementary Fig. 4b and data not shown). However, these observations alone cannot rule out a role for NFAT5 since the embryonic hematopoietic development in this mouse model occurs in the presence of cells that are wild type for NFAT5 and, therefore, produce normal levels of IFN-I. With this said, an interesting scenario would be to analyze the hematopoiesis during embryonic development in a mother infected during pregnancy that is deficient for NFAT5 and therefore produces more IFN-I, which potentially will impact on the embryo.

NFAT5 also emerges as a candidate factor to design improved therapies for different immune disorders. Since there is an intimate link between IFN-I oversignaling and the development of autoinflammatory and autoimmune disorders, is of potential interest to discuss the protective role of NFAT5 in these scenarios. Despite the lack of a direct prove that IFN-I hyperproduction is the cause of several immune disorders such as SLE, AGS or reumathoid arthritis, it is clear that increase IFN-I response correlates with the severity of the symptoms (Rodero and Crow, 2016). Outstanding evidences are the improvement in arthritis prognosis when blocking IFNAR or the accumulation of pDCs in the skin lesions of SLE or psoriatic patients that produce high amounts of IFN-I due to the presence of autoantibodies that recognize both self DNA and RNA (Farkas et al., 2001; Miller et al., 2008; Sozzani et al., 2010; Wang et al., 2011b). Both examples highlight the potential benefits that would cause the targeted upregulation of NFAT5 in the skin area or join affected. Indeed, NFAT5 have been already associated to the prognosis of arthritis. However, probably due to the multifactorial components of this disease, NFAT5 expression could correlate with arthritis chronicity as it is reported to promote macrophage survival and synovial proliferation (Choi et al., 2017). Examples where could be worth to study the potential benefits of NFAT5 are the monogenic autoinflammatory disease called interferonopathies. One the one hand, we could consider NFAT5 expression levels as a possible marker for diagnosis. On the other hand, as we reviewed in the Introduction, the majority of these rare diseases are caused by loss-of-function mutations of endonucleases or exonucleases

leading to the accumulation of nucleic acids, or gain-of-function mutations in some component of the RLR-MAVS or cGAS-STING pathway (Crowl et al., 2017). Therefore, a therapy based on overexpressing NFAT5 could be effective for a great majority of this heterogeneous set of rare diseases. This would not avoid the original problem of the pathway, but it could diminish the pathogenic effects and improve the prognosis of the patients with these diseases that otherwise remain without an effective therapy.

Finally, this project has also pointed to other questions about the mechanism of action of NFAT5. As a result of this project, we now know that NFAT5 is a transcription factor that positively or negatively regulates the expression of several genes in response to PRR activation. Little is known about how NFAT5 becomes active in response to PRRs. In this sense, it was reported that, upon TLR4 activation, the IKKβ-NF-κB axis is necessary to upregulate NFAT5 mRNA and protein levels and also that NF-κB binds to the promoter of NFAT5 and regulates its expression (Buxadé et al., 2012). However, our results based on the activation of diverse PRRs raises the question of whether the expression and activity of NFAT5 could be regulated by other signaling factors that determine its specificity upon different stimuli and in different cell types. For example, it could be reasonable to wonder whether NFAT5 is also activated by IRF3 as IRFs binding sites are present in the promoter region of NFAT5, near to the previously reported NF-κB site. Also, results from our ChIP-seq analysis reveal no differences in the amount of significant NFAT5 binding peaks between non-stimulated and polyIC-stimulated macrophages. Thus, in the

absence of stimulus, and without consequent IKKβ-NF-κB activation, NFAT5 is still translated and able to bind DNA at the same extend as in response to PRRs, but globally in different regions. This suggests that the activity of NFAT5 is not necessarily dependent on pathogen/TLR stimuli and it can act as a transcriptional regulator in homeostasis. In fact, in the supplementary article included in this Thesis, we describe that NFAT5 regulates the basal expression of the *Ciita* gene in macrophages. This work is entitled "Macrophage-specific MHCII expression is regulated by a remote *Ciita* enhancer controlled by NFAT5", and it provides some evidences that help to understand the mechanism of action of NFAT5. Specifically, we found that (1) NFAT5 is able to regulate gene expression in homeostasis. (2) NFAT5 can regulate gene expression through distal regulatory elements. (3) The role of NFAT5 in regulating gene expression can be cell-specific, as we demonstrate that it regulates *Ciita* expression specifically in macrophages but not in dendritic cells.

NFAT5 emerges after this Thesis as a complex master immune regulator. Initially NFAT5 was described as a positive transcriptional regulator in response to osmotic stress and more recently in supporting proinflammatory cytokines, and we have seen here that NFAT5 also acts as a negative transcriptional regulator limiting the IFN-I response. The combination between our results and the discussion provided here highlights the complexity of the NFAT5 functions. The lack of NFAT5 can have either beneficial effects with potential detrimental consequences (antiviral response) or detrimental effects with potential benefits (HSC

activation). The limits of NFAT5 actions are still not defined and new projects could arise from the findings presented here. This work uncovers new exciting research avenues in the connection between NFAT5 and the IFN-I pathway that range from the control of tumor progression to the understanding of the pathophysiology of autoimmune diseases. There and back again for NFAT5.

CONCLUSIONS

1- NFAT5 limits production of IFN-I in different cell types such as macrophages, conventional dendritic cells, plasmacytoid dendritic cells and mouse embryonic fibroblasts.

2- NFAT5 inhibits expression of IFNβ and numerous interferon stimulated genes (ISGs) upon diverse stimuli such as polyIC or VSV, CMV and LCMV infection, which indicates that NFAT5 could act downstream of several PRRs.

3- NFAT5 does not influence the activity of signaling components upstream of IFN-I genes, but it acts to limit IFN-I expression and IFNAR responses.

4- In vivo, NFAT5 limits systemic production of IFN-I in response to polyIC treatment or LCMV infection.

5- Higher systemic IFN-I production in NFAT5-deficient mice has different physiological consequences. It improves antiviral capacity upon LCMV infection, but also causes enhanced HSC activation to exit quiescence and make them susceptible to exhaustion.

6- *IFNB1* promoter contains an NFAT5 consensus site that constitutes a negative regulatory domain (NRD), which we propose to call NRDIII.

7- NFAT5 binds NRDIII in response to PRR activation to inhibit the expression of *IFNB1*.

8- The consensus site of NFAT5 partially overlaps with the consensus site of IRF3 in the positive regulatory domain III (PRDIII) of *IFNB1* promoter.

9- NFAT5 constitutes a previously unidentified regulatory factor of the *IFNB1* enhanceosome. NFAT5 competes and limits IRF3 binding to repress *IFNB1* promoter activity and prevent excessive activation of the IFN-I pathway.

10- Binding of IRF3 to PRDIII is the main contributor to the activation of *IFNB1* expression in response to TLR3 activation. Although NF-κB binding is necessary for maximal promoter activity, enhanced recruitment of IRF3 that occurs in the absence of NFAT5 binding, makes NF-κB dispensable to reach wild type promoter activity.

11- The overlap between NFAT5 and IRF3 consensus sites in IFN beta gene promoter is conserved through evolution and is also present in most IFN alpha gene promoters.

SUPPLEMENTARY ARTICLE

Macrophage-specific MHCII expression is regulated by a remote *Ciita* enhancer controlled by NFAT5

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LIST OF ABBREVIATIONS

CELL TYPES

BM: Bone Marrow..... 84
BMDC: Bone Marrow-derived Dendritic Cell..... 150
BMDM: Bone Marrow-Derived Macrophage..... 150
cDC: conventional Dendritic Cell..... 61
CLP: Common Lymphoid Progenitor..... 40
CMP: Common Myeloid Progenitor..... 40
DC: Dendritic Cell..... 46
EMP: ErythroMyeloid Progenitors..... 36
GMP: Granulocyte-Macrophage Progenitor..... 40
HSC: Hematopoietic Stem Cell..... 34
HSPC: Hematopoietic Stem and Progenitor cell..... 41
MEP: Megakaryocyte-Erythrocyte Progenitor..... 40
MPP: Multipotent Progenitor..... 38
MSC: Mesenchymal Stromal Cell..... 42
LT-HSC: Long-Term HSC..... 39
pDC: plasmacytoid Dendritic Cell..... 61
ST-HSC: Short-Term HSC..... 38

VIRUSES and PRR AGONISTS

CMV: Cytomegalovirus..... 78
LCMV: Lymphocytic ChorioMeningitis Virus..... 75
LPS: Lipopolysaccharide (TLR4 agonist)..... 132
PAM3CSK: Triacylated lipopeptide (TLR2 agonist)..... 132
polyIC: Polyinosinic:polycytidylic acid (TLR3 agonist)..... 132
VV: Vaccinia Virus..... 79
VSV: Vesicular Stomatits Virus..... 61

GENERAL CONCEPTS

AGM: Aorta-Gonad-Mesonephros..... 35
AGS: Aicardi-Goutieres Syndrome..... 77
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats..... 33
DAMP: Damage-Associated Molecular Patterns..... 47
DBD: DNA-Binding Domain..... 93
DD: Dimerization Domain..... 93
DDR: DNA Damage Response..... 83

EHT: Endothelial-to-Hematopoietic Transition..... 36
ESR: Environmental Stress Response..... 86
GAS: IFNγ-Activated Site..... 72
HR: Homologous Recombination..... 86
IRE: Interferon gene Regulatory Element..... 65
Lin: Lineage specific markers (CD3, B220, TER119, CD11b, GR1). 38
NHEJ: NonHomologous End Joining..... 86
NRD: Negative Regulatory Domain..... 69
PAMP: Pathogen-Associated Molecular Patterns..... 47
PRD: Positive Regulatory Domain..... 65
RHR: Rel Homology Region..... 93
ROS: Reactive Oxygen Species..... 83
SMS: Singleton-Merten Syndrome..... 77
SLE: Systemic Lupus Erythematosus..... 77
TRC: Transcription Replication Conflict..... 86
TSS: Transcription Start Site..... 65
VRE: Viral-Responsive Element..... 43
5-FU: 5-Fluorouracil..... 62

PRINCIPAL ACTORS

IFN: Interferon..... 34
IFN-I: Interferon type I..... 54
IFNAR: Interferon type I Receptor..... 70
IRF: Interferon Regulatory Factor..... 48
ISG: IFN-I-Stimulated Gene..... 54
NFAT5: Nuclear Factor of Activated T cells..... 91
NF-κB: Nuclear Factor kappa B..... 48
PRR: Pattern Recognition Receptor..... 34
RLR: RIG-I-like Receptor..... 51
STAT: Signal Transducer and Activator of Transcription..... 71
TLR: Toll-like Receptor..... 50

MOLECULES

ATF3: AMP-dependent Transcription Factor..... 53
CARD: Caspase-recruitment and activation domains..... 51
cGAS: cyclic GMP-AMP Synthase..... 51
HDAC1: Histone deacetylase 1..... 53