MiR-146a: a key microRNA involved in regulating mast cell survival and T lymphocyte differentiation.

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Only one thing makes
a dream impossible:
the fear of failure.
(Paulo Coelho)

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TABLE OF CONTENTS

Summary

Mast cells are long-lived, tissue-resident cells of the innate immune system. Since the identification of mechanisms that regulate mast cell proliferation, survival and overall homeostasis in the tissues may have important implications for the treatment of mast cell-related diseases such as asthma, allergy and mastocytosis, we investigated novel molecular mechanisms at the basis of mast cell biology, and in particular the role of two activation-induced miRNAs, miR-221 and miR-146a. We found that miR-221 has important roles in regulating multiple processes in differentiated primary mast cells, such as degranulation, adhesion, migration and cytokine production. Since miR-221 is expressed at basal level in mast cells but it is also inducible upon stimulation, we proposed a model in which miR-221 has a dual role in these cells: at resting state, it contributes to the regulation of the cell cycle and cytoskeleton, an effect probably common also to other cell types that express basal levels of this miRNA. However, in response to stimulation through IgE-antigen complexes, miR-221 effects are mast cell-specific and activation-dependent, contributing to the regulation of degranulation, cytokine production and cell adherence (Chapter 1).

Mice that lack the p50 subunit of NF-kB (p50KO) are unable to mount airway eosinophilic inflammation due to the inability to produce IL-4, IL-5 and IL-13, which play distinct roles in asthma pathogenesis, and to a defect in the polarization of Th2 lymphocytes. Since mast cells are master effector cells in allergic responses, we evaluated whether the asthma-resistant phenotype observed in p50KO mice could be partially due to a defect in mast cell development or function. While our data indicate that p50KO mast cells may only marginally contribute to the airway inflammation defect of p50KO mice through a slight impairment in cytokine production, p50KO mast cells showed a marked increase in their ability to survive in response to withdrawal of essential cytokines, which likely correlated with a strong increase in the percentage of mast cells that was observed in the tissues of p50KO animals. Such improved survival of mast cells lacking p50 was due to altered expression of several molecules involved in regulating cell survival and cell death, such as Bcl2, A1 and BAX. Importantly, we also found that miR-146a, a miRNA known to regulate NF-kB signalling, was not expressed in IgE- or LPS-stimulated p50KO mast cells, and that in the context of mast cell survival, miR-146a acted as a pro-apoptotic factor, identifying therefore a new molecular network that regulates mast cell survival in response to a variety of signals (Chapter 2).

Previous work from our lab also pointed toward a role for miR-146a in the differentiation and/or activation of murine CD4 T lymphocytes. We therefore continued investigating a possible role for this miRNA not only in regulating mast cell survival, but also in the

differentiation and function of T cells. We found that miR-146a is expressed at high levels in the effector and effector-memory T cell compartment in both mouse and human, and we provide evidences that miR-146a may regulate T cell expansion upon activation and possibly also memory formation (Chapter 3). A review on the role of miR-146a in immunity and disease is provided in Chapter 4.

Overall our work demonstrates that miR-221 and miR-146a play a key role in regulating mast cell activation, function and survival, and that miR-146a also contributes to the extent of T lymphocyte activation. Finally, we provide novel insights on the role of miRNAs in regulating various functions of mast cells and T lymphocytes in the immune response, contributing to the groundwork for a further understanding of the molecular mechanisms that may lead to immune-related diseases such as asthma, allergy, altered inflammatory responses, and mastocytosis.

Mast Cells

Introduction

Paul Ehrlich identified mast cells (MCs) in 1878 (reviewed in Ref.1). These are cells of the immune system that are present in most tissues of the body and especially near the boundaries between the outside world and the internal milieu, such as the mucosa of the lungs, digestive tract, skin, mouth, and nose[2]. For many years, it was believed that MCs were the same cellular type as basophils, present in the connecting tissues rather than in the blood. It was then discovered that MCs exhibit specific markers and respond to factors differently from basophils[2]. Mature MCs have a limited proliferative ability, which is increased during infection. These cells are round with a diameter of 20-30 μm. MCs are present in the connecting tissue and stretch to concentrate themselves particularly along the blood vessels; they are also abundant in the peritoneum. MCs can be divided in two types, mucosal MCs (MC_M) and connective tissue-type (MC_{CT}).

-> **MC_M** are found in mucosal epithelial surfaces of the intestine and respiratory tract. They are typically associated with Th2 responses. The phenotype of these cells is dynamic and defined by inflammatory perturbations at a tissue site, allowing them to play roles in the induction/amplification phase and the resolution/healing phase of the response[3]. The development of MC_M is dependent on T-cell derived factors, such as IL-3 and IL-4.

-> **MC_{CT}** are found in the sub-mucosal connective tissues of the intestine, and throughout the body in association with other connective tissues. Their maturation is T cell-independent, because normal numbers are seen in lymphocyte-deficient mice[4]. Compared to MC_M, their numbers do not increase as significantly in acute reactions in the lung following Th2-mediated inflammation or atopic diseases[3].

MCs take part in allergic reactions and anaphylactic hypersensitivities (anaphylactic shock). When MCs become activated they quickly release the content of their cytoplasmic granules. Such granules (diameter of 0.3-0.8μm) contain pre-stored mediators including proteases, histamine and heparin. The most abundant proteins stored in MC secretory granules are endopeptidases, which are released by exocytosis. The major enzymes are tryptic and chymotryptic peptidases called tryptases and chymases, respectively. The types, amounts and properties of these serine peptidases vary by MC subtype, tissue, and mammal of origin. Because MCs can express certain of the peptidases at a very high level, immunohistochemistry and immunoassay approaches using antibodies directed against these enzymes are useful experimentally as well as clinically in assessing MC numbers, locations,

activation, and disease-association. Tryptases and chymases also can oppose inflammation by inactivating allergens and neuropeptides causing inflammation and bronchoconstriction. Thus, like MCs themselves, MC serine peptidases play multiple roles in host defence.

-> **Tryptase** is the most abundant protease in human MCs, comprising up to 20% of the cell protein content[5]. It has a variety of effects including inducing chemotactic activity in eosinophils[6], inactivation of fibrinogen, activation of tissue matrix metalloproteinase, stimulation of fibroblast proliferation.

-> **Chymases**, like serine endopeptidase and cathepsin-G, are synthesized by MCs, neutrophils and eosinophils. It has a variety of effects including the activation of angiotensin I, which is a vasoconstriction peptide[7].

Other pre-formed mediators of the cytoplasmic granules include histamine and heparin. Histamine is a vaso-active mediator that increases vascular permeability, while heparin is a glycosaminoglycan sulphate, with a strong negative electric charge that has powerful anticoagulation properties.

Apart from the abovementioned pre-formed mediators stored in the cytoplasmic granules, MCs can produce a broad panel of *de-novo* synthesized mediators, including cytokines (e.g IL-6, IL-13, IL-4, IL-5, TGFβ) and chemokines (e.g MIP-1α, MIP-1β, RANTES), as well as nitrogen oxide and leukotrienes.

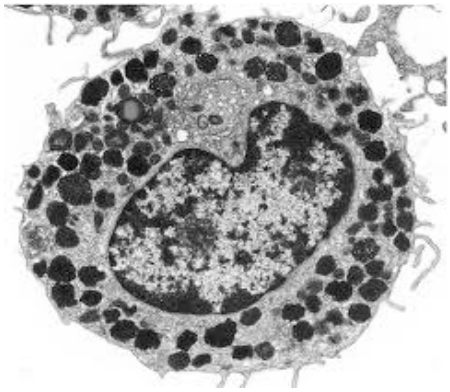

Figure 1: An image of a MC. This image appeared on the cover of the Oct 2007 issue of Laboratory Investigation.

Mast Cell Differentiation, Activation and Migration

The progenitors of the MCs are found in the bone marrow and migrate through the blood stream in an immature state to reach the target tissue[8]. Two models of murine MCs differentiation have been described. The first was published in 2005 by Chen *et al.* in which, based on the expression of a number of surface markers, the MC progenitors (MCPs) were described as Lin^- Kit^+ $Sca\text{-}1^-$ $Ly6c^-$ $Fc\varepsilon RI\alpha^-$ $CD27^-$ $\beta7^+$ - $T1/ST2^+$ [6]. These cells gave rise only to MCs in culture and reconstituted the MC compartment when transferred into *kit* mutant MC-deficient mice[6]. A second model was described (also in 2005) by Arinobu *et al.*[9], in which they found that Lin^- Kit^+ $Fc\gamma RII/III^+$ $\beta7^+$ cells were bipotent progenitors because they could give rise to both basophils and MCs (BMCPs). They concluded that BMCPs could be generated mainly from bone marrow granulocyte/macrophage progenitors (GMPs). Finally, somewhat reconciling all these different models, Franco *et al* in 2010 described two subsets of myeloid progenitors: $Sca\text{-}1^{lo}$ common myeloid progenitors (SL-CMPs, $Sca\text{-}1^{lo}$ Lin^- Kit^+ $CD27^+$ $Flk\text{-}2^-$), and $Sca\text{-}1^{lo}$ granulocyte/macrophage progenitors (SL-GMPs, $Sca\text{-}1^{lo}$ Lin^- Kit^+ $CD27^+$ $Flk\text{-}2^+$ $CD150^{-/lo}$) [10]. They found that MCP potential was present in the SL-CMP fraction but not in the more differentiated SL-GMP population. In conclusion, during hematopoiesis the MC lineage appears to be specified earlier than and independently from other granulocytes[10].

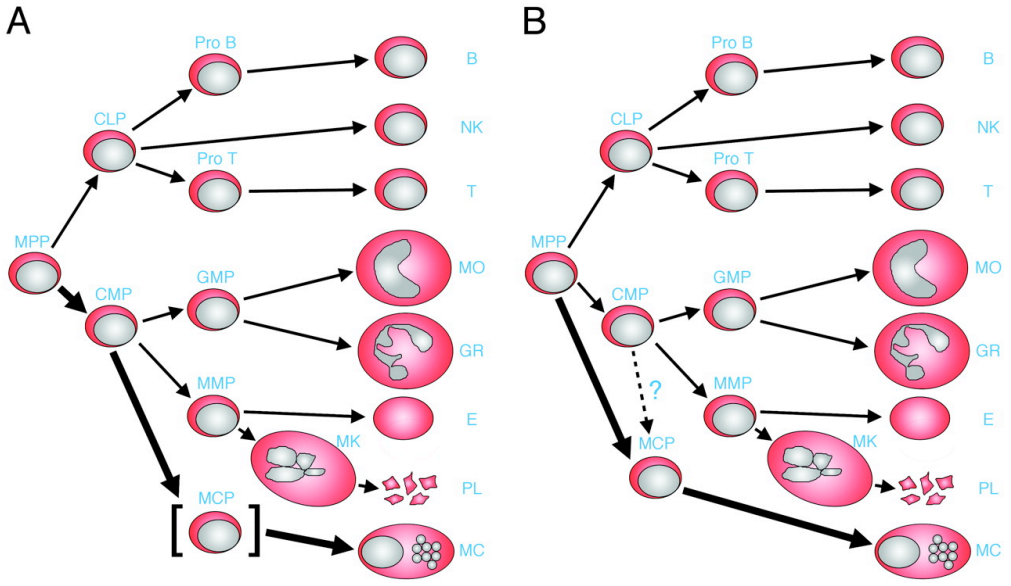

Figure 2: MC hematopoiesis and the change of lineage models. (A) In the old view, the presence of MCPs was not clear, but mast cells were considered to be the progeny of CMPs. (B) In the new view proposed by Chen *et al.*[8] the presence of MCPs is clear. The thick line shows the main differentiation pathway from MPP to mast cell. CLP (common lymphoid progenitor); CMP (common myeloid progenitor); GMP (granulocyte macrophage progenitor); MCP (mast cell-committed progenitor); MEP (megakaryocyte erythrocyte progenitor) [11-12].

Upon final differentiation in the tissues where they will ultimately reside, MCs can

be activated through a variety of receptors present on their surface. Some are activating receptors and others play an inhibitory role. Activating receptors include: the high affinity IgE receptor (FcεRI)[13/14], Kit (stem cell factor receptor)[15-18], the IL-3 receptor[19-21], IgG receptors, complement receptors and Toll-like receptors (TLRs).

During MC activation, such as in allergic reactions, MCs remain inactive until an antigen (or an allergen) is recognized by specific IgE antibodies already associated to the FcεRI on the MC surface, leading to MC degranulation, production of cytokines and other mediators, and to a cascade of responses which may eventually lead to eradication of the infections agent or to allergic responses. Allergic hypersensitivity is associated with both IgE and type-2 (Th2) T cell responses to environmental allergens. As shown in Figure 3, in allergic individuals initiation of type-2 responses to an allergen leads to the production of cytokines, such as IL-13 and IL-4, which favour allergen-specific IgE production from B-lymphocytes. Once such MC-bound allergen-specific antibodies are cross-linked by an allergen, the release of MC mediators determines the subsequent physiological responses to the allergen.

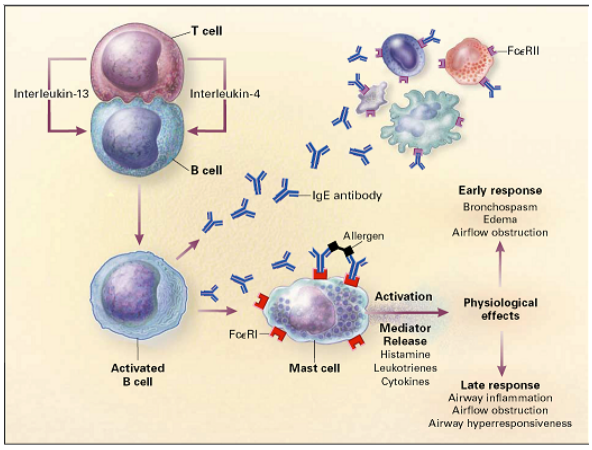

Figure 3: MCs activation and related activity. Upon stimulation via the allergen-induced IgεR, MCs were activated and released mediators that could lead to a variety of physiological effects.[13]

Migration is essential at several stages of the MC differentiation and activation, including: i) progenitor movement towards the sinusoids in the bone marrow;

ii) migration through the sinusoidal endothelium;

iii) recruitment via venules into tissues;

iv) migration during maturation towards their final location within the tissue.

Mechanisms operate under basal conditions to maintain MC numbers in the target tissues. A local MC hyperplasia is likely to involve increased progenitor recruitment in addition to

increased local MC proliferation[22]. MCs are thought to use the same basic migration mechanisms as other granule-containing inflammatory cells, in which chemo-attractant molecules generated in inflamed tissues recruit granulocytes by acting on surface receptors[23].

Interactions with other immune cell types

Not only MCs are key effector cells in allergic and asthmatic responses, but also they are capable of modulating other innate and adaptive immune responses[24-26]. They assist the defence against pathogens by releasing the contents of their granules. These molecules are key for the early defence and recruitment of other effector cells to sites of infection[27-29]. For example, the very broad panel of cytokines and chemokines produced by MCs upon activation can influence the differentiation, recruitment and activation of other innate immune cells as well as of T lymphocytes. Moreover, the release of histamine can have multiple effects on other cells of the immune system, such as activation of IFN-γ -producing Th1 lymphocytes via H1 receptors, or suppression of immune responses through H2 receptors[30].

A role for MCs as antigen-presenting cells was hypothesized based on the fact that expression of MHC class II molecules can be induced on these cells upon activation with LPS, INF-γ and TNF-α[31], however, the *in vivo* role of MHCII expression on these cells remains debated.

In the past few years a number of studies also focused on the interaction between MCs and others cells of the immune system[32/33]. It was found (Pucillo's group 2008) that T regulatory (Treg) cells directly inhibited the FcεRI-dependent MC degranulation through cell-cell contact involving interactions between OX40 on Treg cells and OX40L on MCs. Moreover, it was shown that in the presence of activated MCs, MC-derived IL-6 and Th2 cytokines (IL-4, IL-13) skewed Tregs and effector T cell into IL-17–producing T cells (Th17)[32] .

Mast cell-related pathologies

The release of mediators from MC granules is a common pathological event during allergic responses. Consequently, MC stabilization has become the main treatment for patients with allergic symptoms. Activated MCs contribute to allergic and vascular diseases, but also to rheumatoid arthritis, cancer, atherosclerotic lesions[33-37], and probably many other

untested diseases[38]. Moreover, a dysregulated MC proliferation and activation leads also to mastocytosis[44].

Specifically:

- Allergy: it is a hypersensitivity disorder of the immune system[39/40]. Allergic reactions occur when a person's immune system reacts to normally harmless substances in the environment. In some people, severe allergies to environmental, dietary allergens or to medication may result in life-threatening reactions called anaphylaxis[41]. Food allergies and reactions to the venom of stinging insects, such as wasps and bees, are often associated with these severe reactions. Treatment for allergies includes anti-histamines that specifically prevent allergic reactions, steroids that modify the immune system in general, and medications such as decongestants that reduce the symptoms. Immunotherapy uses injected allergens to desensitize the body's response.

- Asthma: it is the common chronic inflammatory disease of the airways characterized by variable and recurring symptoms, reversible airflow obstruction, and bronchospasm. Asthma is becoming more common each year, especially in children.[42]

- Mastocytosis: it is a tumor characterized by the abnormal proliferation and accumulation of aberrant MCs in tissues. Its clinical course is variable ranging from an asymptomatic state for years to a highly aggressive and devastating one. When mastocytosis develops into an aggressive form, its course is very rapid and often fatal[43].

->Genetic of mastocytosis: A point mutation in the tyrosine kinase receptor gene *KIT* is the most common genetic abnormality found in mastocytosis patients. This mutation results in a substitution of valine for aspartate at codon 816 of exon 17[44].

->Therapy for mastocytosis: patients with mastocytosis are treated symptomatically and no curative therapies for the aggressive disease types are currently available. Some success in reducing the mast cells burden has been reported with alpha-interferon[45/46].

B and T lymphocytes

Introduction

Adaptive or specific immunity recognizes non-self and can eliminate foreign antigens. Unlike innate immunity, adaptive responses display antigen specificity, a big diversity and immunological memory. Moreover, self/ non–self-discrimination is regulated by more sophisticated mechanisms than is the case of innate immunity. The adaptive immune system is capable of distinguishing subtle differences and associating them with a cellular context. It can recognize billions of antigen variants and exhibits a memory for the encountered foreign antigens. An effective adaptive immune response requires the presentation of an antigen by a professional antigen-presenting cell (APC) to a specific lymphocyte. There are two major populations of lymphocytes:

-> B lymphocytes

-> T lymphocytes

B lymphocytes

B lymphocytes mature within the bone marrow[47]. When B cells leave the bone marrow, they already express a unique antigen-binding receptor at the surface. This B cell receptor (BCR) is constituted by a membrane bound immunoglobulin (Ig) molecule. When this molecule encounters a matching antigen, binding induces the proliferation of the B cell bearing it[48]. Its progeny can then differentiate into memory B cells and effector plasma cells. B cell development occurs through several stages, each stage representing a change in the genome content at the antibody loci.

An antibody is composed of two identical light (L) and two identical heavy (H) chains. In each chain the variable (V) region is unique and is specific for an antigen, while the constant (C) region specifies the physiological effect of an antibody once it recognizes its antigen[49]. The C region can change in a process called class switching: this leads to the generation of an antibody that retains the same specificity (specified by the variable domains), but different constant domains. The H chain is the larger of the two types of chains that comprise a normal immunoglobulin or antibody molecule. The H chain portion of an antibody contains 2 regions; the Fab (Antigen-binding portion) and Fc (Constant region; confers biological activity such as phagocytosis of microorganisms) (Figure 4).

For example, naïve (antigen-inexperienced) mature B cells produce both IgM and IgD, which are the first two heavy chain segments in the immunoglobulin locus. After encounter with an antigen, activated B cells undergo antibody class switching to produce

IgG, IgA or IgE antibodies. IgE antibodies are the ones that are mainly involved in allergy and are bound to the FcεRI on the MCs surface.

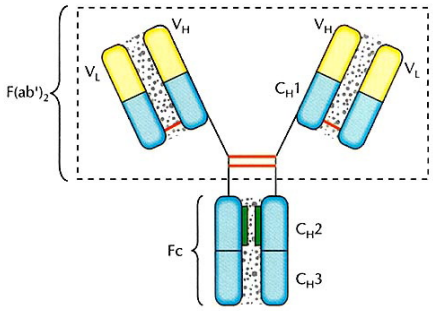

Figure 4: Schematic representation of an immunoglobulin. V_L= Variable domain on the light chain, V_H= Variable domain on the heavy chain , C_H= Constant domain in the heavy chain, Fab= Fragment Antigen Binding, Fc= fragment crystallizable, position of the carbohydrate =green and interchain disulfide bonds =red. Poljsk R, "Production and structure of diapodies", Structure 1994, Vol 2, Issue 12, 1121-1123

Upon encounter with an antigen, B cells differentiate to plasma cells, which are short-lived and dedicated to secrete very high amounts of antibodies, or to memory B cells, which are long-lived and provide faster responses following a second exposure to the same antigen.

T lymphocytes

T cells derive from progenitors in the bone marrow, but unlike B cells, they terminate their maturation in the thymus. Mature T cells express a specific T cell receptor (TCR) at their cell surface, a receptor responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules.

T cells can only recognize an antigen in the context of MHC molecules, meaning that the antigen must be 'presented' to the T cell from the surface of another cell. There are two types of MHC molecules:

-> Class I = present on nearly all nucleated cells

-> Class II = only expressed on professional APCs such as dendritic cells, activated macrophages and B cells.

MHC class I molecules present degradation products derived from intracellular (endogenous) proteins in the cytosol. For example, protein products deriving from an intracellular pathogen such as a virus will be presented by the infected cell on its surface in the context of MHCI molecules. These will be recognized by specific cytotoxic T cells that will kill the infected cell leading to eradication of the infection.

MHC class II molecules present fragments derived from extracellular (exogenous) proteins that are located in an extracellular compartment. These are expressed by professional APCs, which will be recognized by helper T cells (Th) that will be activated and than can help to trigger an appropriate immune response, which may include localized inflammation and swelling due to recruitment of phagocytes, or may lead to a full-force antibody immune response due to activation of B cells.

T lymphocytes fall into different groups based on their response to pathogens:

-> **T cytotoxic cells** (CTL) kill cells that are infected with viruses or other intracellular pathogens, or are otherwise damaged or dysfunctional (such as tumor cells). They produce toxic granules containing enzymes that induce the death of pathogen-infected cells. CTL express TCRs that can recognize a specific antigenic peptide bound to MHCI molecule, which in turn is recognized by the surface protein CD8, that is a trans-membrane glycoprotein that serves as a co-receptor for the TCR.

-> **T helper cells** (Th) have no direct cytotoxic or phagocytic activity, but rather, they activate and direct other immune cells through a combination of cell-to-cell interactions (such as CD40/CD40L) and through cytokine production. They are characterized by the expression of the CD4 receptor that interacts directly with MHC class II molecules on the surface of the APC.

$CD4^+$ T cells priming

Dendritic cells (DCs) can be considered the first actors to enter the game in the process of T cell activation as they capture peptide antigens and present them in complex with MHC for T cell recognition. DCs act as real APCs, as they are able to deliver all signals required to fully activate a T cell.

During an immune response DCs travels from the infection site to the lymph nodes, where they present antigens that are bound to MHCII. The antigen recognition by the TCR is known as **Signal 1** for T cell activation. However, to become fully activated, a T cell needs to receive other co-stimulatory signals, known as **Signal 2**. This is a protective measure to ensure that a T cell is responding to a foreign antigen. The second signal involves co-stimulatory molecules such as, for example CD28 that is expressed by T cells, which interacts with CD80 and CD86 on the membrane of DCs. Based on the cytokine produced by DCs (**Signal 3**), naïve T cells can differentiate to different phenotypes.

For example, IL-12 favours differentiation of naive CD4 T cells towards a Th1 phenotype, characterized by the ability to produce high levels of IFN-γ.

Upon encounter with an antigen in the presence of all co-stimulating signals, a naïve T cell can differentiate into an effector phenotype, characterized by high expression of effector cytokines (such as IFN- γ, IL-2 and IL-4). This population will normally contract and return to basal levels upon eradication of the pathogen, however some antigen-experienced T cells will remain and form the memory compartment. In the case of successive infection, memory cells will be able to quickly induce a more rapid and robust secondary immune reaction.

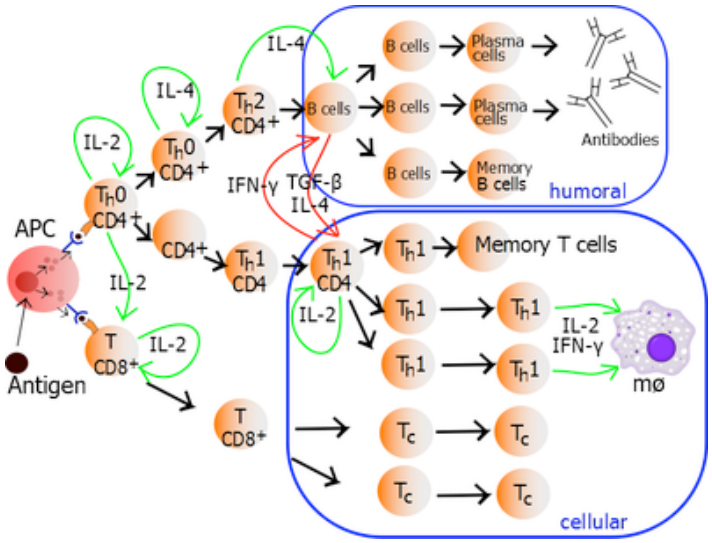

Figure 5: Priming model for Th1/Th2 cells. Rang, H. P. (2003). Pharmacology. Edinburgh: Churchill Livingstone. ISBN 0-443-07145-4. Page 223

Memory T cells (T_m)

Memory cells are characterized by the ability to quickly expand and respond upon secondary exposure to their cognate antigen. Two phenotypic subpopulations of T_m cells have been identified based on the expression of different surface markers and predominant immunological characteristics such as their tissue homing properties: central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells)[50].

T_{CM} => they are characterized by the expression of the following combination of surface markers: high expression of CD62L, CCR7, CD44, CD127, and low expression of CD45RA and CD25. Due to their high expression of CD62L and CCR7 (see below), they have been shown to migrate efficiently to peripheral lymph nodes.

T_{EM} => compared to T_{CM}, they express lower levels of CD62L and CCR7, which make these cells poorly able to migrate to the lymph nodes, and are rather preferentially found in other sites, such as the liver and lungs, where they have immediate effector functions upon encountering antigen.

Specifically:

CD62L (L-selectin) => CD62L acts as a "homing receptor" for leukocytes to enter secondary lymphoid tissues via high endothelial venules. This receptor is commonly found on the cell surfaces of naive T-lymphocytes, which have not yet encountered their specific antigen. Antigen-experienced T_{CM}, also express CD62L to localize in secondary lymphoid organs.

CD44 => This protein participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing[51]. It is commonly considered a marker of T cell activation.

CD45RA => The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. This gene is specifically expressed in hematopoietic cells and it has been shown to be an essential regulator of T- and B-cell antigen receptor signalling. Various isoforms of CD45 exist: CD45RA, CD45RB, CD45RC, CD45RAB, CD45RAC, CD45RBC, CD45R0, CD45R (ABC), each characterized by expression on specific T cell subsets. Naive T cells express the large CD45 isoform, CD45RA, while the shortest isoform (lacking RA, RB and Rc exons), CD45R0, characterizes memory T cells.

CCR7 => It has been shown to control the migration of naïve and memory T cells to secondary lymphoid organs, such as lymph nodes, as well as to stimulate dendritic cell maturation[52]. CCR7 deficiency results in massive accumulation of $CD4^+$ and $CD8^+$ T cells in the peritoneal and pleural cavities[53].

CD4+ lymphocytes differentiation

CD4 T helper (Th) cells can be divided into different subsets characterized as follows by the cytokines they produced and by the expression of 'master' transcription factor essential for their differentiation[54]:

Th1	=>	- signature cytokine	:	IFN- γ
		- master transcription factor	:	T-bet
		- function	:	promote protective immunity against

				intracellular pathogens. By secreting IFN-γ, they induce activation of macrophages and upregulation of iCOS leading to the killing of pathogens.
Th2	=>	- signature cytokines	:	IL-4, IL-5, IL-13
		- master transcription factor	:	GATA3
		- function	:	promote humoral immune responses and host defense against extracellular parasites. However, they can also potentiate allergic responses and asthma. Their development and maintenance is regulated by IL-4, IL-25 and IL-33
Th9	=>	- cytokines production	:	IL-9, IL-10
		- transcription factor	:	PU.I
		- function	:	as opposed to the others, this is not yet considered a separate linage. These cells are involved in host defense against extracellular parasites, primarily nematodes. Despite their production of anti-inflammatory IL-10, they promote allergic inflammation. Their role in other inflammatory diseases remains still unclear.
Th17	=>	- signature cytokines	:	IL-17, IL-22
		- master transcription factor	:	RORγt
		- function	:	promote protective immunity against extracellular bacteria and fungi. Also promote autoimmune and inflammatory diseases. Th17 cells are generated in the presence of TGF-β and IL-6 and/or IL-21 and are maintained by IL-23 and IL-1β.

Th22	=>	- signature cytokine	:	IL-22
		- master transcription factor	:	AHR
		- function	:	Identified in inflammatory skin diseases. Their role in host defense remains unclear as this subset only recently been characterized.
Tfh	=>	- cytokines production	:	IL-21
		- transcription factor	:	BCL6
		- function	:	These cells are involved in the promotion of germinal center responses and provide help for B cell class switching.
Tregs	=>	- signature cytokine	:	IL-10
		- master transcription factor	:	Foxp3
		- function	:	are crucial in the maintenance of immunological tolerance, as their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection. They are characterized by the expression of high levels of CD25 on the surface.

While the subsets shown above are important to define the different classes of Th cells with their different functions, it is important to also underlie that these cells are not only heterogeneous in their phenotype, but they also show considerable plasticity. In other words, even within 'pure' populations, some cells retain the ability to switch to another subset or to a mixed phenotype[55/56]. The cytokines produced by the different subsets of T cells influence isotype switching to different classes of antibodies. Most relevant to MCs and MC-related diseases, Th2-derived IL-4 induces B cells to produce IgE in both mouse (table 1) and human (table 2)

Table 1: Mouse

T cells	Cytokines	Immunoglobulin classes					
		IgG1	IgG2a	IgG2b	IgG3	IgA	IgE
Th2	IL-4	↑	↓	↓	↓	↓	↑
	IL-5					↑	
Th1	IFNγ	↓	↑	↓	↓	↓	↓
Treg	TGFβ			↑	↓	↑	

Table 2: Human

T cells	Cytokines	Immunoglobulin classes					
		IgG1	IgG2	IgG3	IgG4	IgA	IgE
Th2	IL-4				↑		↑
	IL-5					↑	
Th1	IFNγ						
Treg	TGFβ					↑	

Table 1 and 2: Ig class switching in mouse (table 1) and in the human (table 2) for Th1, Th2 and Treg cells[55/56]. The red arrows meaning the up-regulation of the correspondent immunoglobulin in the presence of the indicated cytokine, while the black the down-regulation.

microRNAs

Introduction

MicroRNAs (miRNAs) are small (20-25 nucleotides (nt)), non-coding RNA molecules involved in posttranscriptional gene regulation[57]. They are representative features of all cells, as they regulate large fractions of a cell's transcriptome. In vertebrates, miRNAs constitute about 1% of all genes, with 1048 miRNAs found in humans and 672 in mice (miRBase database, www.mirbase.org)[58]. In 1993, during developmental studies on *C. elegans*, Victor Ambros and colleagues discovered that the gene *lin-4* encoded for 2 small RNAs: the first measured 22nt in length, while the second was long 61nt[59/60]. Moreover, the *lin-4* mRNA sequence showed antisense complementarity for different sites in the 3' untranslated region (3'UTR) of the gene *lin-14*. Briefly, *lin-4* regulated *lin-14*, reducing the amount of transcribed protein without changing the level of the messenger. Since that seminal discovery, the 22nt RNA *lin-4* has been recognized as a prototype for a wide class of regulatory RNA called miRNAs. In 2000, another gene was found in *C.elegans*: *let7*, encoding for a second miRNA involved in the transition from the larval to the adult stage[60]; since then miRNAs have been discovered in most organisms and they have been shown to regulate a large proportion of biological processes. MiRNAs perform their function by binding to the RNA-induced silencing complex (RISC) [61], which constitutes the machinery that modulates target mRNA expression in this endogenous regulatory RNA pathway. Indeed, the RISC complex recognizes complementary mRNA molecules and blocks their translation (and/ or induces mRNA degradation in case of perfect complementary with the miRNA sequence) resulting in substantially decreased levels of protein translation and effectively turning off the gene. Apart from miRNAs, other classes of small RNAs include short interfering RNA (siRNAs, mainly found in plants), piwi RNA (piRNA), trans-acting RNA (transiRNA), small scan RNA (scnRNA) and repeat associated siRNA (rasiRNA)[62].

MiRNA biogenesis

Mature miRNAs derive from long double-stranded (ds) primary transcripts (pri-miRNA), characterized by the presence of a stem-loop secondary structure, called pre-miRNA. The pri-mRNA is usually dependent for its transcription on the RNA polymerase II[62]. From the pri-miRNA, the process to yield mature miRNAs involves two steps and requires the RNase-III enzymes Drosha and Dicer, as well as companion dsRNA-binding proteins (Figure 6). In the nucleus, Drosha processes the newly transcribed pri-miRNA into the pre-miRNA hairpin precursor of approximately 70nt. Pre-miRNAs are exported into the cytoplasm through exportin 5, a protein localized on the nuclear membrane[57]. The

cytoplasmic enzyme Dicer further processes the pre-miRNA into a 19-25nt miRNA duplex[63-65]. The less stable of the two strands in the duplex is incorporated into the RISC complex [63], which regulates protein expression. The RISC complex has as catalytic center in the Argonauts protein (Ago) characterized by two domains: a PAZ domain, that can bind the single stranded 3' end of the mature miRNA, and a PIWI domain that structurally resembles ribonuclease-H and functions to interact with the 5' end of the guide strand. The precise molecular mechanisms that underlie posttranscriptional repression by miRNAs still remain to be fully elucidated.

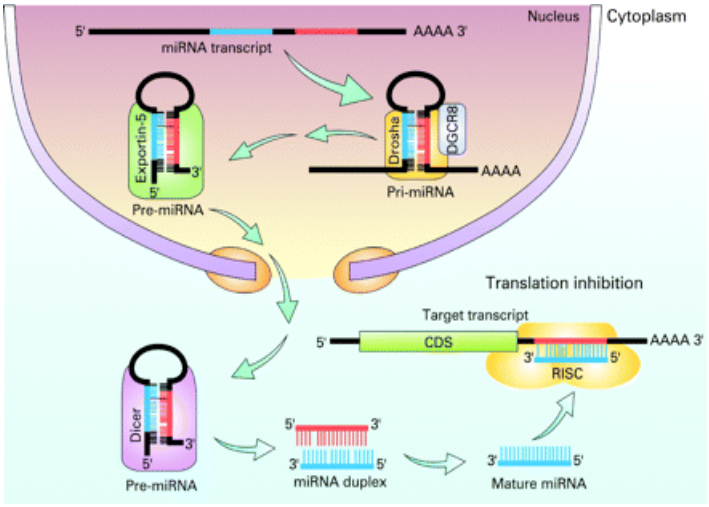

Figure 6: canonical microRNA biogenesis pathway. Adapted from T.Asselah et al, 2008 [66]

Post-transcriptional gene silencing by microRNAs

In human there are four Ago proteins (Ago1- 4). However Ago-2[67] is the only one with a so-called 'slicer' activity, as it is the only Ago family member able to degrade target mRNAs, and it is therefore required for RNA-mediated gene silencing (RNA interference or RNAi) mediated by the RISC. The 'minimal RISC' appears to include EIF2C2/Ago-2 bound to a short guide RNA such as a miRNA or siRNA. These guide RNAs direct RISC to complementary mRNAs that are targets for RISC-mediated gene silencing. The precise mechanism of gene silencing depends on the degree of complementarity between the miRNA or siRNA and its target. Binding of RISC to a perfectly complementary mRNA generally results in silencing due to the cleavage of the mRNA specifically by Ago-2. Such mechanism is usually the one at play with siRNAs in plants and in RNAi experiments. Binding of RISC to a partially complementary mRNA results in silencing through inhibition of translation, which represents instead the most common form of miRNA-mediated regulation for example in mammals and is independent of endonuclease activity.

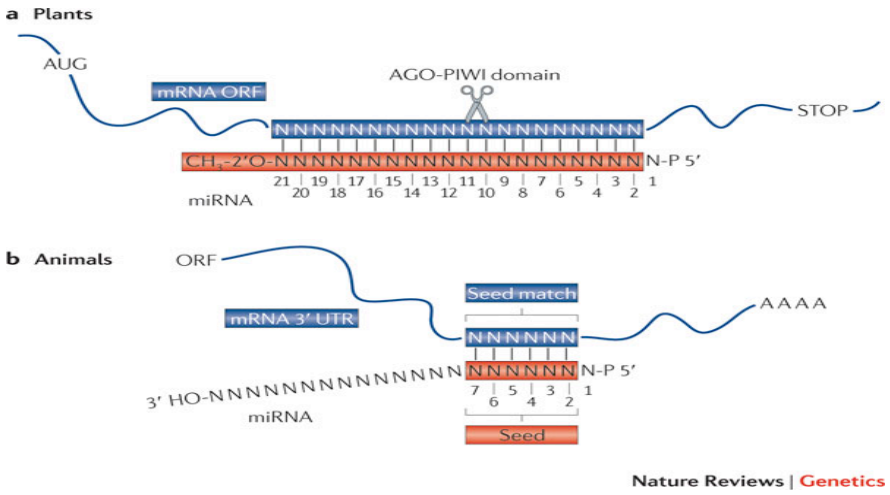

Figure 7: a. Plant miRNAs recognize fully or nearly complementary binding sites, b. Animal miRNAs recognize partially complementary binding sites, which are generally located in 3′ UTRs. Adapted from E Huntzinger at all, Nature, 2011

As mentioned above, in plants the complex constituted from RISC and miRNA is perfectly complementary to the mRNA target, causing the cut of the mRNA[61/68/69]. Similar to the mechanism employed in RNAi, the cleavage of a single phosphodiester bond on the target mRNA occurs between bases 11 and 10[70]. In animals, conversely, the complex constituted from RISC and miRNA is an imperfect match to the 3' UTR of the mRNA target. This association causes a block in mRNA translation with or without destabilization and secondary degradation of the mRNA target. Importantly, miRNAs act in a combinatorial manner, as the same transcript may be regulated by multiple miRNAs, while one individual miRNA may target many different transcripts.

MiRNAs regulate the expression of hundreds of gene[71]. However, identifying the target genes has proven difficult because their effect is often small[72] and they bind to target mRNAs by partial complementarity over a short sequence. Indeed, the part of a miRNA essential for target recognition is the so-called ‘seed’ sequence, which comprises nucleotides 2-to-7 in the miRNA most 5’ portion[73]. However, a number of algorithms to predict miRNA targets have been developed over the years, and although they may contain some false positive and false negative and always need to be related to the specific cellular context, they have been instrumental to identify relevant miRNA targets. Some of the most utilized target prediction databases include TargetScan[73/74],PicTar [75/76] and miRanda [77/78].

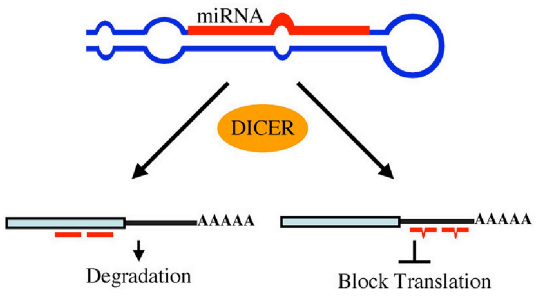

Figure 8: Post-transcriptional gene silencing by a miRNA. miRNAs also bind to targets with imperfect complementarity and block translation. M.Vella et al, 2005 [79]

MicroRNAs and the immune system

In the past few years it became clear that miRNAs play pivotal roles in regulating both adaptive and innate immunity[80]. Chen et al identified miR-181 as a miRNA specifically expressed in hematopoietic cells[81]. It was also demonstrated that miRNAs are involved in the immune system in studies showing selective expression of miR-223 in the bone marrow and the involvement of this miRNA, with miR-155 and miR-146a, in the differentiation of myeloid cells[82].

After these works, many studies identified miRNAs as crucial components of the molecular circuitry that controls differentiation and function of cells of the immune system, including the control of the differentiation of various immune cell subsets as well as their immunological functions.

MiR-146a

During the past several years some miRNA functions in the immune system have been unveiled, among which one miRNA has shown important regulatory functions: miR-146. The murine and the human genome contain two miR-146 genes: miR-146a and miR-146b[58]. MiR-146a and miR-146b are both conserved in all vertebrates and are involved in the regulation of inflammation and other processes in innate as well as adaptive immunity. Some miRNAs, like these two miR-146 molecules, are expressed as a family that share the same seed sequence, but are encoded by different loci in the genome. MiR-146a and miR-146b are located on murine chromosome 5 and 10, respectively, and their products differ only by 2nt in the 3' region, while the 'seed' sequence, that is a conserved heptamer situated in the miRNA 5′-end and important for target recognition, is identical, implicating that both miR-146a and miR-146b should recognize the same targets.

mmu-miR-146a UGAGAACUGAAUUCCAUGGGUU

mmu-miR-146b UGAGAACUGAAUUCCAUAGGCU

Figure 9: Mature form of miR-146a and miR-146b. The regions highlighted in red display the differences in sequence. Shaded in grey is the seed sequence, important for target recognition and identical in both miRNAs.

Most studies examined primarily miR-146a rather than miR-146b, as these two miRNAs not only should recognize the same targets, but miR-146a is often more abundantly expressed in the immune system. As for a role of miR-146a in innate immunity, it was shown that in human monocytes, miR-146a is a primary inflammatory response gene induced by IL-1β and TNFα and transcriptionally regulated by NF-kB[83]. Its targets were shown to be TRAF6 (TNF receptor-associated factor 6) and IRAK1 (IL1-receptor associated kinase)[83], which are adaptor molecules implicated in the NF-kB activation pathway. In the context of human monocytes activated with LPS, miR-146a was shown to act as a negative regulator of NF-kB activation, and specifically to work in a feedback system in which a stimulus induces NF-kB activation through a MyD88-dependent pathway, resulting in the expression of inflammatory genes, but also in the up-regulation of the miR-146a, which in turn down-regulates levels of IRAK1 and TRAF6 expression, reducing the activity of the pathway[83].

As for a role for miR-146a in adaptive immunity, recently it has been found that NF-kB and c-ETS binding sites are essential for the induction of miR-146a transcription upon TCR stimulation in T cells[83], while in a Jurkat T cell line miR-146a was shown to regulate activation-induced cell death (AICD) through modulation of FADD expression[84]. In this model, miR-146a overexpression also led to impaired AP1 activity and as a consequence, reduced IL-2 expression[84].

Importantly, recent studies have revealed important physiological roles of miRNAs in many aspects of mammalian immune cell function and their altered expression has been associated to pathological conditions on the immune system, such as autoimmunity and cancer[85]. Chronic inflammation contributes to cancer initiation and progression. Among the many mechanisms linking inflammation to cancer, NF-kB has been identified as a key mediator of inflammation-induced carcinogenesis[86]. As a negative regulator of NF-kB activation, miR-146a has been shown to be involved in progressive myeloproliferative disorders[87]. Specifically, it was shown that miR-146a KO mice develop, with the age, a chronic inflammatory and carcinogenesis phenotype meaning that miR-146a plays an important role as a tumor suppressor miRNA in hematopoietic lineages[87].

In human T cells, miR-146a is expressed at low levels in naïve T lymphocytes while it is abundantly expressed in memory T cells[88] as well as in Treg[89] and it is induced upon TCR stimulation, consistent with its expression being dependent on NF-kB induction[86/89]. In Treg cells, miR-146a mediated down-regulation of Stat1 (a key transcription factor required for Th1 effector cell differentiation), and was shown to be necessary for their ability to suppress Th1 responses[89].

Overall, these data showed an important role for miR-146a in regulating both innate and adaptive immunity, and a dysregulation of miR-146a expression may lead to a number of diseases, including cancer, making this miRNA also a putative novel attractive target for therapy.

NF-κB

Introduction

The choice between life and death is a crucial event in the regulation of the immune system. A major regulator of this decision is the transcription factor NF-κB. NF-κB was first discovered by Nobel Prize (1975) laureate David Baltimore via its interaction with an 11-base pair sequence in the immunoglobulin light-chain enhancer in B cells[90]. NF-κB is like a master switch because it turns on (or off) the genes that code for mediators of inflammation.

In nature, a variety of mechanisms exist that modulate NF-κB activity. Indeed, NF-κB is a transcription factor involved in the regulation of many aspects of the biological processes such as inflammation and innate immune responses[91-93]. A deficit of NF-κB can lead to an immune dysfunction as well as to autoimmune diseases and cancer[92/93]. NF-κB binds DNA as a dimer that recognizes a discrete nucleotide sequence (5'- GGGRNYYYCC - 3', in which R=purine and Y=pyrimidine) in the upstream region of a variety of cellular and viral response genes. In mammalian cells, the NF-κB/Rel family contains five members: Rel A (p65), c-Rel, Rel-B, NF-κB1 (p50; p105) and NF-κB2 (p52; p100)[94]. P100 and p105 are the precursors for p52 and p50 respectively. These proteins share a conserved 300 amino acid sequence in the N-terminal region, known as the Rel homology domain (RHD).

The RHD is the common DBD (DNA binding domain) ant it is found in a family of eukaryotic transcription factors, which includes NF-κB, Dorsal, NFAT, among others. Some of these transcription factors appear to form multi-protein DNA-bound complexes[95]. RHD acts as regulator of some of these transcription factors, modulating the expression of their target genes[96]. NF-κB proteins bind the DNA as homo- and heterodimers. The most common NF-κB heterodimer is p65-p50; in unstimulated cells, it is sequestered in the cytoplasm through interaction with IκB inhibitory proteins. Upon stimulation, the IκB kinase phosphorylates the IκB. When phosphorylated, the IκB inhibitor molecules are modified by ubiquitination, which then leads them to be degraded by proteasome. With the degradation of IκB, the NF-κB complex is then free to enter the nucleus where it can 'turn on' the expression of specific genes that have DNA-binding sites for NF-κB nearby.

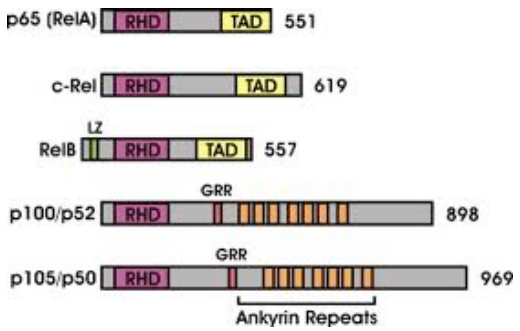

Figure 10: Schematic diagram of NF-κB protein structure with RHD (Rel homology domain) and TAD (trans-activator domain)[97]

P50 can bind DNA as a heterodimer with other NF-κB family members as well as a p50:p50 homodimer ($(p50)_2$). Importantly, since p50 (as well as p52) does not contain the COOH-terminal transactivation domain, it can form inhibitory homodimers, able to function as transcriptional repressors. A physiological role as negative regulators implies that their biogenesis must be highly controlled. Such a regulatory step could involve the release of the inactive precursor (p105). If proteolysis of p105 is triggered and there is not enough p65 available as partner for heterodimerization, $(p50)_2$ should form.

The overexpression of p50 acts to suppress the transactivating effectors of p65 at some NF-kB sites[98], and this mechanism has been implicated in the down-regulation of major histocompatibility complex expression[99] leading to a high malignancy and low immunogenicity of certain murine tumors, such as breast cancer and Hodgkin's lymphoma. The explanation for this down-regulation effect, is that $(p50)_2$ reduces transcriptional activation by competing for binding with p65-p50, since p65, but not p50, carries a transactivating domains. Another explanation could be that $(p50)_2$ interacts with a transcriptional repressor, as suggested by the observation that some proteins (e.g Drosophila dorsal switch protein 1) converts NF-κB from a transcriptional activator to a repressor only in presence of p50[100].

NF-kB activation pathway

In an inactive state, NF-κB is located in the cytosol in complex with the inhibitory protein IκBα. NF-κB can be activated through 2 different pathways: a classical and an alternative pathway.

-> CLASSICAL PATHWAY:

A variety of extracellular signals can activate the enzyme IκB kinase (IKK). IKK, in turn, phosphorylates the IκBα protein, which results in ubiquitination, dissociation of IκBα from NF-κB, and eventual degradation of IκBα by the proteosome. Through this cascade of phosphorylation events, NF-κB becomes therefore able to enter the nucleus, where it binds to response elements in the DNA, inducing gene expression and ultimately resulting in activation of transcription[101-103]. Specifically, this pathway leads to the increased transcription of target genes encoding chemokines, cytokines, and adhesion molecules, perpetuating inflammatory responses and promoting cell survival. The classical pathway is typically triggered by ligand binding to the TCR, BCR, TLR, TNF receptor and interleukin-1 receptor (IL1-R) superfamily members.

-> ALTERNATIVE PATHWAY:

In the alternative pathway, p52/p100 is phosphorylated at two C-terminal sites by the IKKα homodimer and ubiquitinated. This modification targets the inhibitory C-terminus for proteasomal degradation, producing p52. Although they are often activated concurrently, the classical and alternative NF-κB activation pathways have distinct regulatory functions [101/104-105]. The alternative pathway is triggered by the activation of certain TNF receptor family members, including CD40, CD30, LTβR (lymphotoxin β receptor) and B-cell-activating factor belonging to the TNF family receptor (BAFF-R). Activation of the alternative pathway regulates the development of lymphoid organs and the adaptive immune system[106].

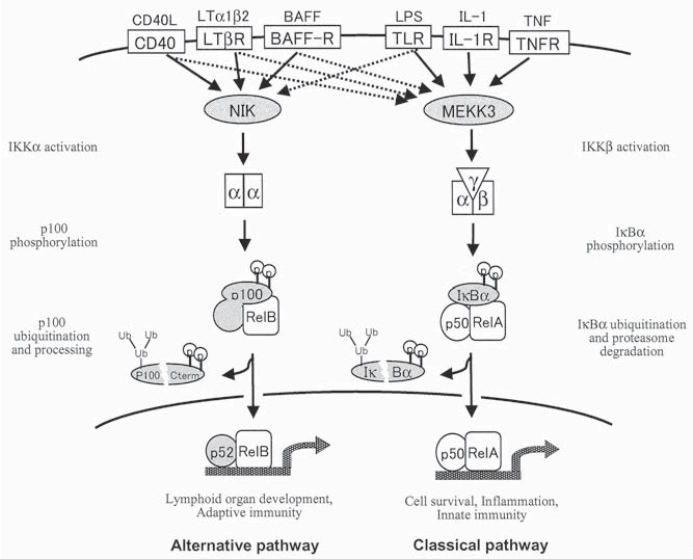

Figure 11: This schema shows the classical and the alternative NF-κB activation pathways. In the classical pathway, IKK frees NF-κB from the inhibitor that holds it in the cytoplasm – so that it may enter the nucleus. Once inside the nucleus, NF-κB is able to bind gene promoters and 'turn on' inflammation[106].

NF-κB and apoptosis

Apoptosis is the process of programmed cell death that may occur in multicellular organisms[104]. Two pathways of initiation of apoptosis are known in mammals[105/106].

- "Extrinsic or death receptor pathway": following activation of "death receptors" (molecules in the TNF receptor superfamily, including TNF-R1, CD95 (Fas)), caspase-8 is recruited into the DISC complex (death-inducing signaling complex) through FADD (Fas-associated death domain)[107] and activated. This cascade then activates caspase-3 and eventually induces apoptosis[108].

- Intrinsic or mitochondrial pathway": various signals may converge at the mitochondrial level to induce a translocation of mitochondrial cytochrome c (cyt c) into the cytosol. Proteins in the Bcl-2 (*B-cell leukemia/lymphoma 2*) family represent the primary regulators in this pathway: anti-apoptotic members (Bcl-2, Bcl-X_L, Bcl-W, Bfl-1 and Mcl-1) act as inhibitors while pro-apoptotic members (Bax, Bak, Bad, Bcl-X_S, Bid, Bik, Bim and Hrk) serve as promoters[109/110] by blocking or enhancing, respectively, the release of cyt c into the cytosol.

NF-κB is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival. As such, many different types of human tumors have dysregulated NF-κB expression and/or activation[111-113]. Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis. Defects in NF-κB results in increased susceptibility to apoptosis leading to increased cell death[111-113].

In tumor condition, such as in Hodgkin's lymphoma or breast cancer, NF-κB is active either due to mutations in genes encoding the NF-κB transcription factors themselves or in genes that control NF-κB activity (such as IκB genes) leading to a down-regulation of NF-κB targeted genes. Blocking NF-κB can lead, in these tumors, to a reduced proliferation, increased cell death, or to increased sensitivity to the action of anti-tumor agents[113].

Since NF-κB is a key player in the inflammatory response and its suppression can limit the proliferation of cancer cells, methods to inhibit NF-κB signalling have potential therapeutic applications in cancer and inflammatory diseases[111-113]. NF-κB is therefore the subject of active research among pharmaceutical companies as a target for anti-cancer therapy[113]. Thus, combined therapy that inhibits NF-κB function in the presence of apoptotic

stimuli may lower the anti-apoptotic threshold of tumors to provide a more effective treatment against resistant forms of cancer.

NF-κB p50 KO mice

Contrary to mice deleted for other NF-κB family members, such as p65, which is embryonically lethal[114], mice lacking the NF-κB p50 subunit show no developmental abnormalities but exhibit multifocal defects in immune responses involving B-lymphocytes, including non-specific responses to infection[115] as well as defective proliferative responses to LPS[116]. These mice also show a tumor-resistant phenotype characterized by the presence of M1-like tumor-infiltrating macrophages[117/118]. M1 macrophages are considered potent effector cells that kill intracellular organisms and tumors cells, and are characterized by the ability to produce high levels of pro-inflammatory cytokines such IL-12, IFN-γ and TNF-α. Another important phenotype of p50KO mice is their inability to mount airway eosinophilic inflammation (figure 12) in response to challenge with aerosolized OVA[119]. Such reduced eosinophilic inflammation was correlated to the inability of T lymphocytes to polarize to Th2 and as a consequence to a failure to induce production of IL-4, IL-5 and IL-13, all cytokines with important roles in asthma pathogenesis[119]. However, the possibility of a defect in MC differentiation and function in these mice was never investigated.

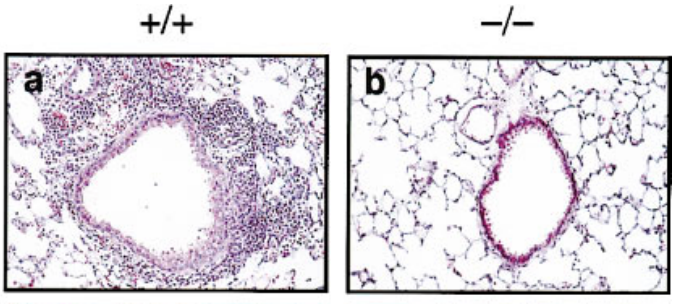

Figure 12: Immunohistochemical slides of a lung section from a WT (a) and p50KO (b) mouse sensitized with OVA . Here we can see the total absence of the airway inflammation in p50KO mice[119].

Aim of this work

Mast cell-related diseases heavily affect a high percentage of the world population. For example more than 3% of the population in US and 9.4% in UK suffer from asthma, while more than 40% is affected by some kind of allergy[120]. Moreover, mastocytosis is a mast cell tumor that ,although rare and often mild, has no cure, and when becomes aggressive it also becomes very rapidly devastating and fatal. A better understanding of how mast cell differentiation and function are regulated within normal immune responses as well as disease can lead us to better treatments of diseases such as asthma, allergy and mastocytosis. Importantly, miRNAs have emerged as important regulators of many aspects of cell differentiation, proliferation and interaction with the environment and effector functions.

They are also quickly becoming useful biomarkers and possible targets for manipulation for therapeutic purposes. However, the role of miRNAs in the regulation of mast cell differentiation, proliferation and effector functions is almost completely unexplored, and it is exactly the Aim of this study.

Importantly, we found that some of our newly identified mechanisms of mast cell regulation are also at play during the acute activation of T lymphocytes, indicating that they may represent more general mechanisms of regulation of the immune response.

The identification of new molecular networks that regulate T lymphocytes and mast cell biology and homeostasis could lead us to the identification of novel points of entry for diagnosis and possibly therapy of mast cell-related diseases.

This thesis is divided into 4 chapters:

Chapter 1) MiR-221 is an activation-induced miRNA in MCs, that our lab identified as important in regulating mast cell proliferation[121]. Following up on that work, we found that miR-221 has important roles in regulating multiple processes in differentiated MCs, such as degranulation, adhesion and migration. Since miR-221 is expressed at basal level in MCs but it is also inducible upon stimulation, we proposed a model in which miR-221 has a dual role in these cells: at resting state, it contributes to the regulation of the cell cycle and cytoskeleton. However, in response to stimulation through IgE-antigen complexes, miR-221 effects are MC-specific and activation-dependent,

contributing to the regulation of degranulation, cytokine production and cell adherence.

This part of the work was published in PLoS ONE (2011); 6:1-13: "MiR-221 Influences Effector Functions and Actin Cytoskeleton in Mast Cells".

Chapter 2) The specific aim of this part of the work was to investigate the role of NF-kB p50 in regulating MC function. Indeed, mice lacking NF-kB p50 (and p105) show reduced eosinophilia and airway inflammation upon challenge, but differentiation and functionality of MCs in these mice were never evaluated. Our data show that p50KO MCs differentiated normally *in vitro* and expressed reduced cytokine levels in response to different stimuli, such as IgE-crosslinking or LPS, indicating that although the airway inflammation defect originally observed in p50KO mice might be predominantly Th2-dependent, reduced cytokine production from MCs may also contribute to this phenotype. Importantly, we found that p50KO MCs showed a marked increase in their ability to survive in response to withdrawal of essential cytokines, which likely correlated with a strong increase in the percentage of MCs that were observed in the tissues of p50KO animals. Since the identification of novel mechanisms that regulate MC proliferation, survival and overall homeostasis in the tissues may have important implications for the treatment of MC-related diseases such as asthma, allergy and mastocytosis, we investigated the molecular mechanisms at the basis of such enhanced survival.

This part of the work was published in Mol Cell Biol. (2012) Nov;32(21):4432-44: "miR-146a and NF-κB1 Regulate Mast Cell Survival and T Lymphocyte Differentiation.

Chapter 3) Despite intense investigation, the requirements for the acquisition and maintenance of the human T memory phenotype have yet to be completely elucidated. MiR-146a is a well-known regulator of the immune system: it has an established role in regulating NF-kB activation and tolerance in innate immunity, it acts as an onco-suppressor, and it modulates T regulatory (Treg) cell functions. However, the role of miR-146a (and miRNAs in general) in

the establishment of immunological memory remains unexplored. Here, we found that miR-146a was expressed at high levels in the memory T cell compartment from both human and mouse. Although expression of miR-146a in primary human T cells determined no significant effect on cytokine production and Fas-mediated cell death, miR-146a expression lead to increased expansion in response to TCR stimulation pointing towards a role for this miRNA in human T cell activation and possibly memory formation.

This part of the work was published in Mol Cell Biol. (2012) Nov;32(21):4432-44: "miR-146a and NF-κB1 Regulate Mast Cell Survival and T Lymphocyte Differentiation.

Chapter 4) MiRNAs are regulatory molecules able to influence all aspects of the biology of a cell, in particular is important as a modulator of differentiation and function of cells of the immune system. Here we summarize the most recent progress in understanding the role of a specific miRNA, miR-146a, in innate and adaptive immune responses, as well as in disease.

This part of the work was published in Mol. Biol. Int. (2011);2011:437301 "miR-146a in Immunity and Diseases".

Chapter 1

"MiR-221 Influences Effector Functions and Actin Cytoskeleton in Mast Cells"

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PLoS one

MiR-221 Influences Effector Functions and Actin Cytoskeleton in Mast Cells

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Abstract

Mast cells have essential effector and immunoregulatory functions in IgE-associated allergic disorders and certain innate and adaptive immune responses, but the role of miRNAs in regulating mast cell functions is almost completely unexplored. To examine the role of the activation-induced miRNA miR-221 in mouse mast cells, we developed robust lentiviral systems for miRNA overexpression and depletion. While miR-221 favored mast cell adhesion and migration towards SCF or antigen in trans-well migration assays, as well as cytokine production and degranulation in response to IgE-antigen complexes, neither miR-221 overexpression, nor its ablation, interfered with mast cell differentiation. Transcriptional profiling of miR-221-overexpressing mast cells revealed modulation of many transcripts, including several associated with the cytoskeleton; indeed, miR-221 overexpression was associated with reproducible increases in cortical actin in mast cells, and with altered cellular shape and cell cycle in murine fibroblasts. Our bioinformatics analysis showed that this effect was likely mediated by the composite effect of miR-221 on many primary and secondary targets in resting cells. Indeed, miR-221-induced cellular alterations could not be recapitulated by knockdown of one of the major targets of miR-221. We propose a model in which miR-221 has two different roles in mast cells: in resting cells, basal levels of miR-221 contribute to the regulation of the cell cycle and cytoskeleton, a general mechanism probably common to other miR-221-expressing cell types, such as fibroblasts. Vice versa, upon induction in response to mast cell stimulation, miR-221 effects are mast cell-specific and activation-dependent, contributing to the regulation of degranulation, cytokine production and cell adherence. Our studies provide new insights into the roles of miR-221 in mast cell biology, and identify novel mechanisms that may contribute to mast cell-related pathological conditions, such as asthma, allergy and mastocytosis.

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Introduction

Mast cells are cells of the innate immune system that reside in most tissues and are characterized by cytoplasmic granules containing active mediators such as histamine and proteases, which can be rapidly released upon activation. A broad panel of cytokines and chemokines is also rapidly synthesized upon acute stimulation, although some cytokines (such as TNFα) can be pre-stored in the granules [1,2]. On the surface, mast cells are characterized by the expression of KIT and FcεRI (high-affinity IgE receptor). KIT, the receptor for the stem cell factor (SCF), is a critical regulator of mast cell differentiation, proliferation, activation and survival. Although mast cells have been considered for a long time detrimental to the host, mainly because of their major role as master effector cells in allergic responses, it is now becoming clear that depending on the context, mast cells can either positively or negatively regulate innate or adaptive immune responses to pathogens or allergens (for a recent review see [3]).

MicroRNAs (miRNAs) are small, non-coding RNAs that are emerging as major players in the regulation of endogenous gene expression in immune homeostasis [4]. Specifically, miRNAs regulate the expression of target genes by interacting with sites in the 3′ untranslated region (3′UTR) of their target mRNAs. MiRNAs regulate all aspects of a cell, including differentiation, function, proliferation, survival, metabolism, and responses to environmental changes. A single miRNA can potentially modulate the expression of hundreds of transcripts in a cell, both by direct and indirect effects, and as many as 90% of human genes may be regulated by miRNAs [5]. MiRNAs are implicated in various pathologic conditions as well as in tumorigenesis [4,6], and within the immune system, deletion or overexpression of specific miRNAs can impair innate or adaptive immune responses [7].

While the importance of miRNAs in various types of normal and diseased cellular processes is by now well established, very little is known about the role of miRNAs in mast cell development, function and disease. Our lab identified miR-221/-222 as a family of miRNAs that is transcriptionally induced upon mast cell activation, and we showed that expression of miR-221 and/or miR-222 to levels similar to the endogenous of activated mast cells, led to reduced mast cell proliferation [8]. MiR-221 and miR-222

derive from the same primary transcript and share the same seed sequence, implying that they should recognize the same targets [9]. Following up on our previous studies, we investigated the role of miR-221 in mast cell differentiation and function. Specifically, we found that although miR-221 does not seem to affect mast cell differentiation, it has important roles in regulating multiple processes in differentiated mast cells, such as degranulation, adhesion and migration, some of which may be linked to a dysregulation in the actin cytoskeleton. Indeed, we found that alteration of miR-221 expression in mast cells and fibroblasts led not only to a reduction in cell proliferation similar to what we previously described using a different expression system, but also to an alteration of actin content and overall cellular shape in both cell types. Transcriptional profiling and bioinformatics analysis using the Sylamer algorithm [10], indicated that miR-221 effects in mast cells were mediated by the alterations of the level of expression of many primary and secondary targets. Importantly, such miR-221-mediated alterations of the cell phenotype could not be recapitulated by knockdown of one of the most prominent target for miR-221, suggesting that the observed effect of miR-221 in resting mast cells and fibroblasts is likely to be composite, due to the alteration of many genes. Moreover, we also observed mast cell-specific, activation-dependent effects of miR-221. Since miR-221 is expressed at basal level in mast cells, but it is also inducible upon stimulation, we propose a model in which miR-221 has a dual roles in these cells: at resting state, it contributes to the regulation of the cell cycle and cytoskeleton, a housekeeping effect that can be observed also in different cell types expressing this miRNA. However, in response to stimulation through IgE-antigen complexes, miR-221 effects are mast cell-specific and activation-dependent, contributing to the regulation of degranulation, cytokine production and cell adherence. Overall, our studies provide insights on the role of miRNAs in mast cells, and lay the groundwork for understanding some of the mechanisms underlying pathological conditions caused by mast cells, such as allergy and mastocytosis.

Materials and Methods

Ethics statement

All animal studies were performed in accordance with the Swiss Federal Veterinary Office guidelines and were approved by the Dipartimento della Sanita' e della Socialita', authorization number 18/2010.

Plasmids

About 400bp of the mouse miR-221 or miR-222 genomic sequences were cloned into the pAPM lentiviral vector [11]. Point mutations in the seed sequence of miR-221 were introduced using the Quick Change Site-Directed Mutagenesis kit (Stratagene) and the following primers (mutations underlined): *miR-221mFW*: 5′-GTTTGTTAGGCAACATCGCGATTGTCTGCTGGGTTTC-AGG; *miR-221mRV*: 5′- CCTGAAACCCAGCAGACAATCGC-GATGTTGCCTAACAAAC. The control vector pAPM-shLuc, expressing a miR-30-based shRNA against luciferase, was obtained from Thomas Pertel and Jeremy Luban and contained the following sequence: 5′- CACAAACGCTCTCATCGACAAG. The miRNA target (miRT) vectors containing four sequences fully complementary to miR-221 and/or miR-222, were provided by Bernhard Gentner and Luigi Naldini [12].

Cell cultures

For bone marrow-derived mast cells (BMMCs) differentiation, bone marrow cells from C57Bl/6 mice (6-8 weeks old) were differentiated and maintained in IMDM with 10% FBS, 2 mM L-glutamine, 0.1 mM non essential amino acids, 50 μMβ-mercaptoethanol, antibiotics and 50% WEHI-3 conditioned supernatant as a source of IL-3 [13,14]. The IL-3-dependent mast cell line MC/9 (ATCC) was cultured as for primary BMMCs. 3T3 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 μMβ-mercaptoethanol and antibiotics.

Transductions and transfections

Lentiviral transductions of mast cells were performed exactly as described [8,13]. 3T3 cells were transduced with concentrated lentiviruses prepared exactly as described [8,13] and selected with 2 μg/mL puromycin for several days before performing experiments. For transient transfections with oligonucleotides, 3T3 cells were transfected with Lipofectamine-2000 (Invitrogen) and 20pmol of siRNAs against $p27^{Kip1}$ (siGENOME *Cdkn1b*) or control siRNAs (siGENOME non-targeting siRNA #2 and/or siGLO) (all from Thermo Scientific) following manufacturer's instructions. Cells were analyzed 48–72 h after transfection. Efficiency of transfection was assessed in each experiment by transfecting a fluorescent oligonucleotide (siGLO) and analyzing the percentage of siGLO+ cells by FACS, which usually ranged between 50 and 70%. The efficiency of protein knockdown was assessed by Western blot.

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) according to manufacturer's instructions. To analyze miRNA expression, qRT-PCR was performed using a miRNA reverse transcription kit and TaqMan miRNA assays from Applied Biosystems, following exactly manufacturer's instructions.

Immunostainings and microscopy

Anti-KIT-APC, anti-FcεRIα-PE and anti-CD25-PE were purchased from eBioscience. For microscopy, 10^5 BMMCs were labeled with CFSE (Invitrogen) and added to a monolayer of 3T3 cells on coverslips. After 12 hours, coverslips were washed to eliminate non-adherent cells, and cells were fixed with 3.7% p-formaldehyde, permeabilized with 0.1% Triton-X100 and stained with 160nM phalloidin-AlexaFluor-594 (Invitrogen). Glass slides were mounted using Gelvatol (20% polyvinyl alcohol, 100mM Tris-HCl pH 8.5, 2.5% DABCO). The same protocol was used to stain 3T3 cells grown directly on coverslips. Bright field images were captured with a Nikon Eclipse E800 and analyzed with the Openlab software (Improvision). For FACS-based quantification of F-Actin, $3{\cdot}10^5$ cells were fixed 15–30 min in 4–5% p-formaldehyde, permeabilized 2min with 0.1% Triton-X100, and stained with phalloidin at a 1:100 dilution.

Degranulation, adhesion and migration assays

For degranulation assay, BMMCs ($5{\cdot}10^4$) were resuspended in 50 μL OptiMEM, 1% FBS and stimulated for 1 h with 1.5 μg/mL IgE-anti-DNP (clone SPE-7, Sigma) and 0.2 μg/mL DNP-HSA (Sigma). After stimulation, cell pellets were lysed in 50 μL of 0.5% Triton-X100 in OptiMEM, 1% FBS, and 50 μL of a 3.8 mM solution of the β-hexosaminidase substrate 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma) were added to both cell lysates and supernatants. After incubation for 2 h at 37°C, the reaction was stopped with 90 μL glycine 0.2 M, pH 10.7, and absorbance was read at 405 nm. The percentage of degranulation was calculated as the ratio between the absorbance of supernatants and the total absorbance of supernatants and cell lysates [15]. Alternatively, degranulation was assessed using the same annexin V-PE kit used to

detect apoptosis (BD-Pharmingen) [16,17]. Briefly, cells were stimulated for 30 min with 1.5 μg/mL IgE-anti-DNP and 0.2 μg/mL DNP-HSA, in the presence or absence of 20 ng/mL SCF (Peprotech). Cells were then washed and stained with annexin V-PE following manufacturer's instructions. For adhesion experiments, 10^5 BMMCs were added to a monolayer of 3T3 cells in 24-well plates. Floating cells were harvested 8h later, and adherent cells were detached using 10mM EDTA. In order to distinguish BMMCs from fibroblasts, cells were stained for KIT, and numbers of KIT+ cells were evaluated by FACS. The percentage of adherent cells was calculated as the ratio between adherent cells versus total. Cell migration was assayed using 24-well transwell chambers (Corning) with 8.0 μm pores in polycarbonate membranes. $2{\cdot}10^5$ BMMCs were seeded in the upper chambers and allowed to migrate for 2 h. In some experiments, 20 ng/mL of SCF was used as chemoattractant, while in others BMMCs were first sensitized with 300 ng/mL IgE-anti-DNP for 12 h and then allowed to migrate towards 0.2 μg/mL DNP-HSA. The percentage of migrated cells was calculated as the ratio between the number of cells in the lower chamber versus total.

Cell-cycle analysis and Western blots

For propidium iodide staining and DNA content analysis, non-confluent 3T3 cells were fixed in 70% ethanol for 45 min on ice, followed by incubation for 30 min at 37°C with 100 μg/mL RNaseA and 40 μg/ml propidium iodide. Cells were analyzed by FACS immediately afterwards. Total protein extracts for Western blot were prepared by cell lysis in Laemmli sample buffer. Samples were run on 12% SDS-polyacrylamide gels and immunodetection was performed with p27 C-19 and β-tubulin H-235 antibodies (Santa Cruz Biotechnologies).

Microarrays and Sylamer analysis

Gene arrays were performed at Miltenyi Biotec using Agilent dual-color whole-genome oligo arrays and total RNA from three independent biological replicas. Genes found to be commonly regulated were functionally annotated. Sylamer analysis was performed through a Sylarray web server [18,19]. Specifically, the Agilent probes were mapped to the 3′zUTRs of genes stored in the Sylarray database. The sequences were previously masked from low complexity regions and redundant UTR sequences with DUST algorithm and RSAT purge-sequence interface to Vmatch [20,21]. As a control we also analyzed the mutant seed sequence to check for any evidence of its influence in the experiment and no significant association was found. Array data are MIAME compliant and are deposited in the MIAME compliant database Gene Expression Omnibus (GEO) with accession number GSE24462.

Statistical analysis

Results are expressed as mean ± standard deviation. Comparisons were made using the Student's unpaired t-test and the GraphPad Prism Software.

Supporting Information

Additional information can be found in the Supplementary Methods S1.

Results

A robust lentiviral system for manipulating microRNA expression in mast cells

As we previously described for acute stimulation of mast cells [8], BMMCs stimulated with IgE-antigen complexes upregulated miR-221 expression (Figure 1A). While stimulation-dependent upregulation of this miRNA could be favored by SCF co-stimulation, SCF alone had no effect on miR-221 expression (not shown). To investigate the role of miR-221 in regulating primary mast cell functions, we developed a lentiviral system to manipulate miRNA expression in primary BMMC and used it to alter miR-221 expression. The pAPM/pAGM vectors were used to overexpress miR-221 or miR-222; as control, we used a mutant version of miR-221 (miR-221m), containing mutations in the seed region to abrogate target recognition, as well as a vector expressing an irrelevant hairpin (shLuc) (Figure 1B). The miR-221m mature sequence had no predicted targets as assessed by TargetScan [9]. The 'miRNA target' (miRT) vectors contain four miRNA binding sites (miR-bs) cloned downstream a GFP reporter gene, and they were used to functionally ablate miR-221/-222 [12]. Transcription from such vectors results in accumulation of decoy mRNAs that divert miRNAs from their physiological targets [22].

To assess expression from these vectors, BMMCs were transduced with the indicated vectors, and miRNA expression was assessed by qRT-PCR (Figures 1C and 1D). Compared to untransduced, unstimulated cells (expression set to 1 in Figures 1C and 1D), transduction of primary mast cells with pAGM/pAPM-miR-221 increased miR-221 expression by ~60-fold, whereas transduction with miRT-221 decreased expression by ~10-fold (Figure 1D). Transduction with the mutant miR-221m had no effect (Figure 1C). Initial experiments were performed using a vector (Tween) that induced only modest overexpression (~4-fold), similar to the levels of endogenous miR-221 observed upon cell stimulation (Figure 1A) [8]. However, both types of vectors (weaker and stronger expression) gave similar results qualitatively, although the stronger vector provided bigger quantitative differences, and was therefore used in most of the subsequent experiments. To assess the functional effects of miRNA overexpression/ablation, the mast cell line MC/9 was transduced to overexpress miR-221 or the mutant miR-221m. Transduced cells were selected with puromycin, subjected to a second round of transduction with the miRT vectors, and monitored for GFP expression (Figure 1E). As a result of binding of the overexpressed miRNAs to their cognate sites in the 3′ UTR of the GFP reporter mRNA expressed from the miRT, GFP expression was strongly reduced specifically in cells expressing miR-221 but not the mutant miR-221m. We therefore used both validated systems (overexpression and ablation) to study mast cell differentiation in the presence or absence of miR-221.

MiR-221/-222 as well as the transcriptional repressor PLZF are both known important regulators of hematopoietic cell differentiation [23,24,25]. We previously showed that binding sites for PLZF were enriched in mast cell-specific DNaseI hypersensitive sites found upstream of the miR-221-222 genomic sequence [8]. To address the possible relation between PLZF and miR-221, we analyzed expression of both *Plzf* mRNA and miR-221 during mast cell differentiation (Supplementary Figure S1). We observed an inverse relation between *Plzf* and miR-221 expression during mast cell differentiation, and ectopic expression of PLZF in mast cells diminished miR-221 expression in response to acute stimulation, suggesting that PLZF is able to repress miR-221-222 induction either directly or indirectly, and possibly through PLZF-binding regulatory elements in the miR-221-222 locus [8]. However, ectopic expression of PLZF in differentiated mast cells had no effect on the basal levels of endogenous miR-221, indicating that other factors regulate basal expression of this miRNA in mast cells.

To assess whether miR-221/-222 may have a direct role in regulating the differentiation process in mast cells, we transduced bone marrow-derived hematopoietic progenitors with lentiviruses

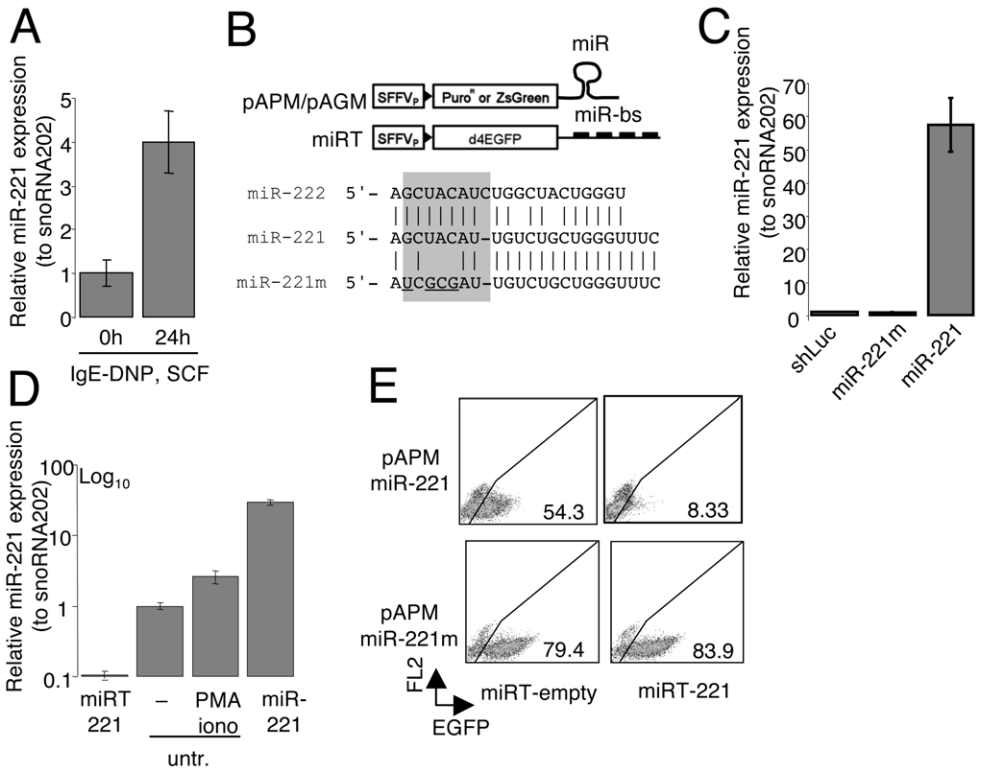

Figure 1. MiR-221 is upregulated upon mast cell activation and its expression levels can be altered using lentivirus-based systems. **A**) Differentiated BMMCs were either left resting or were stimulated with 1.5 μg/mL IgE anti-DNP, 0.2 μg/mL DNP-HSA and 20ng/mL SCF prior analysis of miR-221 expression by TaqMan qRT-PCR. SnoRNA202 was used as endogenous control. **B**) Schematic representation of the lentiviral vectors used to stably overexpress or functionally ablate miR-221 and miR-222 in BMMCs. Reporter genes were either puromycin or ZsGreen in the pAPM or pAGM vectors, respectively. Sequences corresponding to the mature murine miR-221, miR-222 and miR-221m are also shown. The miRT vectors contain 4 sequences fully complementary to miR-221 and/or miR-222 cloned downstream the reporter gene. SFFVp: spleen focus-forming virus promoter; d4EGFP: destabilized GFP; bs: binding sites. **C**) BMMCs were transduced with the control vector shLuc, miR-221 or miR-221-mutant. After selection with 2 μg/mL puromycin for 48h, miR-221 expression levels were assessed by TaqMan qRT-PCR. Expression levels are referred to the ones of the shLuc-transduced cells, which are set to one. **D**) BMMCs were transduced with miRT-221 (depleting) or miR-221 (overexpressing) vectors, and miR-221 expression levels were assessed by TaqMan qRT-PCR. Cells transduced with miR-221 were selected with 2 μg/mL puromycin for 48h prior analysis, while cells transduced with miRT-221 were sorted to >90% GFP+ prior RNA extraction. Untransduced cells were either left unstimulated or were stimulated with PMA and ionomycin for 24 h prior RNA extraction. Expression levels are referred to the untransduced and unstimulated cells, which are set to one. **E**) MC/9 cells were transduced with miR-221 (*upper panels*) or miR-221m (*lower panels*) and selected with 1 μg/mL puromycin prior a second transduction with the indicated miRT vectors (miRT-empty or miRT-221). GFP levels were determined by FACS. Transduction efficiency and levels of miR-221 expression or depletion are representative of tens of experiments, including all the experiments shown hereafter.
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to either overexpress (pAPM) or ablate (miRT) miR-221 and/or miR-222 early during mast cell differentiation (Supplementary Figure S1). Differentiation was monitored over a period of at least three weeks by assessing the percentage of FcεRIα+ KIT+ cells. Interestingly, the percentage of BMMCs increased steadily over time in all samples, and mast cell differentiation was not significantly affected by either overexpression or ablation of miRNAs. Moreover, there was no obvious alteration in cell granularity or in the content of the granules (data not shown).

MiR-221 regulates degranulation, migration and adherence in differentiated BMMCs

Since there was no effect of miR-221 in mast cell differentiation, we set out to investigate its role in mast cell functions, especially the ones connected to signaling through the FcεRI, given that miR-221 expression is inducible upon stimulation. Differentiated BMMCs were lentivirally transduced to force expression of miR-221, followed by analysis of the effects on mast cell degranulation, migration and adherence (Figure 2). Upon activation, mast cells release an array of enzymes that are pre-stored in cytoplasmic granules. We analyzed the ability of miR-overexpressing BMMCs to degranulate upon stimulation using β-hexosaminidase activity in the supernatant of activated cells as a measure of degranulation (Figure 2A). In resting conditions, cells did not degranulate, regardless of miRNA expression (Figure 2A, *top panel*) but, upon stimulation with IgE and antigen, BMMCs overexpressing miR-221 degranulated more compared to the controls (Figure 2A, *lower panel*), although they also showed a slightly reduced content of β-hexosaminidase in the granules to begin with (Figure 2A, *top panel*). To further confirm these results and to assess the effect of different conditions of stimulation (namely IgE crosslinking with or without SCF co-stimulation), we assessed degranulation of cells overexpressing miR-221 or controls by using a staining with annexin V. This staining takes advantage of the fact that mast cells do not die upon stimulation (which is instead a survival factor [26]) and that during the membrane fusion process of degranulation, annexin V binding occurs at sites of secretory granule exposure to the cell surface [16,17]. In general, BMMCs stimulated with a combination of IgE-antigen and SCF degranulated more strongly compared to cells stimulated without SCF (Figure 2B). However,

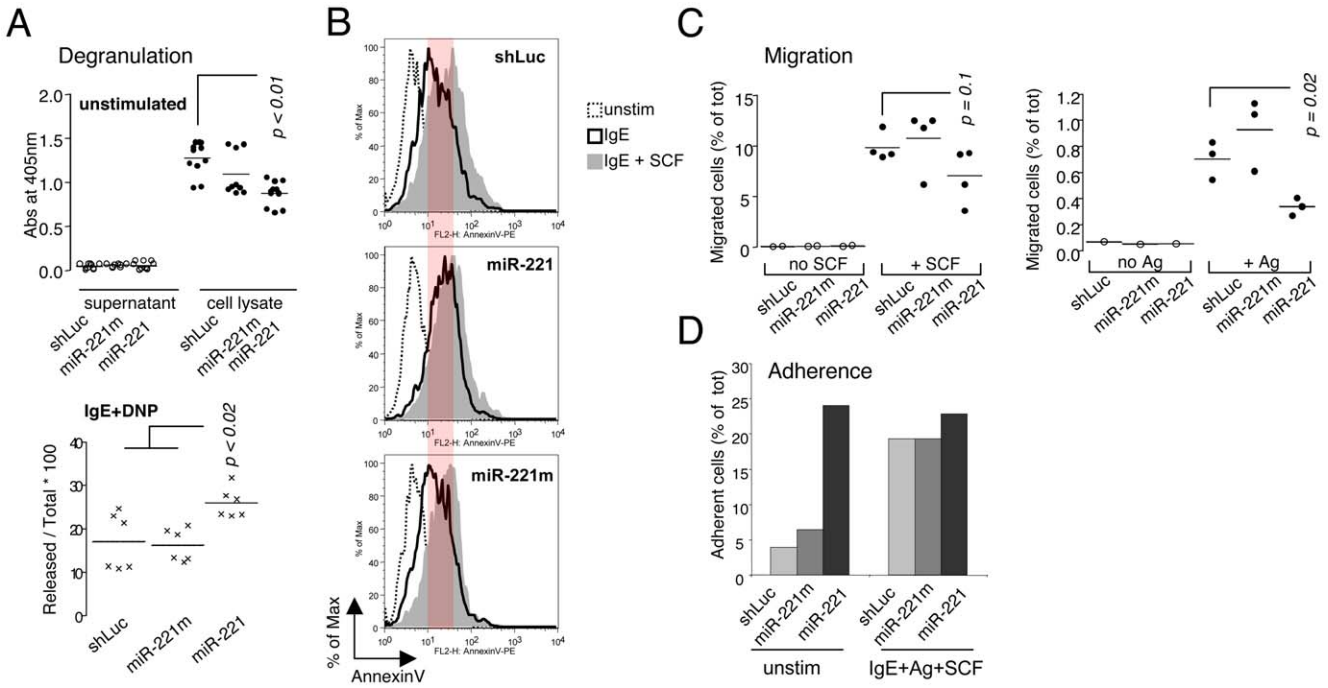

Figure 2. Degranulation, migration and adherence of cells overexpressing miR-221. A) Transduced BMMCs were either left unstimulated (*upper panel*; n = 5 experiments, each performed in triplicate) or were stimulated with IgE anti-DNP and DNP-HSA for 1h (*lower panel*; n = 2 experiments, each performed in triplicate) prior measurement of β-N-acetylhexosaminidase release to assess degranulation. **B**) Transduced BMMCs were either left unstimulated or were stimulated for 30min with IgE-antigen complexes alone or in combination with 20ng/mL of SCF. Cells were then stained with annexin V-PE to assess the extent of degranulation (representative of 2 independent experiments). The red shading in the figure indicates the difference between IgE versus IgE+SCF stimulation in control cell, which is reduced in miR-221-expressing cells). **C**) *Right panel*: BMMCs transduced with indicated vectors were seeded in the upper well of a transwell chamber and then allowed to migrate towards the lower chamber containing no chemoattractant (empty circles) or SCF (black circles). Each circle represents one independent experiment. *Left panel*: same as above, except that cells were sensitized with anti-DNP IgE overnight, and allowed to migrate towards the antigen (DNP-HSA). **D**) BMMCs transduced with the indicated vectors were seeded on a monolayer of 3T3 cells, and were either left resting or were stimulated for 8h with IgE-antigen complexes and SCF. Cells were then detached by treatment with 10mM EDTA, and the percentage of adherent cells versus total was assessed by surface staining for KIT and FACS analysis. Shown is one experiment out of at least six.
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compared to the controls (shLuc and miR-221m) miR-221 overexpression increased degranulation in response to IgE, as shown already by β-hexosaminidase assay, but miR-221-overexpressing cells did not further degranulate in response to the combination of both SCF and IgE crosslinking (middle panel). This could be due to the fact that the SCF receptor KIT is expressed at lower levels on these cells (see below), or to the fact that in the presence of miR-221 cells are activated more strongly upon IgE crosslinking, and cannot be further activated by the combination of IgE and SCF.

Next, we investigated the capability of BMMCs to migrate in a transwell system (Figure 2C). We found that cells overexpressing miR-221 migrated significantly less towards SCF as compared to the controls (Figure 2C, *right panel*). KIT is a target for miR-221 [24], therefore, to understand whether the reduced migration was due to an intrinsic feature of cells overexpressing miR-221, or to a reduced ability to 'sense' SCF in the environment due to lowered expression of KIT, we repeated the same experiment sensitizing BMMCs with IgE-anti-DNP prior inducing migration towards DNP-HSA (Figure 2C, *left panel*). The discernible, albeit modest, migration of control-transduced cells towards the antigen was significantly impaired if miR-221 was overexpressed, indicating that the reduced migration was due to effects of miR-221 on targets other than KIT.

Another process promoted by the stimulation through the FcεRI is adherence of mast cells to the substrate. Since mature mast cells do not normally circulate *in vivo*, but reside in tissues, we explored whether miR-221 had any role in regulating cell adherence and migration, as these are essential processes not only under normal homeostatic conditions, but also during inflammation and tumorigenesis. As *in vitro*-differentiated mast cells grow in suspension, we assessed the ability of BMMCs to adhere to a feeder layer of fibroblasts in a co-culture system (Figure 2D). Increased adherence is a normal process observed upon stimulation of mast cells with IgE and antigen, however, BMMCs expressing miR-221 adhered at a higher percentage compared to the controls even in resting, unstimulated conditions. Vice versa, upon stimulation all cells were able to adhere to the feeder layer of fibroblasts at comparable levels, regardless of miRNA expression. Since miR-221 overexpression was sufficient by itself to increase adherence, our data indicate that endogenous miR-221/-222 upregulation upon cell activation may contribute to the increased adherence of mast cells observed upon stimulation.

These results may point towards a role for miR-221 in regulating the signaling cascade originating from the FcεRI. In this context, it is important to highlight that miR-221 overexpression did not alter surface expression of FcεRI (see below). To evaluate whether signaling from the FcεRI could be affected in presence of miR-221, we assessed the levels of ERK phosphorylation in response to IgE crosslinking (Supplementary Figure S2). In all conditions and time-points tested, there was no significant difference in ERK phosphorylation in cells overexpressing miR-

221 compared to the controls, suggesting that miR-221 might not affect directly the signaling cascade from the FcεRI. However, in miR-221-expressing cells, in addition to increased degranulation (Figure 2, panels A and B), we also observed increased cytokine production (IL-6 and TNFα) in response to IgE crosslinking, but not to LPS (Supplementary Figure S2 and data not shown). This observation may indicate that miR-221 expression favors mast cell activation in response to IgE-antigen complexes, however in a way that doesn't seem to grossly affect ERK phosphorylation.

Microarray analysis identified genes affected by miR-221

To gain insight into the mechanisms underlying such pleiotropic effects of miR-221 in mast cells, we performed a microarray analysis of BMMCs overexpressing miR-221 or miR-221m, as it has been reported that indeed the impact of a miRNA on protein production can be closely approximated using mRNA arrays [27]. Out of ~42.000 transcripts analyzed, we found 397 significantly upregulated genes in miR-221-expressing BMMCs, as well as 343 significantly downregulated genes as compared to cells transduced with miR-221m (Figure 3A). Importantly, the known miR-221 targets *Cdkn1b* ($p27^{Kip1}$) and *Kit* were found to be downregulated with a mean fold-change repression of -3.2 and -2.0, respectively. While the upregulated genes presumably reflect secondary changes due to changes in the expression of primary targets, the downregulated gene subset may contain both primary targets as well as secondary effects.

Of note, when we compared the list of our downregulated genes with the targets predicted by TargetScan [9] and microCosm [28], we found very few overlapping genes, which could indicate either that only few downregulated genes are the real primary targets, or that many targets are relevant only in the specific mast cell context (Figure 3B). To better understand how miR-221 could regulate gene expression specifically in mast cells, we therefore performed a Sylamer analysis [19] on the complete gene list from the arrays, which was ranked from most downregulated to most upregulated (left to right in the graph in Figure 3C). Sylamer is an algorithm for determining whether specific 6, 7 or 8nt motifs corresponding to miRNA seed sequences are enriched or depleted in a ranked gene list [19]. The Sylamer plots can be used to analyze influence of a miRNA on an expression set profile. Sylamer calculates enrichment of seed words in 3′UTRs of a sorted gene list, providing a p-value for genes left of each rank versus the genes on the right. In this case, the upper left portion of the plot represents an influence of miRNAs on downregulated genes (Figure 3C), indicating a specific influence of miR-221 on a subset of downregulated genes. Any point of a line provides a p-value that a word is enriched in the UTRs left of it on the X-axis. Although in some cases most of the effect of a given miRNA in a particular cellular context goes

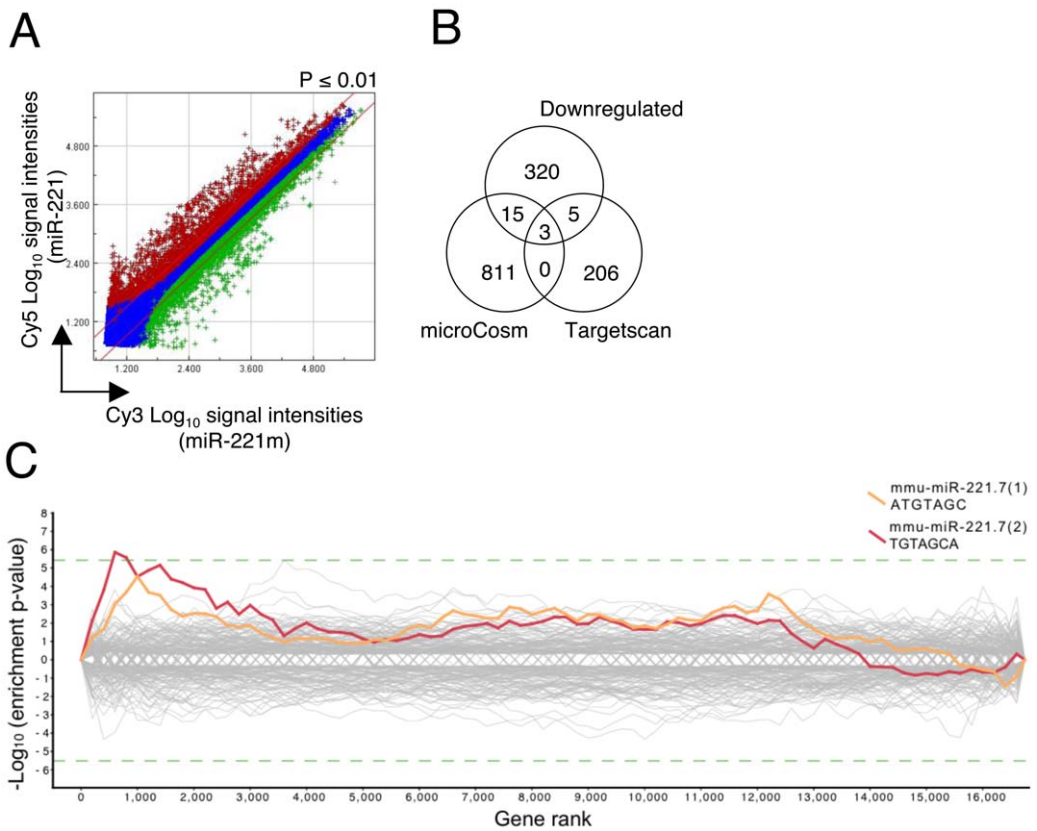

Figure 3. Identification of genes dysregulated upon miR-221 overexpression in BMMCs. A) Double-log scatter plot comparing the differential expression of mRNAs in BMMCs transduced with the miR-221 and miR-221m. Red diagonal lines define the areas of 2-fold differential signal intensities. Blue cross: unchanged genes. Red cross: significantly upregulated genes (p-value <0.01). Green cross: significantly downregulated genes (p-value <0.01). **B**) Comparison between significantly downregulated genes and genes predicted to be miR-221 targets by microCosm and Targetscan. **C**) Sylamer plot analysis for the Seed Complementary Region (SCR) words corresponding to the seed of miR-221 (red) and its one-nucleotide shifted sequence (orange). Log_{10}- transformed and sign-adjusted enrichment P values for each SCR word, relative to P values of all other words, are plotted on the Y-axis, against the ranked gene list on the X-axis (left, downregulated genes; right, upregulated genes). The dashed green line defines the Bonferroni corrected p-value cutoff for significance of 0.05.
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primarily through one or few molecular targets, in other cases this does not provide the complete picture of miRNA effect on the transcriptome and proteome of a cell [27,29,30]. Our array analysis showed that the transcriptomes of miR-221-overexpressing cells differed by the expression of 740 genes compared to the controls, and the Sylamer analysis indicated that many of the downregulated genes in mast cells were likely to be directly affected by miR-221 expression, suggesting that the effect of this miRNA goes through the fine modulation of a multitude of targets.

Some of the targets that were up- or downregulated were also confirmed at protein level; among others, we selected two of the already known targets for miR-221, *Kit* and *Cdkn1b* [8,24,31], as well as one upregulated gene (*Il2Ra*) that is particularly relevant in mast cell biology as a marker for systemic mastocytosis [32,33]. As assessed by surface staining, levels of KIT, but not of FcεRI, were significantly diminished (~2-fold) in BMMCs overexpressing miR-221 (Figure 4A). In contrast, as we previously described [8], levels of $p27^{Kip1}$ protein were especially decreased in stimulated cells (Figure 4B, compare lane 4 with lanes 2 and 6). While CD25 (*Il2Ra*) is normally not expressed by human mast cells in non-pathogenic conditions [32,33], it is normally expressed by a subset of *in vitro*-differentiated murine BMMCs (Figure 4C) as well as by a subset of peritoneal and tissue mast cells in the mouse (Deho' and Monticelli, unpublished observation). The mean fold increase for *Il2Ra* from the arrays was 2.1, which strongly correlated with the increased surface expression of this marker in cells overexpressing miR-221 (Figure 4C).

Although some of the targets for miR-221 were individually confirmed, our arrays experiments and Sylamer analysis indicated that miR-221 determines the downregulation of more than 200 primary targets and the subsequent secondary alterations of many genes in the transcriptome. Therefore, to gain insights into the function of the genes that showed altered expression in the presence of miR-221, we performed functional grouping and gene ontology

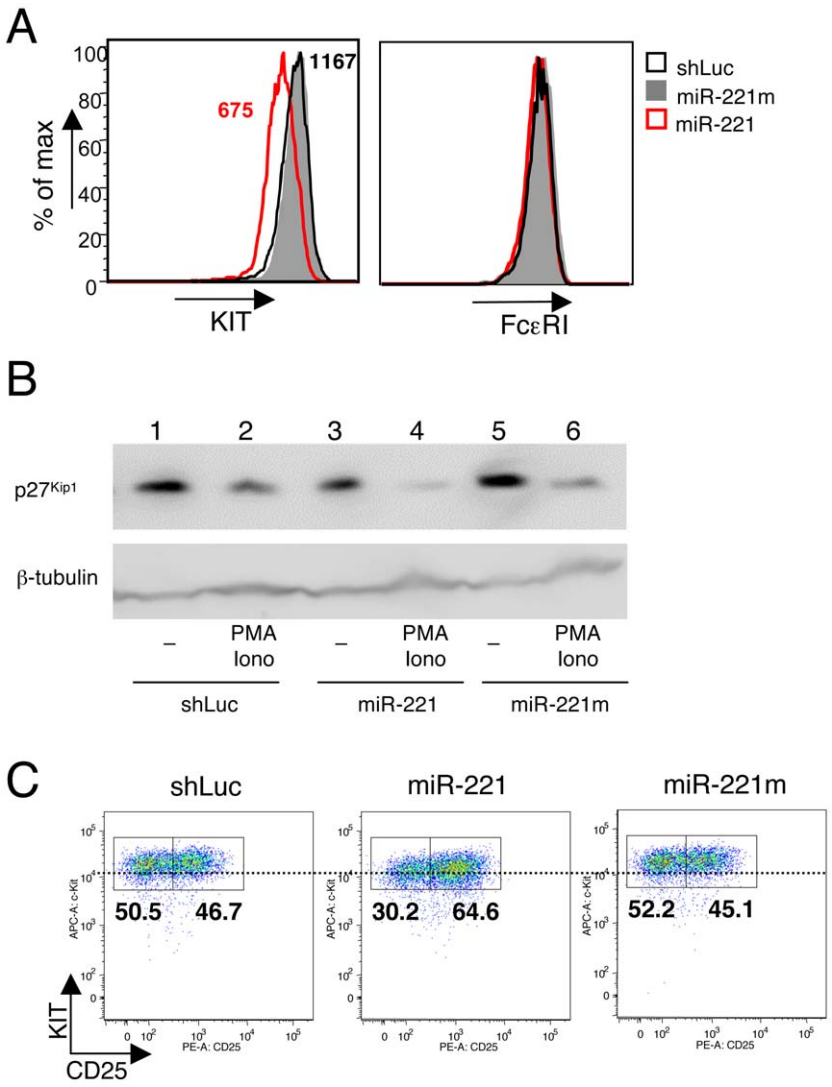

Figure 4. MiR-221 primary and secondary targets are altered also at protein level. A) Differentiated BMMCs were either left untransduced or were transduced with the indicated vectors. After selection with puromycin, surface expression of KIT (*left*) and FcεRI (*right*) was assessed by FACS. The mean fluorescence intensity (MFI) for KIT expression in miR-221- (red) and shLuc- (black) expressing cells is also indicated. **B**) Differentiated BMMCs as in A) were either left untreated or were stimulated with 20nM PMA and 1 μM ionomycin for 24h prior lysis and Western blot analysis of $p27^{Kip1}$ expression. β-tubulin was used as loading control. **C**) BMMCs as in A) were stained and FACS-analyzed to assess surface expression of KIT and CD25. The horizontal dotted line was added as a reference to appreciate the expected KIT dowregulation in miR-221 expressing cells.
doi:10.1371/journal.pone.0026133.g004

(GO) analysis, and found that many basic biological processes were affected (Table 1). Among the downregulated genes, the categories that were statistically significant were surprisingly few, and belonging for the most part to very general metabolic pathways, such as protein folding, transcription, lipid metabolism and cell differentiation. This finding may suggest that in resting, unstimulated cells, miR-221/-222 regulate basic metabolic processes that are common to many different cell types and/or species, and correlates with the fact that these miRNAs belong to a family very conserved in evolution, down to at least zebrafish [8]. Although they are most likely due to secondary changes in the transcriptome, the GO categories of the upregulated genes included several pathways that we also observed altered experimentally, such as cell proliferation, cell adhesion and cell migration. Among these latter GO categories, we focused our attention on one of the most statistically significant one, namely the cytoskeleton (which was also present in the GO list of terms for the downregulated genes, although with a p-value of 0.1), as we hypothesized that an alteration in the ability of the cells to properly regulate the cytoskeleton could to some extent explain why these miRNAs affect many different biological processes, at least in unstimulated cells.

MiR-221-dependent alterations of the cytoskeleton and actin content in mast cells and 3T3 cells

To assess whether miR-221 expression could affect the cytoskeleton, we stained transduced BMMCs with phalloidin (Figure 5A). We used the same co-culture system with fibroblasts used in Figure 2D to assess cell adherence, and to distinguish mast cells from the underlying layer of feeder cells (also stained by phalloidin), BMMCs were first labeled with CFSE. Strikingly, not only miR-221-overexpressing cells showed increased numbers of adherent cells (as shown also in Figure 2D), but while the actin ring underneath the plasma membrane was barely visible in control cells, cells overexpressing miR-221 (or miR-222, not shown) showed the presence of a much thicker ring (Figure 5A). Moreover, when we quantified the overall cellular amount of F-actin in cells depleted for miR-221 (using the miRT-depleting vectors), we observed a small but reproducible decrease in the amount of F-actin present in these cells (Figure 5B), further indicating that these miRNAs might be important regulators of the actin organization in mast cells.

Table 1. Gene Ontology (GO) frequency distribution for downregulated and upregulated genes.

DOWNREGULATED – Categories	Times Observed	Probability
Cell migration	15	5.30E-05
Protein folding/modification	30	1.80E-04
Transcription	38	5.70E-04
Lipid metabolism	17	2.30E-03
Cell differentiation	28	3.20E-03
Other metabolism	29	8.00E-03
UPREGULATED – Categories	**Times Observed**	**Probability**
Receptor signaling	73	9.40E-09
Cell proliferation	26	4.60E-08
Cytoskeleton	35	4.70E-08
Cell cycle	27	6.40E-08
Cell adhesion	26	3.30E-07
Cell differentiation	37	1.10E-05
Kinase/Phosphatase signaling	23	1.30E-05
Other metabolism	39	1.90E-05
Protein folding/modification	35	2.50E-05
Development	46	2.90E-05
G-protein signaling	43	8.80E-05
Cell migration	15	3.10E-04
Nucleotide metabolism	26	3.70E-04
Inflammation	11	1.00E-03
Intracellular trafficking	20	5.08E-03

Gene ontology (Bonferroni corrected) of mRNAs that were downregulated or upregulated in the microarrays. A cutoff of P = 0.01 was used for the categories to be considered statistically significant.
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To independently confirm these results, and to investigate whether the observed effect was a general feature of this miRNA or a cell type-specific effect due to alterations of targets relevant only in the mast cell context, we transduced 3T3 fibroblasts with the same lentiviral vectors used on mast cells. 3T3 cells expressed low levels of endogenous miR-221 that were increased ~20-folds upon transduction with a miR-221 expressing vector (Figure 6A). MiR-221 overexpression in 3T3 cells led to a strong downregulation of endogenous $p27^{Kip1}$, even more remarkable than the one observed in mast cells (Figure 6B). Despite such strong downregulation of the cell-cycle inhibitor $p27^{Kip1}$, 3T3 cells overexpressing miR-221 showed the same reduced proliferation that we previously described for mast cells (Figure 6C) [8]. Moreover, 3T3 cells overexpressing miR-221 showed overall altered morphology, with odd, elongated and/or irregular shapes (Figure 6D), as well as a slightly increased content of F-actin (Figure 6E), indicating that the miR-221-dependent effects on the cytoskeleton and cell cycle observed in resting mast cells are likely to be due to the dysregulation of targets that are ubiquitously expressed and are therefore cell type-independent. However, FcεRI stimulation led to mast cell-specific (or at least not present in fibroblasts) effects of miR-221, with increased degranulation and cytokine production.

Analyzing the data from our transcriptome profiling, we found that in the 'cytoskeleton' group of downregulated genes, the top candidate, most downregulated gene was *Cdkn1b* ($p27^{Kip1}$), and specifically the one splice variant that can be regulated by miR-221/-222 (as we previously described in [8]). While $p27^{Kip1}$ is a cell cycle inhibitor with a well established role in cell cycle progression at the G1-S transition, it has also been shown that cytoplasmic $p27^{Kip1}$ plays an important role in cell motility and migration, and that $p27^{Kip1}$-deficient fibroblasts fail to form long cellular protrusions, assume an overall rounded shape [34] and show reduced migration [35]. To assess whether miR-221-dependent down-regulation of $p27^{Kip1}$ may have a role in regulating 3T3 and mast cells shape and cytoskeleton, we therefore performed a knockdown of $p27^{Kip1}$ in 3T3 cells using siRNAs (Figure 7). Efficiency of transfection and $p27^{Kip1}$ knockdown were evaluated by transfection and FACS analysis of a fluorescent ds-oligo (siGLO) and by Western blot, respectively (data not shown and Figure 7A). It has to be noted that the efficiency of transfection was at the most ~70%, so that the residual protein observed in Western blot may in part be due to the fact that some cells still expressed significant levels of $p27^{Kip1}$. However, the knockdown of $p27^{Kip1}$ did not alter the overall cell-cycle profile of 3T3 cells (Figure 7B), and the cells did not show any particularly altered shape, apart from a slight increase in the percentage of cells that were smaller and more rounded (Figure 7, panels C and D). Although this effect was fairly modest (even in the experiments

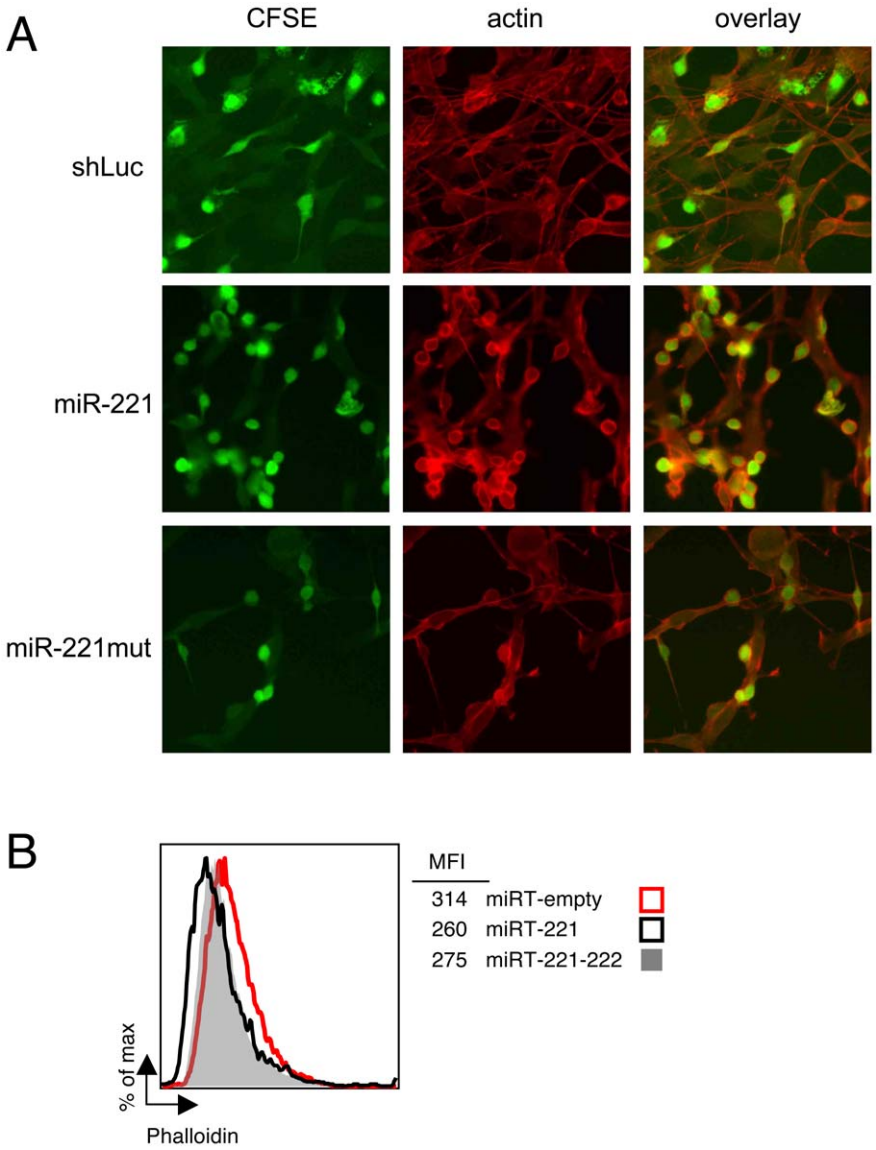

Figure 5. MiR-221 influences BMMCs actin cytoskeleton. A) Transduced BMMCs were loaded with CFSE (green) and allowed to adhere to a monolayer of 3T3 cells. After washing to remove non-adherent cells, F-actin was stained with phalloidin-AlexaFluor-594 (red) prior imaging with a fluorescence microscope. Shown is one representative experiment out of three. **B**) BMMCs were transduced with the indicated miRT vectors, and overall F-Actin content was assessed by FACS staining with phalloidin. Shown is one representative experiment out of three.
doi:10.1371/journal.pone.0026133.g005

with the strongest downregulation of $p27^{Kip1}$ only <15% of the cells were counted as 'small and round', compared to the controls), it was in line with what was previously reported for *Cdkn1b*-deleted fibroblasts. Indeed, $p27^{Kip1}$ KO fibroblasts were shown to have a rounded shape with no alterations in the cell cycle [34]. Most importantly, the knockdown of $p27^{Kip1}$ did not recapitulate the phenotype we observed in miR-221 overexpressing 3T3 cells, as cell cycle and cellular shape were either unaltered or completely different from what we observed in miR-221-transduced cells, suggesting (as indeed indicated by our Sylamer analysis), that the effect of this miRNA is composite and goes through the down-modulation of multiple targets.

Although the mechanisms underlying the role of miR-221 specifically in mast cells in both resting and stimulated conditions will require further investigation and will be the subject of future work, our data show that the effect of this miRNA goes through the alteration of the levels of many targets in the mast cell transcriptome, that it has important roles in regulating mast cell physiology, and finally that at least some of its biologic effects in resting cells may be explained by alterations in the actin cytoskeleton of mast cells.

Discussion

Although mast cells have a long lifespan, accumulation of a large mast cell burden *in vivo* is usually not observed. Thus, a homeostatic mechanism must exist to limit differentiation and accumulation of mast cells in peripheral tissues, both during basal maintenance, and during mast cell hyperplasia in inflammatory processes [36]. MiR-221 is a likely candidate as a regulator of mast cell functions: we previously showed that it is transcriptionally induced upon mast cell activation, and that it contributes to the modulation of proliferation in unstimulated mast cells [8]. We now showed that miR-221 may have more ubiquitous effects to fine-

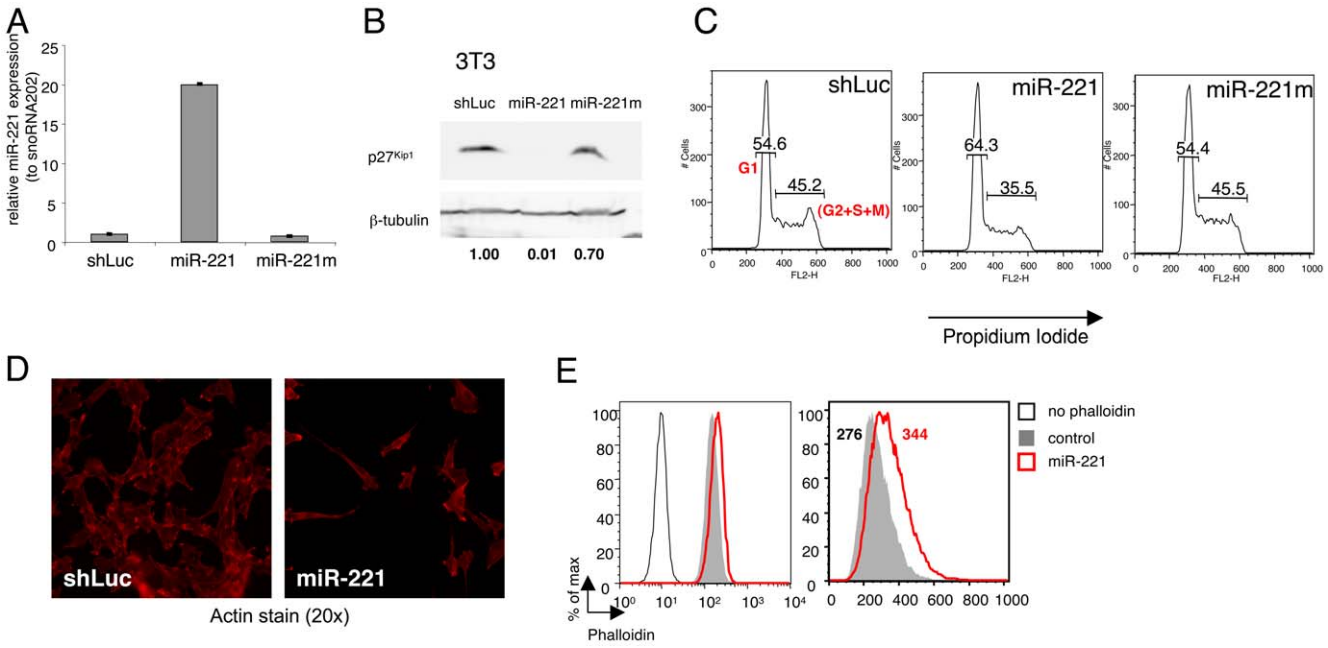

Figure 6. MiR-221 affects cell proliferation and actin cytoskeleton in fibroblasts. A) 3T3 cells were transduced with lentiviral vectors to express either miR-221 or controls shLuc and miR-221m. After selection with 2 μg/mL for at least 2 days, total RNA was extracted and levels of miR-221 expression were assessed by TaqMan qRT-PCR. SnoRNA202 was used as endogenous control; for comparison, levels of miR-221 expression in the shLuc-transduced sample were set to one. Representative of two independent experiments. **B**) 3T3 cells as in A) were lysed in Laemmli sample buffer and $p27^{Kip1}$ expression was analyzed by Western blot. Expression of β-tubulin was used for normalization in the quantification of bands intensity (numbers below the blot). One representative experiment out of 3 is shown. **C**) Cells as in A) were collected during exponential-phase growth and the DNA content was assessed with propidium iodide staining as a measure of the number of cells in each stage of the cell cycle. For simplicity, only the percentage of G1 cells (left in each plot) versus the percentage of dividing cells (S+G2+M) are shown. One out of three independent experiments is shown. **D**) Cells as in A) were seeded on a coverslip and allowed to adhere overnight, after which they were fixed in 4% paraformaldehyde and stained with phalloidin-AlexaFluor-594 prior imaging with a fluorescent microscope. Shown is one representative experiment out of two. **E**) Cells as in A) were trypsinized to single cell suspension, fixed and stained as in B) and analyzed by FACS. The left plot shows (on a log scale) the unstained control compared to stained samples, while on the left the scale was changed to linear to better show the differences among samples. The MFI for each sample is also provided. Depending on the experiment, control cells were either untransduced cells, cells transduced with shLuc- or the miR-221m-expressing vector, or all of the above. Shown is one representative experiment out of four.
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tune proliferation and actin cytoskeleton in cells as different as resting mast cells and fibroblasts. On the other hand, miR-221 also influenced many other features of differentiated mast cells, including cytokine production, migration, adhesion and survival upon withdrawal of essential cytokines (Supplementary Figure S3), all mechanisms that may be involved in regulating tissue accumulation and resolution of hyperplasia upon eradication of inflammation *in vivo*.

While we were able to show that at least some of these effects may be linked to a miR-221-dependent regulation of the actin cytoskeleton in mast cells, this miRNA may have both 'house-keeping' functions, active in different cell types, like the regulation of the cytoskeleton and cell cycle, but also cell-specific effects on targets such as KIT and CD25 that are expressed by mast cells but not fibroblasts. As for targets, our arrays data and bioinformatics analysis showed that expression of miR-221 led to the downregulation of 343 genes, many of which are likely to be primary targets, containing the miR-221 seed-complementary sequence in their 3′UTR. For this reason, we think that the effect of miR-221 in resting mast cells may be composite and due to small alterations of many genes in the transcriptome. However, since we did not perform knockdown of every individual potential target, the possibility remains that the effect of miR-221 in mast cells and fibroblasts might be mediated by the downregulation of one or few predominant genes that we were so far unable to identify. Yet, although levels of $p27^{Kip1}$ in miR-221 overexpressing fibroblasts were very low, the individual knockdown of this protein led to a phenotype that somewhat resembled the phenotype of $p27^{Kip1}$-deleted fibroblasts, but did not resemble in any way the phenotype of miR-221 overexpressing cells, suggesting that miR-221 target regulation is more complex then the downregulation of one predominant target.

While it is clear that miRNAs regulate many different cellular processes, understanding the details of the functions of individual miRNAs remains challenging. Indeed, as in the case of miR-221 and -222, miRNAs are frequently present as families of redundant genes. MiR-221 and -222 share the same seed sequence (Figure 1B), and should recognize the same targets [9]; although most of the experiments shown here were performed primarily with miR-221, we also performed experiments using miR-222-expressing vectors, which always gave results similar to miR-221, both in mast cells and in fibroblasts (not shown). Another difficult aspect of dealing with miRNAs is that each miRNA has many potential targets with disparate functions, with no means to decide a priori which is the most meaningful and therefore worthy of experimental validation. Finally, the degree of target downregulation is typically less than 50% [29,37], and understanding which fraction of the miRNA:-target interaction is actually relevant for a biological response remains a challenging task. In our hands, $p27^{Kip1}$ was a striking example of this phenomenon, as while it was downregulated very strongly upon miR-221 expression, its knockdown did not recapitulate the complex effect of miR-221 expression.

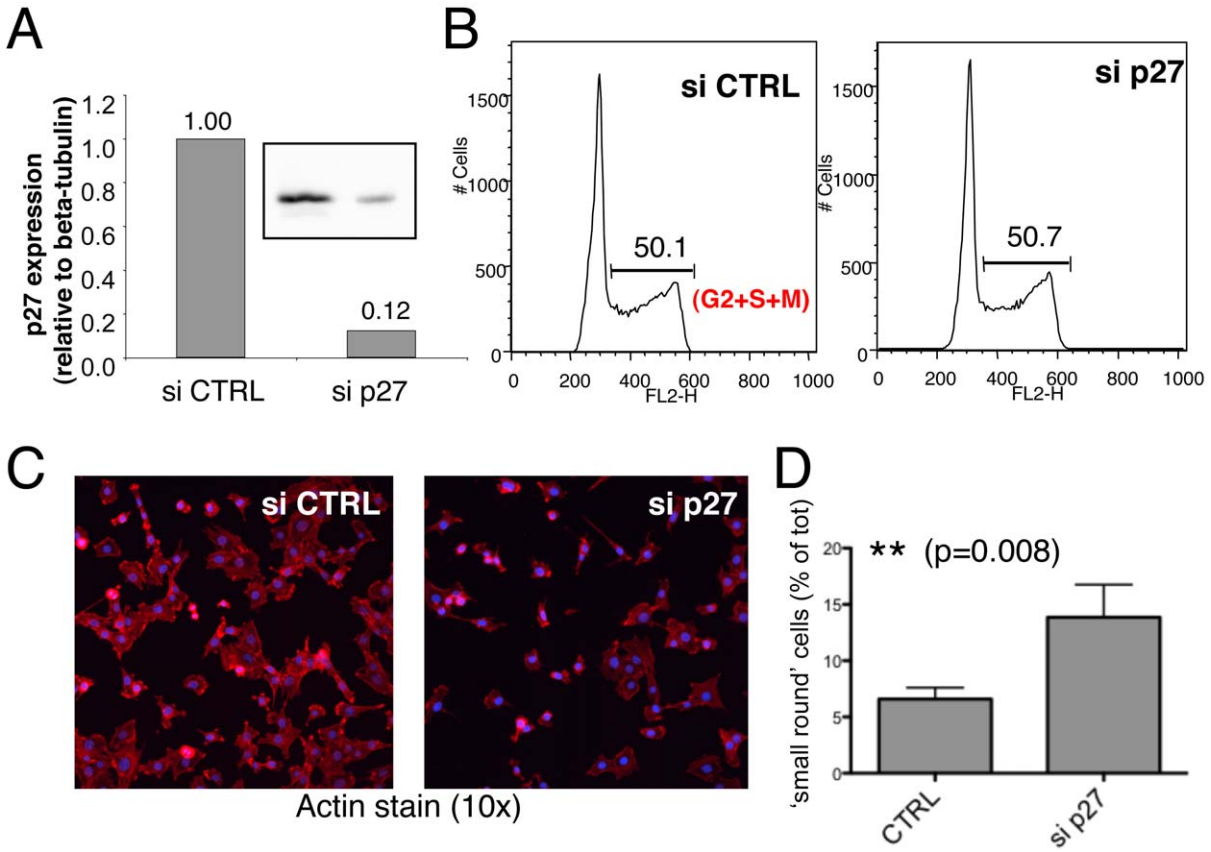

Figure 7. Knockdown of the miR-221 target p27^{Kip1} does not recapitulate the composite effects of miR-221 expression. A) 3T3 cells were transfected with siRNA oligonucleotides against p27^{Kip1} (si p27) or with a non-targeting control (si CTRL). 48–72 h later cells were collected and lysed for Western blot analysis. Shown is the quantification (against β-tubulin, used as loading control) of one representative blot; the actual blot for p27^{Kip1} is shown in the inset. Efficiency of transfection ranged between 50–70% depending on the experiment, with a downregulation of protein expression as assessed by Western blot that ranged widely between 0.05–80% of the levels of control, with the latter case (highest expression, minimal knockdown) showing no phenotypic effect. **B**) 3T3 cells as in A) were trypsinized to single-cell suspension, fixed in ethanol and stained with propidium iodide for cell-cycle analysis. **C**) Cells as in A) were seeded on coverslips and allowed to adhere overnight prior fixation with 4% paraformaldehyde and staining with phalloidin-AlexaFluor-594. Nuclei were counterstained with DAPI (blue). **D**) Quantification of the percentage of 'small, round' cells observed in an experiment as in C). For each sample, 8 random fields were counted, at a magnification of 10x.
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Although clearly able to regulate some general cellular features, such as cell cycle and cytoskeleton in resting mast cell, we speculate that miR-221/-222 may also be part of a mechanism that contribute to mediate many of the changes that occur in mast cells upon stimulation. Specifically, miR-221 may have a 'housekeeping' function in resting mast cells, where it is expressed at low, basal levels, and contributes to the regulation of the cell cycle and cytoskeleton. Vice versa, miR-221 is also transcriptionally activated upon stimulation, and in this case it would contribute to the regulation of cell-type specific, FcεRI-dependent mechanisms, such as cytokine production, degranulation and cell adhesion. In this perspective, it is important to highlight that although transcription of *pri-miR-221-222* starts early upon IgE stimulation, mature miR-221 accumulation occurs with a 'slow' kinetic (the peak of mature miRNA expression is reached at ~24h after initial stimulation) [8]. We therefore speculate that the slow kinetic of accumulation of the mature form of miR-221 may actually contribute to a resting, but 'activation-ready' cellular state, that favors increased degranulation, adherence and cytokine production upon challenge (Supplementary Figure S4).

Indeed, mast cell stimulation normally leads to increased adherence and increased survival of the cells, which can be further activated by a secondary challenge [26,38]. Upon secondary encounter with the antigen mast cells respond with increased degranulation and cytokine production (reviewed in [38]). Although such higher levels of degranulation and cytokine production are usually considered to be the result of increased levels of FcεRI, the fact that miR-221-expressing cells showed no perturbation of FcεRI expression and at the same time increased adherence (even in the absence of stimulation), increased degranulation as well as cytokine production suggests that miR-221 may contribute to such intensified cellular response upon secondary challenge. The processes mediated by this miRNA may also have a role in regulating mast cell homeostasis in tissues and possibly also in pathologic conditions. It will be therefore interesting to assess what is the role of miR-221/-222 *in vivo* in mouse models, both in terms of cell homeostasis at steady-state or upon sensitization and challenge with an antigen or with a pathogen, and this will be the subject of future work.

Along the line of a possible role of these miRNAs in mast cell-related diseases, we showed that miR-221 and miR-222 regulate levels of KIT expression in mast cells. Of note, KIT levels are often abnormally low in patients with indolent systemic mastocytosis, although the D816V gain-of-function mutation in KIT is

present in most of these patients and is sufficient to cause indolent mastocytosis [39]. This downregulation of KIT expression is even more pronounced in poor prognosis systemic mastocytosis (i.e. systemic mastocytosis associated with a clonal hematopoietic disease, aggressive systemic mastocytosis and mast cell leukemia) [39], suggesting that multiple layers of regulation of this receptor may be acting in diseased cells. In this context, it will be interesting to assess whether miR-221/-222 could be part of a molecular mechanism involved in KIT regulation specifically in mastocytosis. Indeed, although the D816V mutation may be sufficient to cause indolent mastocytosis, other additional defects, that remain to be identified, are required to induce aggressive mast cell disorders, and may very well include dysregulated miRNA expression [40,41,42]. This is even more intriguing considering that CD25, which is so far the best available diagnostic marker for systemic mastocytosis with bone marrow involvement [32,33], is also regulated by these miRNAs. While miR-221/-222 were already implicated in various human cancers for their effect on proliferation, we show here for the first time that these miRNAs also regulate mast cell adhesion, migration, and survival, all processes that may have implications in mastocytosis.

In summary, although miR-221 doesn't seem to affect mast cell differentiation, it influences many features of the biology of differentiated cells. Specifically, at basal levels, in resting conditions, these effects are likely to be linked, at least in part, to the regulation of the actin cytoskeleton and cell cycle, two features that are regulated by miR-221 independently of the cell type. However, upon mast cell stimulation, miR-221 may have some more cell type-specific, activation-dependent effects, influencing the extent of degranulation, adherence and cytokine production in response to IgE-antigen complexes. Although still speculative, we propose a model in which miR-221 would have two different roles in mast cells: in resting cells, it contributes to normal cell homeostasis through the regulation of the cell cycle and cytoskeleton, while upon induction following acute stimulation, it contributes to increase the strength of the response to antigenic challenge. Overall, our work provides new insights into previously unknown effects of miR-221 in mast cell biology, and may have important implications for our understanding of the molecular mechanisms underlying normal and pathologic mast cell conditions.

Supporting Information

Figure S1 MiR-221 expression can be regulated by the transcriptional repressor PLZF, but it has no role in BMMC differentiation. A) Lineage depleted (Lin–, lacking surface expression of CD5, CD45R, CD11b, Gr-1, 7-4 and Ter-119) and Lin+ bone marrow cells were either immediately used for RNA extraction or differentiated to mast cells in IL-3 containing medium [43]. Total RNA from Lin– derived mast cells was used to assess *Plzf* mRNA expression (*upper panel*) and miR-221 (*lower panel*). **B**) Differentiated BMMCs were lentivirally transduced to ectopically express PLZF. After puromycin selection for 48h, cells were either left untreated or were stimulated with 20nM PMA and 1 μM ionomycin for 24h, prior RNA extraction and analysis of *Plzf* and miR-221 expression. **C**) Lin– cells were transduced with the indicated vectors to either force (pAPM) or ablate (miRT) miR-221 expression, and were cultured for three weeks in the presence of IL-3 to allow mast cell differentiation. Cultures were analyzed weekly for the presence of mast cells (FcεRIα+ KIT+) by surface staining. Each point represents one independent experiment. Cells transduced with shLuc, miR-221 and miR-221m vectors were selected with 2 μg/mL puromycin, while cells transduced with the miRT vectors (empty, T-221 and T-221-222) were FACS-sorted for GFP expression. **D**) Total RNA was extracted from cells treated as in C) at the end of the differentiation period (percentage of FcεRIα+ KIT+ cells was greater than 90%), and expression of miR-221 was assessed by TaqMan qRT-PCR. SnoRNA202 was used as endogenous control, with levels of miR-221 expression set to one in the shLuc-transduced sample. Cells transduced with shLuc and miR-221 vectors were selected with 2 μg/mL puromycin, while cells transduced with the miRT vectors (empty, T-221 and T-221-222) were FACS-sorted for GFP expression.
(TIF)

Figure S2 MiR-221 expression does not significantly alter ERK phosphorylation in mast cells, but favors cytokine production. A) Differentiated BMMCs transduced with the indicated vectors were sensitized with 1.5 μg/mL of IgE-anti-DNP for 15min on ice. After washing to remove unbound IgE, 200ng/mL of DNP-HSA were added, and the cells were immediately moved to a 37°C water bath for 5, 15 and 45min. Cells were subsequently fixed, permeabilized and stained with biotinylated anti-phospho-p44/42 MAPK (Erk1/2). The mean fluorescence intensity for each sample is indicated on the left. **B**) Cells as in A) were either left untreated or were stimulated for 5min with 1 μM ionomycin and 20nM PMA at 37°C, after which cells were fixed, permeabilized and stained with an anti-Erk1/2 antibody. The mean fluorescence intensity for each sample is indicated next to the histograms. Shown is one experiment out of two. **C)** BMMCs as in A) were stimulated with 1.5 μg/mL IgE-anti-DNP and 200ng/mL DNP-HSA for 3.5h at 37°C. To block export from the Golgi, brefeldin A (10 μg/mL) was added in the last two hours of stimulation. Cells were subsequently fixed, permeabilized and stained with anti-IL-6-PE and anti-TNF-α-PE-Cy7. One representative experiment out of three is shown.
(TIF)

Figure S3 MiR-221 expression favors mast cell survival in response to withdrawal of essential cytokines. BMMCs were transduced with the indicated vectors and differentiated in the presence of IL-3 only (*top panel*) or IL-3 + 10ng/mL SCF (*bottom panel*) for three weeks, after which all cytokines were washed out of the culture medium for at least 24h prior evaluation of early cell death with annexin V and 7AAD staining. Shown is the percentage of cells in early apoptosis (annexin V+ 7AAD–).
(TIF)

Figure S4 A 'dual' role for miR-221 in mast cells. Speculative model of the possible roles of miR-221 in mast cells. At resting state, basal levels of miR-221 expression would regulate homeostatic mechanisms such as the cell cycle and cytoskeleton. These effects are not necessarily cell type-specific, as they can be active also in fibroblasts, which also express miR-221. Upon mast cell activation, 'early' effects include the release of preformed mediators from the cytoplasmic granules and the *de novo* synthesis of other mediators, including a broad panel of cytokines. The peak of accumulation of mature miR-221 is instead a 'late' event upon cell stimulation, and we speculate that it may contribute to the strength of the response upon secondary challenge, with increased degranulation, cytokine production and cell adherence.
(TIF)

Methods S1
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: SM. Performed the experiments: RJM LD NR SM. Analyzed the data: SM. Wrote the paper: SM. Performed Sylamer analysis: NB HKS AJE.

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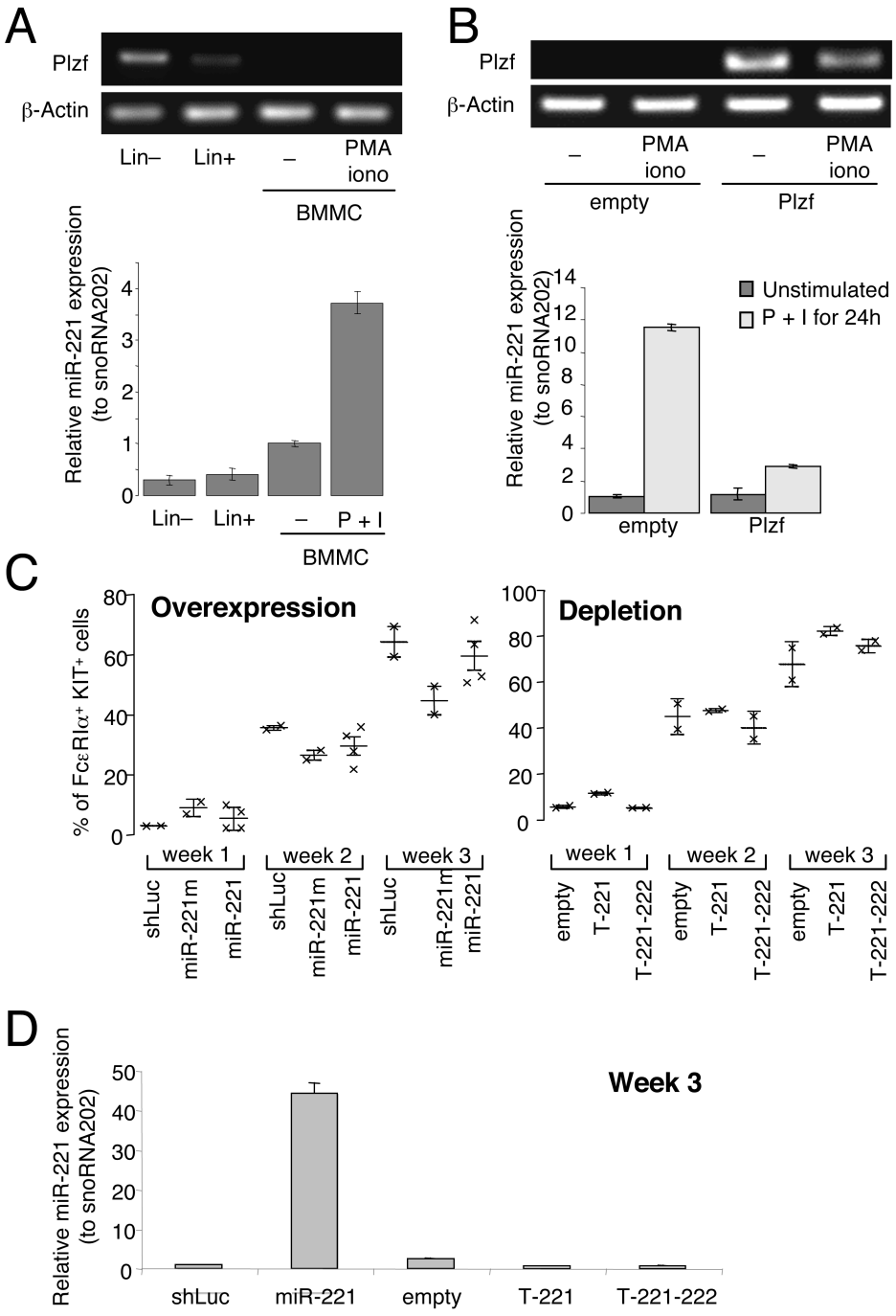
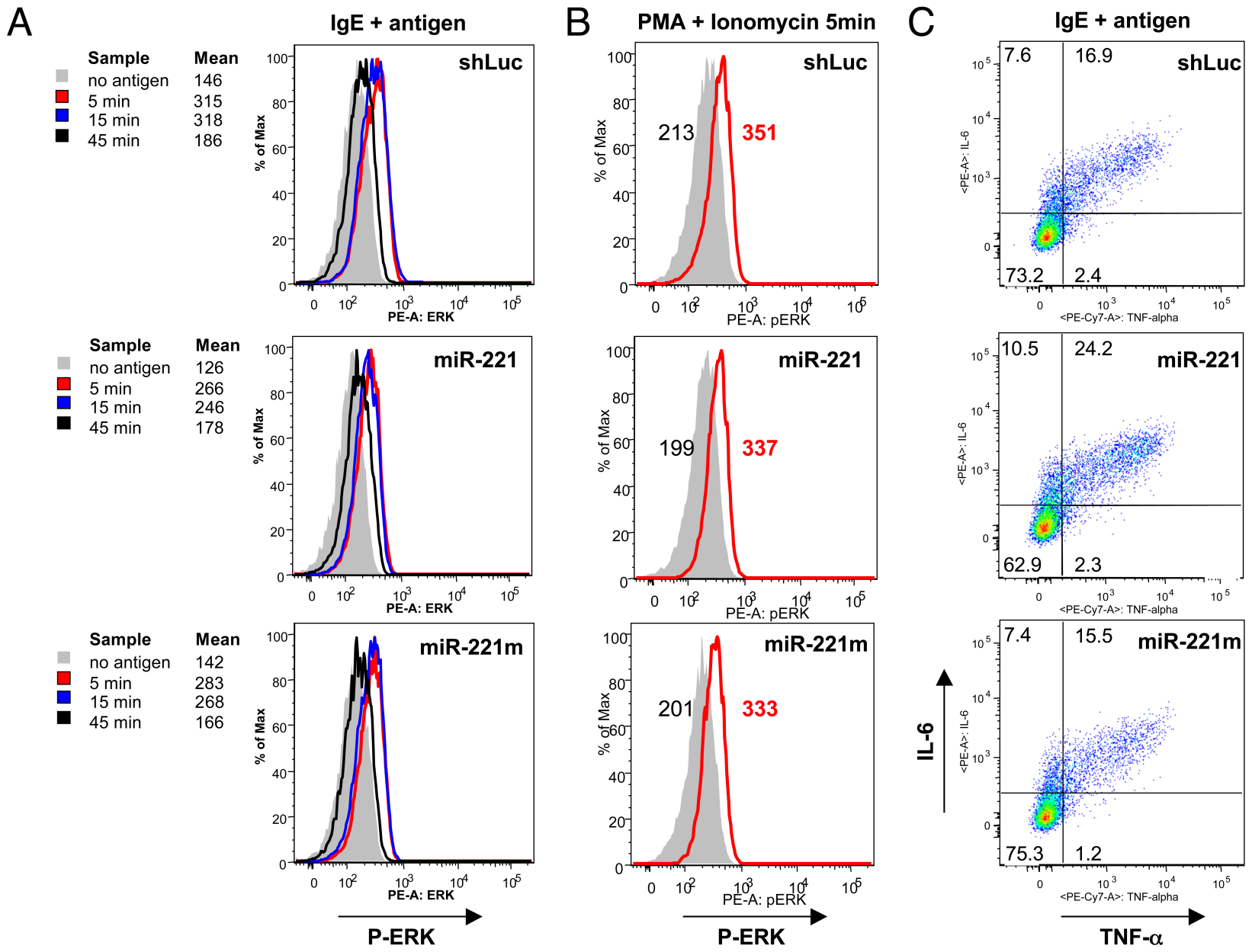
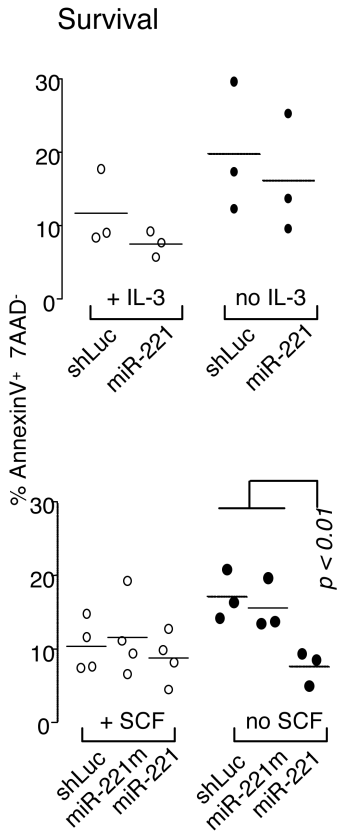


Mayoral *et al.* Supplementary Figure S1



Mayoral *et al.* Supplementary Figure S2



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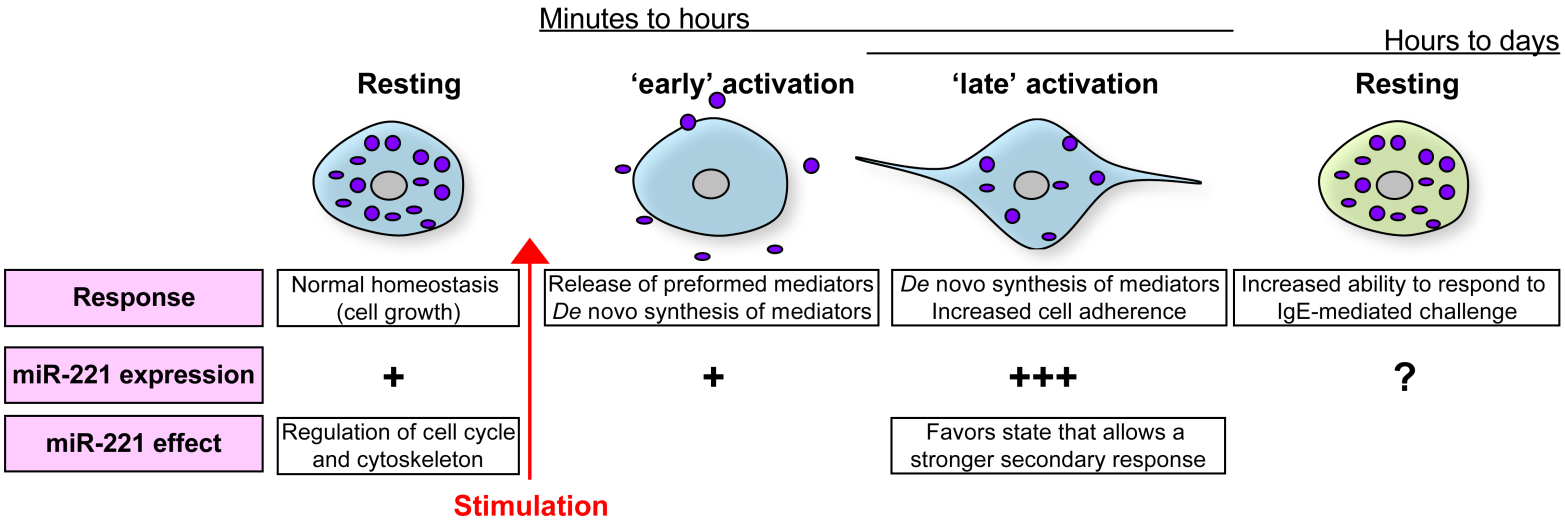

Mayoral *et al.* Supplementary Figure S4

SUPPORTING INFORMATION

SUPPLEMENTARY METHODS

Plasmids. Murine Plzf was provided by Wilfried Ellmeier [1], and it was subcloned into the scALPS vector [2] using standard cloning techniques.

Cell cultures. Lineage-negative (Lin–) cells [3] were enriched from bone marrow using a lineage-depletion kit (Miltenyi Biotec) and cultured for 48h in serum-free media (StemCell Technologies) containing 100ng/mL SCF, 100ng/mL Flt3L, 50ng/mL TPO, 20ng/mL IL-3 (all from Peprotech), antibiotics; media was then changed to regular BMMC media.

RT-PCR. For semi-quantitative RT-PCR, total RNA (1μg) was reverse transcribed using the iScript kit (Bio-Rad), before PCR amplification using the following primers: *PlzfFW*: 5'- CCTTTGTGTGTGATCAATGCGGTG; *PlzfRV*: 5'- TGGTGCTTGAGGCTGAACTTCTTG; *β-actinFW*: 5'- GCAGCTCCTTCGTTGCCGGT; *β-actinRV*: 5'- GGCTTTGCACATGCCGGAGC.

Apoptosis detection. Apoptosis was evaluated using the annexin V-PE apoptosis detection kit (BD-Pharmingen) following exactly manufacturer's instructions.

Intracellular staining. For intracellular cytokine staining, cells were stimulated with 1.5μg/mL IgE anti-DNP and 200ng/mL DNP-HSA in complete media for 3,5h at 37°C. Brefeldin-A (10μg/mL, Sigma) was added in the last 2h of stimulation. Cells were then fixed in 4% paraformaldehyde for 10min at RT, and permeabilized with 0.5% saponin, 1% BSA in PBS, prior staining with fluorochrome-conjugated anti-cytokines antibodies (anti-IL-6, anti-TNF-α, both from eBioscience) for 30min at RT. For intracellular staining of phospho-ERK, cells were sensitized with 1.5μg/mL of IgE-anti-DNP for 15min on ice. After washing to remove unbound IgE, 200ng/mL of DNP-HSA were added, and the cells were immediately moved to a 37°C water bath for an incubation time of 5, 15 and 45min. Alternatively, cells were stimulated with 1μM ionomycin and 20nM PMA at 37°C. Cells were subsequently fixed in 4% paraformaldehyde, permeabilized with 0.5% saponin, 1% BSA in PBS and stained intracellularly with biotinylated anti-phospho-p44/42 MAPK (Erk1/2) (Cell Signaling).

SUPPLEMENTARY REFERENCES

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Chapter 2

"NF-kBp50 regulates mouse mast cell survival in part through upregulation of miR146a"

Summary

Mice that lack the p50 subunit of NF-kB (p50KO) are unable to mount airway eosinophilia inflammation due to the inability to produce IL-4, IL-5 and IL-13, and to a defect in the polarization of Th2 lymphocytes. Since MCs are master effector cells in asthmatic and allergic responses, we evaluated whether the asthma-resistant phenotype observed in p50KO mice could be partially due to a defect in MCs development or function. Our data showed that p50KO MCs differentiated normally *in vitro* and produced reduced cytokines levels in response to different stimuli such IgE-crosslinking or LPS, indicating that although the airway inflammation defect observed in p50KO mice might be predominantly Th2-dependent, reduced cytokine production by MCs could also contribute to this phenotype.

Interestingly, we also found that p50KO MCs showed a marked increase in their ability to survive in response to withdrawal of essential cytokines, which likely correlated with a strong increase in the percentage of MCs that was observed in the tissues of p50KO animals. Since the identification of novel mechanisms that regulate MC proliferation, survival and overall homeostasis in the tissues may have important implications for the treatment of MC related diseases such as asthma, allergy and mastocytosis, we investigated the molecular mechanisms at the basis of such enhanced survival. Compared to normal controls, we found that MCs lacking p50 showed altered expression of several molecules involved in cell survival and cell death. Specifically, expression of the anti-apoptotic molecules Bcl2 and A1 was increased, while levels of the pro-apoptotic factor BAX were decreased.

Importantly, we also found that miR-146a, a miRNA known to regulate NF-kB signalling, was not expressed in IgE- or LPS-stimulated p50KO MCs, and that in the context of MC survival, miR-146a acted as a pro-apoptotic factor.

Introduction

MCs are derived from hematopoietic progenitor cells that enter nearly all vascularized tissues, where they complete their maturation. MCs are regarded as key effector cells in immediate hypersensitivity reactions and allergic disorders such cutaneous/mucosal allergy, asthma and mastocytosis. Asthma is a chronic inflammatory disease of the airways characterized by variable and recurring symptoms, reversible airflow obstruction, and bronchospasm[39-42]. When activated, MCs secrete mediators such as cytokines, histamine, chemokines, proteases and leukotrienes. These cells can be activated through a variety of stimuli, including direct injury, cross-linking of IgE or IgG receptors, TLRs or by activated complement proteins. If MC proliferation and homeostasis in the tissues is not properly regulated, it can lead to a myeloproliferative disorder known as mastocytosis, where an abnormal accumulation of pathogenic MCs can be observed in various organs such as skin, liver, spleen, lymph nodes and most commonly the bone marrow. The symptoms of the disease are caused by the release of MC mediators in the tissues, resulting in a progressive loss of function on the organ involved. This disorder can have a clinical course variably ranging from asymptomatic to highly aggressive and devastating, and understanding the molecular basis that regulate MC differentiation and function in normal as well as diseased conditions may lead to new treatments of MC-related disases[39-42].

NF-kB is a family of transcription factors involved in the regulation of many biological processes such as inflammation and innate immune responses[122]. Dysregulated NF-kB activation can have important outcomes in both allergy and tumor transformation. For example, mice that lack the NF-kB p50 subunit are unable to mount airway eosinophilic inflammation due to the inability to produce IL-4, IL-5 and IL-13, and to a defect in the polarization of Th2 lymphocytes[119].

Here, we investigated whether NF-kB p50 may have a role in regulating MC proliferation, differentiation, overall homeostasis in the tissues and activation, as it could improve our understanding of the molecular mechanisms at the basis of MC-related diseases such as asthma, allergy and mastocytosis. We provide evidence that p50KO MCs showed increased survival upon withdrawal of essential cytokines, which may explain the increased percentage of MCs observed in the tissues of p50-deleted animals. We also show that the underlying mechanism for such increased

survival involved increased expression of pro-survival factors such as Bcl2 and A1, as well as reduced expression of pro-apoptotic factors such as Bax and miR-146a. The latter in particular acted in this context as a modulator of NF-kB signalling by targeting TRAF6 and reducing MC survival.

Material and Methods

Cell cultures and stimulation

Bone marrow-derived MCs (BMMCs) from C57Bl/6 mice and p50KO mice[123] (6-8 weeks old) were differentiated *in vitro* by culturing total bone marrow cells in IMDM medium containing 10% FBS, 2mM L-glutamine, 0.1mM non essential amino acids, 50 μM β-mercaptoethanol, antibiotics and 50% WEHI-3 conditioned supernatant as a source of IL-3. WEHI-3 conditioned supernatant was prepared exactly as described[124]. Cell differentiation was assessed by surface staining for FcεRI and Kit receptor and by toluidine blue staining as described[124]. Briefly, $5x10^5$ BMMCs were resuspended in PBS and cytospun on glass slides at 300-500rpm for 3-5min, after which they were stained for 5min at RT with a solution of 0.1% basic toluidine blue. Toluidine blue stains nuclei and cells other than MCs in pale blue, while MCs show a characteristically purple cytoplasm full of dark purple granules.

For acute activation, MCs were stimulated with 1.5 μg/ml IgE-antiDNP (clone SPE7, Sigma), 0.2 μg/ml DNP-HSA (Sigma) for different times, or with 20-100 μg/ml of LPS, depending of the experimental conditions. In some specific cases, indicated in the text, cells were stimulated with 20nM PMA and 2 μM ionomycin.

Plasmids

The control lentiviral vectors were previously described and expressed either a synthetic hairpin against the luciferase gene (shLuc) or a non-targeting hairpin (NT) or GFP alone[125]. The 394bp PCR fragment encompassing the pre-miR-146a hairpin plus 150bp of surrounding genomic sequence was obtained from a BAC clone and cloned using standard cloning techniques. Figure I and II show a schematic representation of the vectors used throughout this study. Overall, the plasmids used were the following:

Packaging vectors:

1) psPAX2 (Addgene plasmid 12260): this is the packaging vector containing the CAG promoter (a combination of chicken beta-actin promoter and CMV enhancer) driving the expression of packaging proteins Gag and Pol.

2) pMD2G (Addgene plasmid 12259): this vector expresses the envelope protein –G of vesicular stomatitis virus (VSV-G), which has a high stability and confers broad tropism to the virus.

Transfer vectors:

1) pAPM-shLuc: this is a control vector in which the expression of the reporter gene (puromycin resistance) is driven by the spleen focus-forming virus promoter (SFFVp). An shRNA hairpin directed against an irrelevant gene (luciferase) is cloned downstream the reporter gene and it is expressed in the context of the miR-30 surrounding genomic sequences[126]. We also used a version of this vector (pAGM-shLuc) that contains GFP instead of the puromycin resistance as a reporter gene.

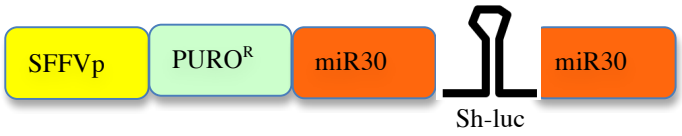


Figure I: schematic representation of the pAPM-shLuc vector

2) pAPM-miR146a: this vector is identical to pAPM-shLuc except that the miR-146a murine genomic sequence is cloned downstream the reporter gene.

Figure II: schematic representation of the pAPM-miR146a vector

HEK 293T cells transfection and transductions of BMMCs

A transfection of three plasmids (two packaging and one transfer vector) was used to generate recombinant viral particles. The detailed method is described in ref 124. Briefly, the following cocktail was prepared in Opti-MEM: 5µg of pMD2G, 15µg psPAX and 20µg of lentiviral vector. After addition of 90 µl of 1mg/ml polyethylenimine pH 7.6 (Polyscience, Inc), the solution was incubated for 10min at RT and then added to the cells. Medium was replaced 12-16h after transfection and viral particle-containing supernatant was collected 24 and 48h later. Viral particles

were concentrated by ultra centrifugation on a sucrose gradient (4°C, 2h at 24'300 rpm) in a Beckman Coulter Optima LE-80 K ultracentrifuge and SW-32-Ti buckets rotor.

$1x10^6$ BMMCs were transduced with lentiviral particles produced as described above at a multiplicity of infection (MOI = Transduction Units/cell) of about 100. The transduction efficiency of MCs under these conditions is usually around 50%. After transduction cells were either selected with 2μg/ml puromycin for 2 days or sorted for GFP expression, depending on the reporter used.

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To analyse miRNA expression, qRT-PCR was performed using a miRNA reverse transcription kit and TaqMan miRNA assays from Applied Biosystems, following exactly the manufacturer's instructions. To analyse Bcl2 family member expression, total RNA (1μg) was reverse transcribed using the iScript kit (Bio-Rad), before PCR amplification with the primers listed below. β-actin was used as an endogenous control.

Bcl2 = FW 5'-TTCGCAGCGATGTCCAGTCAGCT

RV 5'-TGAAGAGTTCTTCCACCACCGT

A1 = FW 5'-GATTGCCCTGGATGTATGTGCTTA

RV 5'-AGCCATCTTCCCAACCTCCATTC

Bcl-XL= FW 5'-CAGTGCCATCAATGGCAACCCATC

RV 5'-CGCAGTTCAAACTCATCGCCTGC

BAX = FW 5'-ACTGGACAGCAATATGGAGCTG

RV 5'-CCCAGTTGAAGTTGCCATCAG

Degranulation assay

$5x10^4$ BMMCs were resuspended in 50μL OptiMEM, 1% FBS and stimulated for 1h with either PMA and ionomycin or IgE-antiDNP and DNP-HSA. The supernatant was collected and the cell pellet was lysed in 50μL of 0.5% Triton-X100 in OptiMEM, 1% FBS. The β-hexosaminidase substrate (4-nitrophenyl N-acetyl-β-D-

glucosaminidate (Sigma)) was then added to both the cell lysates and supernatants (50µL of 3.8mM solution). After incubation for 2h at 37°C, the reaction was stopped with 90µL glycine 0.2M, pH 10.7, and the absorbance was read at 405nm. The percentage of degranulation was calculated as the ratio between the absorbance of supernatants and the total absorbance of supernatants and cell lysates[127].

Thymidine incorporation assay

$1x10^5$ BMMCs were stimulated as described above for 24h, and in the last 16 h of incubation, 1 µCi/ml [^{3}H]thymidine (GE Healthcare) was added. Cells were then collected with a BUCHER (BIOTEC) and levels of thymidine incorporation evaluated with a scintillation beta-counter (MicroBeta TRULUX 1450-021).

Intracellular cytokine staining

$1x10^6$ BMMCs were stimulated with either PMA and ionomycin, IgE and antigen complexes or LPS for 3h. In the last 2 hours of incubation, 10µg/ml brefeldin-A was added to the cultures. The cells were fixed for 10min with 4% paraformaldehyde at RT and permeabilized in 0.5% saponin/ 1% BSA. After a short pre-incubation with 0.5µg/µl of FcBlock (eBioscience) cells were stained with directly conjugated anti-cytokine fluorescent antibodies (eBioscience) for 30min at RT, prior washing and FACS analysis.

Analysis of apoptosis

For cell death analysis, cells were washed extensively to remove all IL-3 and/ or SCF from the culture medium and were either left resting or were stimulated with IgE and antigen. Apoptosis was evaluated at different time points using the Annexin-V-PE apoptosis kit (BD-Pharmingen) following the manufacturer's instructions.

Adoptive transfer, peritoneal lavages, and passive cutaneous anaphylaxis

For adoptive transfer experiments, BMMCs were differentiated from C57Bl/6 and p50KO mice as described above and injected either intradermally (i.d.) (10^6 cells/mouse) in the ear pinna or in the peritoneal cavity (i.p.) of mice lacking MCs (C57Bl/6Kit $^{W\text{-}sh/W\text{-}sh}$ from Jackson Laboratory)[8]. Tissue reconstitution was allowed to proceed for 4 weeks for i.d. injections and 6 weeks for i.p. injections.

For passive cutaneous anaphylaxis reactions, four weeks after i.d. reconstitution, transferred MCs were sensitized by intradermal injection in the ear pinna of IgE-antiDNP (1.5 μg/ml), and than challenged 24h later with intravenous injection of 250 μg/ml DNP-HSA and 5mg/ml Evans-blue in 250μl PBS to assess extravasation. Mice were sacrificed 30min after challenge and the blue ear intensity (measuring extravasation and therefore the extent of MC activation) was analysed by spectrophotometer (OD=600) after extraction from the tissues by incubation in formamide at 63°C O/N. Presence of MCs in the reconstituted ears was assessed by embedding the tissues in paraffin and performing toluidine-blue staining on the tissue slides, which were then manually counted at the microscope for the presence of MCs.

Peritoneal lavages of either WT C57Bl/6 mice, p50KO mice or C57Bl/6Kit $^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with *in vitro* differentiated WT or p50KO MCs, were performed by injecting 2-3mL of PBS in the peritoneal cavity of the animal and recovering as much liquid as possible after extensive washing. The total number of recovered cells was assessed by manual counting and was normalized to the volume recovered, while percentage of MCs was evaluated by surface staining and FACS analysis for cells expressing Kit and FcεRIα.

Western Blots and Immunofluorescence staining

Total protein extracts for Western blot were prepared by direct lysis of the cells in Laemmli sample buffer and boiling for 10min. Samples were run on 12% SDS-polyacrylamide gels and immune-detection was performed with NF-kB p50 antibodies (NLS, sc-114, Santa Cruz Biotechnologies), TRAF6 H-274 antibodies (sc-7221, Santa Cruz Biotechnologies) and β-Tubulin H-235 antibodies (Santa Cruz Biotechnologies) as a loading control. Quantification was performed by ImageQuant LAS 4000.

Tissue slides of paraffin-embedded organs from WT and p50KO mice were kindly provided by Antonio Sica, Istituto Clinico Humanitas (Rozzano, Italy), de-paraffinized in xylol and rehydrated with decreasing concentrations of ethanol. Antigen retrieval was performed with an antigen retrieval solution (pH 6.1, Dako) for 30 min at 95°C. After blocking with serum–free blocking agent (Dako) for 10 min at RT, tissue sections were incubated over-night with 2μg anti-MC tryptase (FL-275, Santa Cruz Biotechnologies). After extensive washes, slides were then incubated for

30 min with an anti-rabbit AlexaFluor 594 secondary antibody (Invitrogen) and mounted with a DAPI-containing mounting medium. Images were captured with Nixon Eclipse E800 and analyse with the Openlab software (Improvision).

Hematopoietic stem cell differentiation

Bone marrow was harvested by flushing the femurs and tibias from young adult mice (4 to 8 weeks). Murine hematopoietic stem cells (HSCs) were enriched by FACS-sorting Lin- Kit+ ScaI+ cells and plated in 96-wells plate by limiting dilution (1 and 5 cells/well) in the presence of IL3. After three weeks the surviving colonies were counted and analysed for the expression of the following surface markers: FcεRIα, Kit, Mac-1 and Gr-1, in ordere to assess MC, macrophage and granulocyte differentiation.

Statistical analysis

Results are expressed as a mean with the standard deviation. Comparisons were made using the unpaired t-test and the GraphPad Prism Software**.**

Results

- MCs lacking p50 can be differentiated *in vitro* and are morphologically similar to control cells.

To assess whether MCs could differentiate normally even in the absence of p50, bone marrow from p50KO and control animals was cultured in the presence of IL-3 and/or SCF for at least 3 weeks (**Figure 1A**). During differentiation (between week 2 and 3) we observed a slightly reduced percentage of differentiated cells in cultures from p50KO bone marrow compared to controls, however at the end of the differentiation period (between week 3 and 4), p50KO MCs were homogenously differentiated and looked phenotypically normal. Specifically, p50KO and WT MCs expressed similar levels of FcεRI and Kit (**Figure 1B, top**), as assessed by surface staining, and showed comparable morphology and presence of cytoplasmic granules upon toluidine blue staining (**Figure 1B, bottom**). To assess proliferation, we performed a thymidine incorporation assay in resting and stimulated conditions, and found that p50KO MCs proliferated similarly to the controls also in response to IgE crosslinking, which normally stimulates MC proliferation (**Figure 2A**). Having established that p50KO cells could differentiate and proliferate normally even in response to antigen, we evaluated their ability to perform their effector functions (degranulation, cytokine production) in response to different stimuli, such as PMA and ionomycin, IgE-Ag complexes and LPS. To assess the extent of MC degranulation, we measured the release of β-hexosaminidase, an assay widely used to characterize cellular components and mechanisms involved in stimulated exocytosis, including those initiated by crosslinking of IgE receptors on MCs. As expected, since this is not supposed to be an NF-kB-dependent process, p50KO MCs degranulated normally in response to either IgE crosslinking or PMA and ionomycin stimulation (**Figure 2B**). We therefore investigated more specifically possible effects on NF-kB-dependent genes in cells lacking p50. In fact, NF-kB regulates the expression of several hundred genes, including IL-13, TNF-α and IL-6, all cytokines produced at high levels by activated MCs[128]. We therefore stimulated WT and p50KO cells with either LPS or IgE crosslinking, and assessed expression of IL-6, TNF-α and IL-13 by intracellular cytokine staining (**Figure 2C**). MCs lacking p50 showed a modest reduction in cytokine production in response to both LPS and IgE-Ag complexes. This reduction was statistically significant for IL-13, which is the cytokine necessary

and sufficient for asthmatic responses in models of experimental asthma[129]. While this defect in IL-13 production in the absence of p50 may contribute to the asthma resistant phenotype observed in p50KO animals[119], it is however rather modest and probably not sufficient to explain the complete lack of airway hyper-resistance and eosinophilia of these mice, which therefore remains predominantly Th2-dependent[119].

- MCs lacking p50 showed a marked increase in their ability to survive apoptosis in response to withdrawal of essential cytokines

NF-kBp50 is also implicated in cell survival[130/131]. Overexpression of p50 has been observed in a number of human cancer including non-small cell lung carcinoma, colon cancer, prostate cancer, breast cancer, bone cancer and brain cancer[98-100]. The rearrangement of p50 gene, however, has been identified only in certain acute lymphoblastic leukemias[122]. We therefore investigated whether p50KO MC are significantly affected in their ability to survive in response to IgE crosslinking and/ or withdrawal of essential cytokines. In order to do this, MCs cultured in the presence of IL-3 or IL-3 and SCF were extensively washed to remove the survival cytokines and then cultured in the absence of IL-3 or IL-3 and SCF for several days, with of without concomitant stimulation with IgE-Ag complexes. Indeed, IgE crosslinking has been shown to be an important survival factor for MCs, by favouring induction of pro-survival factors such as A1[132]. Cell death was assessed daily by Annexin-V staining, and in all conditions tested p50KO cells showed consistently an increased ability to survive in response to withdrawal of essential cytokines compared to the controls (**Figure 3A-B**). As expected, stimulation with IgE partially rescued the cell death of control cells, but once again, this effect was even more pronounced in the absence of p50 (**Figure 3C**). NF-kB has been detected in most cell types, and specific NF-kB binding sites have been identified in promoters and enhancers of a big number of inducible genes involved in cell death, including Bcl-2, A1 and Bcl-xL[133]. In particular, Bcl-2 is a known regulator of IL-3 withdrawal-dependent apoptosis in MCs[134], while A1 is a specific regulator of IgE-dependent survival in MCs[132]. We therefore investigated whether Bcl-2, A1 and other pro- and anti-apoptotic genes may be involved in the observed increased survival of p50KO MCs. To this end p50KO MCs and control cells were either left resting or were stimulated for 24h with IgE and antigen or LPS, after which total RNA was extracted and a qRT-PCR was performed to assess levels of expression of pro- and anti-apoptotic gene candidates.

Interestingly, both Bcl-2 and A1 were upregulated in cells lacking p50. Such up-regulation was already present at basal levels, but it became even more evident upon stimulation with IgE-DNP or LPS (**Figure 4A-B**). Another pro-survival factor, Bcl-XL, was slightly reduced (about 18%) in p50KO cells (**Figure 4C**), while the pro-apoptotic gene Bax was downregulated (**Figure 4D**). Since Bcl-2 and especially A1 were already shown to be involved in regulating MC survival[132], it is likely that the overall net increase of pro-survival factors is at the basis of the observed enhanced survival of p50KO cells. Our data therefore show that p50KO cells are able to survive much better in response to a variety of stimuli, and that such enhanced survival is likely due to a profound alteration in the balance between pro- and anti-apoptotic factors, with the latter being overall favoured.

-MiR146a regulates mast cells survival

Since miRNAs are known to be involved in the regulation of a variety of cell functions, and p50KO cells showed altered cytokine production and survival in response to stimulation, we assessed whether miRNAs might also be involved in these processes. We assessed in particular expression of two miRNAs (miR-146a and miR-221) that we already knew to be inducible in MCs and to be dependent on NF-kB activation[121] .To assess whether miRNA expression was altered in the absence of p50, p50KO and control MCs were either left resting or were stimulated with PMA and ionomycin for 24h, after which total RNA was extracted and levels of miR-146a and miR-221 were assessed by qRT-PCR. Levels of miR-27a were also measured as a control of a miRNA that should not change in MCs in response to stimulation. Strikingly, p50KO MCs showed strongly reduced expression of miR-146a, but not of miR-221 or miR-27a, in response to PMA and ionomycin stimulation (**Figure 5A**). Interestingly, in the absence of p50, MCs were unable to induce miR-146a expression in all conditions tested, including IgE-Ag or LPS stimulation for various time points (**Figure 5B-C**), indicating that p50 is indeed absolutely required for the expression of this miRNA. In contrast, expression of miR-221, also known to be inducible upon MC-stimulation in a NF-kB-dependent manner[121], was comparable between p50KO cells and controls, indicating that for miR-221, but not miR-146a expression, the lack of p50 can be compensated by the presence of other NF-kB subunits.

Since p50KO cells are completely unable to induce miR-146a expression, and it is known that miR-146a is itself a regulator of NF-kB activation[83], we asked whether at

least part of the phenotype observed in MCs in the absence of p50 could be due to the inability of these cells to induce miR-146a expression. Therefore, we forced expression of miR-146a by lentiviral transduction of WT and p50KO MCs. Importantly, we did not overexpress miR-146a, but rather achieved physiological levels of expression that were comparable or lower to the levels of endogenous expression induced by the cells upon stimulation (**compare Figures 6A and figure 5A and 5C**). To assess whether our lentivirally-derived miR-146a was also biologically functional, we assessed levels of expression of the known miR-146a target, TRAF6[83]. In order to do this, p50KO MCs and controls were transduced with either a control vector (shLuc, expressing an irrelevant hairpin), or miR-146a. After selection of the transduced cells, cells were lysed and TRAF6 expression evaluated by Western blot (**Figure 6B**). Such analysis showed a significant decrease in TRAF6 expression whenever miR-146a was expressed, in both p50KO and control cells. We therefore assessed whether miR-146a expression led to altered survival in MCs, somehow mimicking a general reduction in NF-kB activation. Cell death was assessed in cells expressing miR-146a by Annexin-V staining, and in these conditions MCs transduced with miR-146a showed consistently an increased ability to survive compared to the controls, even when cultured in full medium, supplemented with all survival factors (**Figure 6C**). Interestingly, forced miR-146a expression led to a modest reduction of Bcl-2 expression in p50KO cells, indicating that although miR-146a works in the same pathway as p50, forced miR-146a expression is not sufficient to completely compensate and revert the phenotype induced by the lack of p50 (**Figure 6D**). Along the same line, we did not observe any particular effect of miR-146a on IL-6 and TNFα expression, cytokines that were also modestly altered, even by the absence of p50 (**Figure 6E**).

- MCs can be tolerized regardless of p50 expression.

MiR-146a is known to regulate LPS-tolerance in macrophages[135]. Since we found that p50KO cells cannot induce miR-146a in response to LPS, we assessed whether p50KO MCs were impaired in their responses to sequential stimulations with LPS. In order to do this, p50KO and control cells were either left unstimulated (‘medium’ sample, M/M), treated with LPS for 3h (‘stimulation’ conditions), which was then replaced by normal culture medium (L/M), treated with LPS for 20h (also, a stimulation condition, M/L), or treated repeatedly with LPS (‘tolerance’ conditions)

4h+20h (L/L) or even 3h+24h+48h (L/L/L). The expression of IL-6 and TNF-α was assessed by intracellular cytokine staining (**Figure 7A**), while in a parallel experiment the expression of miR-146a was evaluated by qRT-PCR (**Figure 7B**).
Interestingly, MCs lacking p50 showed a reduction in cytokine production in response to acute LPS stimulation while they became unresponsive to subsequent stimulations with LPS similarly to control cells. This result indicates that p50KO MCs have reduced activation in response to LPS but similar tolerance ability. Moreover, in the absence of p50, MCs were unable to induce miR-146a expression also upon multiple stimulations, indicating not only that p50 is absolutely required for the expression of this miRNA even in tolerized condition, but also that in these cells miR-146a is not required for LPS unresponsiveness (**Figure 7B**).

- Immunofluorescence analysis shows elevated numbers of MCs in the tissues of p50KO mice

To assess whether the increased survival observed in p50KO MCs *in vitro* might have an effect also in the normal homeostasis of MCs *in vivo*, we investigated the numbers of MCs in the tissues and peritoneal cavity of mice lacking p50. Peritoneal lavages of WT and p50KO mice were performed and total cell numbers and percentages of MCs were assessed by surface staining and FACS analysis of FcεRIα+ Kit+ cells. The number of MCs recovered from the peritoneal cavity in the absence of p50 was significantly augmented compared to control animals (**Figure 8A**). A similar increase in the percentage of MCs was also observed in the intestinal tissues of p50KO animals, as assessed by immunofluorescence staining using two different MC markers (tryptase and Kit) and blind counting (**Figure 8B**). These results indicate that there is an overall increase of the number of MCs in the tissues of p50KO animals, without however any alterations in the homing properties of these cells. Indeed, MCs were found in all tissues and compartments where they are normally found also in control animals, but not in organs, like the spleen or liver, where MCs are not usually found (not shown).

We hypothesized that such increase in MC numbers could be due to the enhanced survival of p50KO MCs in the tissues. To test whether there was a cell-intrinsic ability of p50KO cells to repopulate tissues in higher numbers, we performed adoptive transfer experiments of *in vitro* differentiated p50KO MCs and control cells into MC-deficient recipient mice (C57Bl/6 $Kit^{W\text{-}sh/W\text{-}sh}$). Adoptive transfer and MC

reconstitution was performed either intradermally in the ear pinna (**Figure 9A-C**) or in the peritoneal cavity (**Figure 9D**). First, we performed passive cutaneous anaphylaxis experiments in which MCs in the ear were sensitized with IgE and then challenged with antigen in the presence of Evans blue dye to measure extravasation induced by the release of vasoactive mediators upon MCs degranulation. As expected since p50KO MCs also degranulated normally *in vitro* (as shown in **figure 2B**), *in vivo* p50KO MCs responded to IgE activation to levels comparable to controls **(Figure 9A and B)**. Reconstitution of the ear pinna with MCs was confirmed by toluidine blue staining of paraffin-embedded ear sections (**Figure 9C**). Manual counting of MCs in ear sections, as well as FACS analysis of MC reconstitution of the peritoneal cavity (**Figure 9C and figure 9D** respectively), showed that p50KO MCs were able to reconstitute the tissues at levels that were similar or just slightly lower compared to control cells. However, in adoptive transfer experiments, *in vitro* differentiated MCs did not repopulate tissues at a higher percentage compared to WT cells, and therefore did not recapitulate in a cell-autonomous manner the observation that in the tissues of p50KO animals there is a significant increase of MCs. This could be due to the fact that the adoptive transfer of fully differentiated, resting cells does not activate the NF-kB pathway and we were therefore unable to observe any difference between WT and KO cells. It is also possible that in an *in-vivo* situation where IgE are present, there is less of the difference between the ability of WT and KO cells to survive, as we could show in *in vitro* experiments in cells stimulated with IgE-Ag complexes (**Figure 3C**).

One other possibility would also be that *in vivo* there is an increased differentiation to MCs from p50KO hematopoietic progenitors that leads to an unbalanced homeostasis of MCs with increased numbers in the tissues. To evaluate the latter possibility we set up an *in vitro* differentiation system in which the fraction of murine bone marrow containing hematopoietic stem cells (HSCs) was enriched by FACS-sorting of Lin-Kit+ ScaI+ cells. These cells were then plated in 96-well plates at one- or five-cells/ well, and allowed to differentiate in MC conditions (presence of IL-3) for three weeks. After this time total surviving colonies were counted and analysed for the expression of FcεRIα, Kit, Mac-1 and Gr-1, to assess percentages of colonies that contained MCs (FcεRIα+ Kit+), granulocytes (Gr-1+) and macrophages (Mac-1+). As shown in **Figure 10**, p50KO and WT HSCs were comparable in their ability to give rise to MCs and other types of myeloid cells, when assessed as

percentage of specific colonies over the total. However, when we counted the overall number of myeloid colonies that were able to grow from p50KO HSC cells gave rise to 1,9-fold more colonies indicating an increased ability of p50KO HSC to differentiate to myeloid cells.

Overall, our findings indicate that p50 acts both at the level of HSCs to favour myelopoiesis and also in a cell-autonomous manner in differentiated MCs to favour survival especially in response to withdrawal of essential cytokines and in response to antigenic stimulation. This novel molecular network comprising NF-kB p50 and miR-146a and regulating MC survival and tissue homeostasis may have important implications for our understanding of the physiologic MC expansion and activation occurring during responses to helminths or allergens, and possibly in MC hyper-proliferative disorders such as systemic mastocytosis.

Discussion

NF-kB is involved in regulating many aspects of cellular activity, in stress, injury and especially in pathways of the immune response. Known inducers of NF-kB activity are highly variable and include, among others, TNFα, interleukin 1-beta (IL-1β), LPS[136/137]. Moreover, NF-kB is involved in many aspects of cell growth, differentiation and proliferation via the induction of other transcription factors such as c-myc, ras and p53[137].

Here, we found that in the absence of one specific NF-kB family member, p50, MCs showed an increased ability to survive in response to a variety of signals. The underlying mechanism for such increased survival involved increased expression of pro-survival factors such as Bcl2 and A1, as well as reduced expression of pro-apoptotic factors such as Bax and miR-146a, which in this context acted as a pro-apoptotic factor and as a modulator of NF-kB signalling by targeting TRAF6. Activation of NF-kB must be a tightly regulated event. In normal cells, NF-kB becomes activated only after the appropriate stimuli, and upregulates the transcription of its target genes. However, regulatory mechanisms must be in place to return NF-kB to its inactive state. NF-kB activation is therefore an inducible and transient process.

In **Figure 11** we propose a model for the role of miR-146a and NF-kB p50 in regulating MC survival and activation. In MCs, NF-kB activation can occur as a result of FcεRI crosslinking or TLR4 engagement. In normal conditions this leads to nuclear translocation of p65-p50 heterodimers that activate transcription of both anti-apoptotic factors (such as Bcl2 and A1) as well as of pri-miR-146a, which helps shutting down NF-kB activation in a negative feedback loop (**panel A**). Our data showed that in the absence of p50 not only some survival factors are strongly upregulated (Bcl-2 and A1 predominantly), but also the negative feedback of NF-kB activation is lost, as miR-146a cannot be expressed (**panel B**). The fact that in the context of NF-kB activation and MC survival miR-146a acts as a pro-apoptotic factor is highlighted by the finding that forced expression of miR-146a (therefore artificially increasing the negative part of the network) led to increased cell death (**panel C**).

What remains to be investigated is the mechanism by which p50 may act as a positive regulator of some genes (including miR-146a, for which p50 is essential), and as a negative regulator of other genes (namely Bcl-2 and A1, whose expression is increased in both basal and activated conditions). One possible explanation could rely

on a difference in the binding activity of NF-kB at the promoters of these specific genes: our current working hypothesis is that p50 homodimers may negatively regulate expression of Bcl-2 and A1 in resting conditions, which is overcome by other activating heterodimers upon activation. MiR-146a expression on the other hand, remains exquisitely dependent on p50 in all conditions.

Therefore, further experiments will be necessary to assess the molecular mechanism responsible for the pro-survival effect observed in a p50 KO background. The difficult task of elucidating the exact mechanisms of action of p50 may be due to this key transcription factor's ability to act as both a transcriptional repressor when homodimerized and a transcriptional activator when heterodimerized with p65.

Indeed, negative regulation of genes by p50 homodimers has been reported to reduce expression of TNFα by tolerant macrophages[138]. Interestingly, macrophages from p50KO mice were resistant to tolerance induced by LPS [138], and overexpression of p50 in the presence of constant amounts of p65 inhibited LPS-induced transcription of the TNF-α promoter, thus mimicking LPS-induced tolerance[130/140]. LPS-induced tolerance in human monocytes is also associated with a predominance of p50-p50 homodimers binding to the TNF-α promoter, resulting in reduced TNF-α production [140/141]. Moreover, it is knows that different NF-kB binding sites can have different binding affinities for the different family members homo- and heterodimers[141].

Finally, our preliminary data on hematopoietic differentiation from p50KO and C57Bl/6 bone marrow-derived HSCs showed a comparable ability to generate MCs and other types of myeloid cells in terms of relative percentages. In other words, the percentage of MC colonies over the total number of colonies observed was similar in the p50KO and in control cells. However, p50KO HSCs were able to produce 1.9-fold more myeloid colonies compared to the control, suggesting that in these mice myeloid differentiation may be overall increased, a hypothesis that we will continue to investigate in future experiments.

Although still completely speculative, an interesting aspect of our findings is also related to aging. Indeed, the activation of NF-kB signaling is also associated with the aging process[142]. Cellular aging involves a progressive accumulation of damaged and defective cellular components, which cause a decline in physiological function of tissues and body fitness. Interestingly, aging cells show an increased resistance to apoptotic stimuli and increased Bcl2 expression[143]. Moreover, old mice show altered HSCs differentiation with increased myelopoiesis[142]. Since these phenotypes are

similar to the ones we observed in p50KO mice, it is tempting to speculate that the absence of p50 may also lead the cells towards a premature aging program. This aspect will be the topic of future work.

In conclusion, our findings indicate that p50 acts both at the level of HSCs to favour myelopoiesis and also in a cell-autonomous manner in differentiated MCs to decrease cell death.

Figure Legends

Figure 1: MCs can be differentiated from murine bone marrow even in the absence of p50 A) BMMCs were cultured in presence of IL-3 with or without addition of SCF (10ng/ml) and the percentages of FcεRIα+ Kit+ cells (differentiated MCs) was assessed weekly. Each dot represents one independent experiment. **B)** (Top panel) Surface staining of fully differentiated MCs, showing comparable expression of FcεRIα and Kit between WT and p50KO cells. (Bottom panel) Toluidine blue staining of cytospins of differentiated WT and p50KO MCs, showing overall similar morphology and similar content of cytoplasmic granules.

Figure 2: p50KO MCs responses to a variety of stimuli. **A)** The ability of MCs to proliferate at steady state and in response to stimulation was assessed by thymidine incorporation assay. p50KO and control BMMCs were either left resting or were stimulated for 24h with IgE-Ag complexes or LPS prior addition of ^{3}H-thymidine. Shown is one representative experiment out of three **B)** To assess MC degranulation in response to acute stimulation, BMMCs were either left untreated or were stimulated with IgE-Ag complexes or LPS for 1h prior measurement of β-N-acetylhexosaminidase release in degranulation assay. Shown is one representative experiment out of three. **C)** To assess cytokine production, cells were stimulated with IgE-Ag complexes or LPS for 5-6h and the productions of IL-6, TNF- α and IL-13 was analysed by intracellular cytokine staining. Each dot in the graphs represents one independent experiment.

Figure 3: BMMCs lacking p50 showed a marked increase in their ability to survive apoptosis in response to withdrawal of essential cytokines. A-B) BMMCs were cultured with IL-3 or IL3+SCF prior removal of IL-3 or SCF for 4 and 6 days. Cell death analysis was performed by Annexin-V staining. The graph in panel **A**) shows the mean of four independent experiments, while panel **B**) is representative of at least two experiments. **C)** Same experiment as in panel A, except that cells were either left unstimulated or were stimulated with IgE and antigen at the time of initial IL-3 withdrawal. Shown is one representative experiment out of at least two.

Figure 4: Expression levels of some pro- and anti-apoptotic NF-kB depending genes. p50KO MCs and control cells were either left resting or were stimulated for 24h with IgE-Ag complexes or LPS. Total RNA was extracted and a qRT-PCR was performed to assess the expression levels of the pro- and anti-apoptotic gene candidates Bcl2, A1, Bcl-XL and BAX. Shown is one representative experiment out of at least four for Bcl2, A1 and BAX, and at least three for Bcl-XL.

Figure 5: MiR-146a is not expressed in p50KO MCs. A) Differentiated BMMCs were either left resting or were stimulated with PMA and ionomycin (P+I) prior analysis of the expression of three different miRNAs (miR-146a, miR-221 and miR27a) by qRT-PCR. SnoRNA 202 was used as endogenous control. Shown is one representative experiment out of three. **B)** WT and p50KO BMMCs were stimulated for the indicated times with IgE-Ag complexes, and expression levels of miR-146a were assessed by qRT-PCR. Shown is one representative experiment out of at least three. **C)** Same as in B) except that cells were stimulated with LPS and analysed at different time points.

Figure 6: MiR-146a regulates MCs survival. A) WT BMMCs were transduced with lentiviral vectors pAPM-shLUC (as a control) and pAPM-miR146a. MiR-146a expression level in transduced cells was assessed by qRT-PCR. **B**) TRAF6 expression levels in WT and p50KO BMMCs transduced with pAPM-shLUC and pAPM-miR146a were analysed by Western blot. β-tubulin was used as loading control and quantification was performed by LAS 4000. Representative of two independent experiments. **C)** BMMCs transduced as in A) were analysed for levels of apoptosis by annexin-V staining. **D)** Total RNA from BMMCs transduced as in A) was extracted and levels of *Bcl-2* expression were assessed by qRT-PCR. β-actin was used as endogenous control. Shown is one representative experiment out of three. **E**) BMMCs transduced as in A) were stimulated with LPS and intracellular cytokine staining was performed to assess expression of the indicated cytokines. Each dot represents one independent experiment.

Figure 7: Tolerization of BMMCs upon LPS stimulation and expression level of miR-146a A) WT and p50KO BMMCs were either left unstimulated (medium only, M/M), treated with LPS for 3h (L/M), treated with LPS for 20h (M/L),

treated repeatedly with LPS for 3h+20h (L/L) or for 3h+20h+24h (L/L/L). After stimulation an intracellular cytokine staining was performed to assess expression of the indicated cytokines. Representative of at least four independent experiments **B**) WT and p50KO BMMCs were treated as in A) and the expression level of miR-146a was assesses by qRT-PCR.

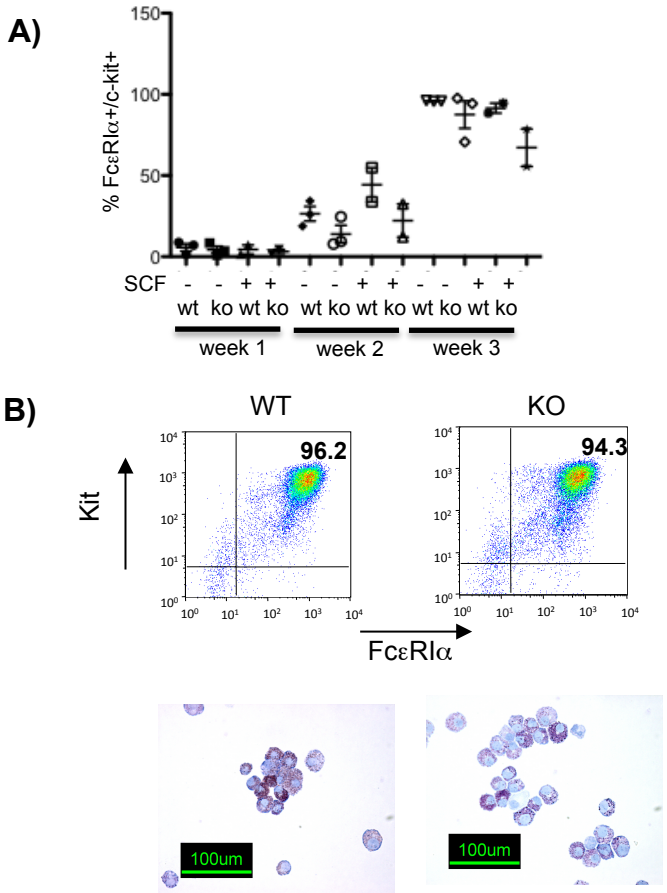
Figure 8: Peritoneal lavage and immunofluorescence analysis show elevated numbers of MCs in the tissues of p50KO mice. A) Peritoneal lavage was performed on WT and p50KO animals, and the resulting cells were analysed for Kit and FcεRIα expression by surface staining. The top graph shows one representative staining, while the compiled results of several mice are plotted in the bottom graph. At least 7 animals per group were analysed. **B**) Immunofluorescence staining using two different MC markers (tryptase or Kit) and blind counting on intestinal tissues of p50KO animals and WT (top). At least 10 fields per tissue were observed and counted blindly (the operator counting the cells was not aware of the type of sample, which was coded). The percentage of tryptase + or Kit+ cells are plotted in a histogram chart (bottom).

Figure 9: p50KO MCs are able to reconstitute the tissues similarly to the controls. BMMCs were differentiated from C57Bl/6 and p50KO mice and injected either intradermally (i.d.) in the ear pinna or in the peritoneal cavity (i.p.) of C57Bl/6Kit $^{W\text{-}sh/W\text{-}sh}$ mice. **A)** Passive cutaneous anaphylaxis. After 4 weeks of i.d. reconstitution of MCs in the ear pinna, mice were sensitized with i.d. injections of IgE-anti-DNP and challenged intravenously 24h later with DNP-HSA together with Evans blue dye to measure extravasation. Blue color appeared only in the presence of activated MCs. The 'empty' ear received no MC injection and didn't become blue, while the control, WT and p50KO ear all became blue as an index of MC activation. **B)** Compiled results of passive cutaneous anaphylaxis experiments in WT and p50KO ears. Blue ear intensity was analysed by spectrophotometer (OD=600). Each dot represents one ear. **C)** Effective ear reconstitution was evaluated by embedding in paraffin 'empty' (C57Bl/6Kit $^{W\text{-}sh/W\text{-}sh}$) ears and ears reconstituted with p50KO or WT (not shown) MCs. Staining of paraffin-embedded ears was performed by toluidine blue. Shown is one representative experiment. MCs present in reconstituted ear stain purple (arrows) as compared to other cell types that stain blue. Imaging was

performed with a Nikon Eclipse E800 microscope. **D)** C57Bl/6Kit $^{W\text{-}sh/W\text{-}sh}$ mice were reconstituted i.p. with *in vitro* differentiated WT or p50KO MCs. Six weeks after i.p. injections, peritoneal lavages were performed and the percentage of MCs was evaluated by Kit and FcεRI α surface staining. Each dot in the graph represents one individual mouse.

Figure 10: p50KO animal shows increased myeloid differentiation activity. Lin- Kit+ ScaI+ cells (enriched in HSCs) from bone marrow of WT and p50KO animals were plated in 96-wells plate at 1 or 5 cells/well in the presence of IL3. After three weeks the surviving colonies were counted and analyzed for the expression of the following surface markers: FcεRIα, Kit, Mac-1 and Gr-1. The overall number of colonies (compared to the number of seeded wells) was 1.9-times higher in p50KO cells compared to WT. Individual colonies were analyzed for the presence of MCs (FcεRI α + Kit+), macrophages (Mac-1^{+}, Kit^{-}, FcεRI α^{-}, Gr-1^{-}), granulocytes (Gr-1^{+}, Mac-1^{-}, Kit^{-}, FcεRI α^{-}). Plotted are the percentages of colonies over the total that contained either MCs alone (MC only), macrophages alone (Mac only), or mixed colonies (mixed).

Figure 11: Summary of the role of p50 and miR-146a in NF-kB activation pathways. Hypothesis on the role of miR-146a, pro/anti survival factors and NF-kBp50 subunit in regulating MC survival. **A)** In normal conditions, upon cell stimulation NF-kB enters the nucleus where it activates the expression of specific genes, leading to a physiological response. **B)** In the absence of p50, the upregulation of pro-survival genes (A1 and Bcl2) together with the impaired expression of the negative regulator miR-146a leads to increased survival. **C)** Forced expression of the negative regulator miR-146a leads to reduced activity of the entire pathway with increased cell death in WT cells, slight reduction of Bcl-2 expression levels, and partial compensation for the lack of p50.



Toluidine Blue staining

Figure 1 : differentiation of WT and p50KO BMMCs

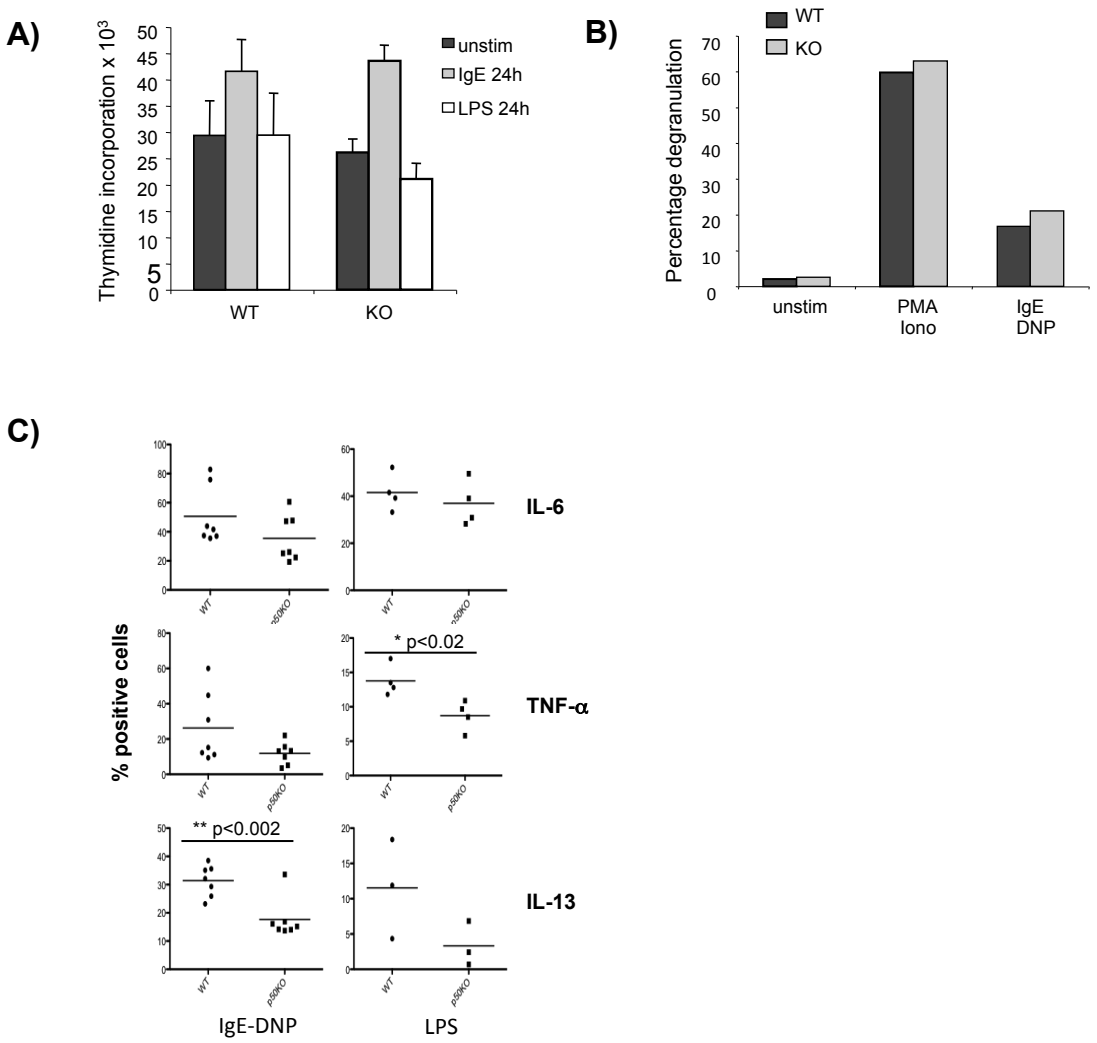


Figure 2 : proliferation and activation

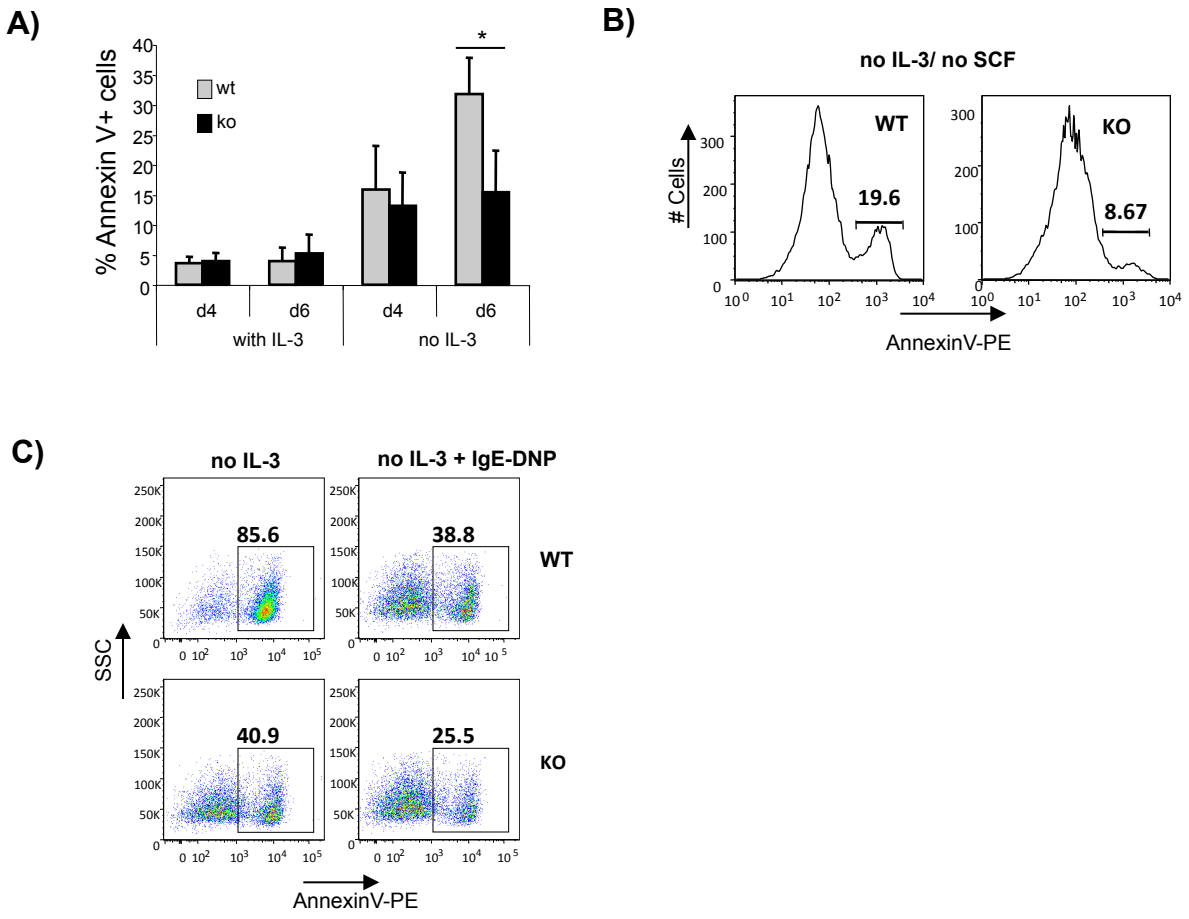


Figure 3 : cell survival

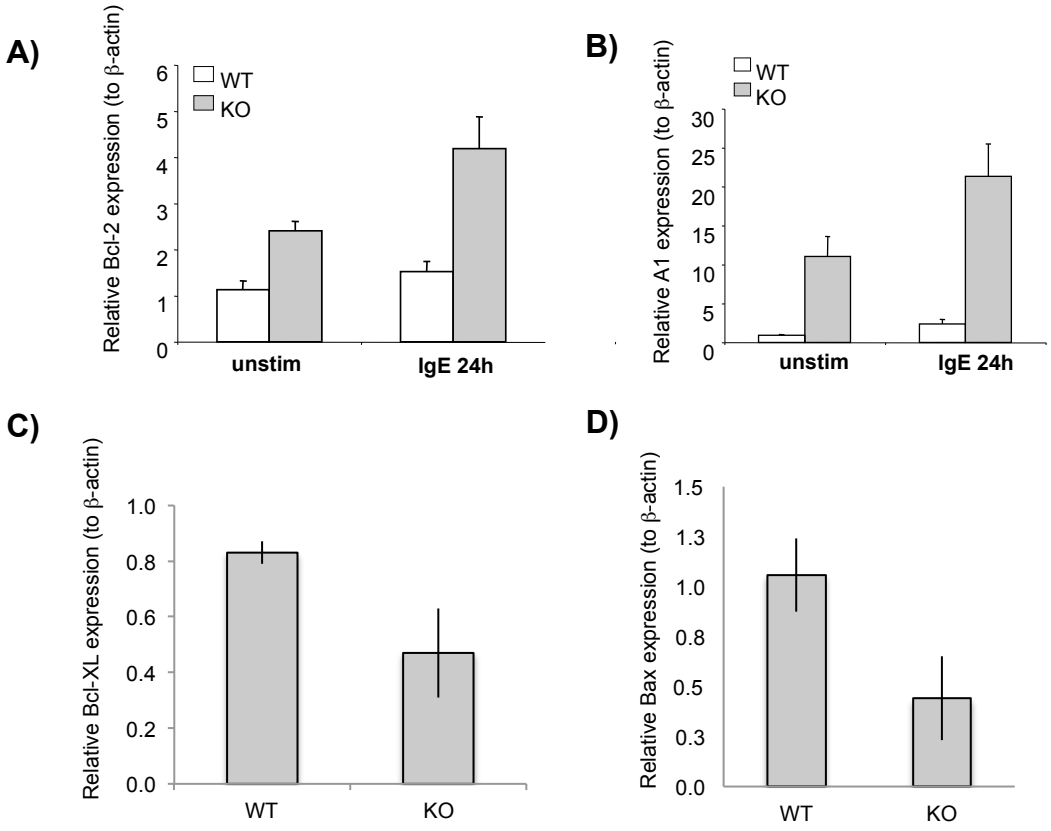


Figure 4 : pro/anti apoptotic genes

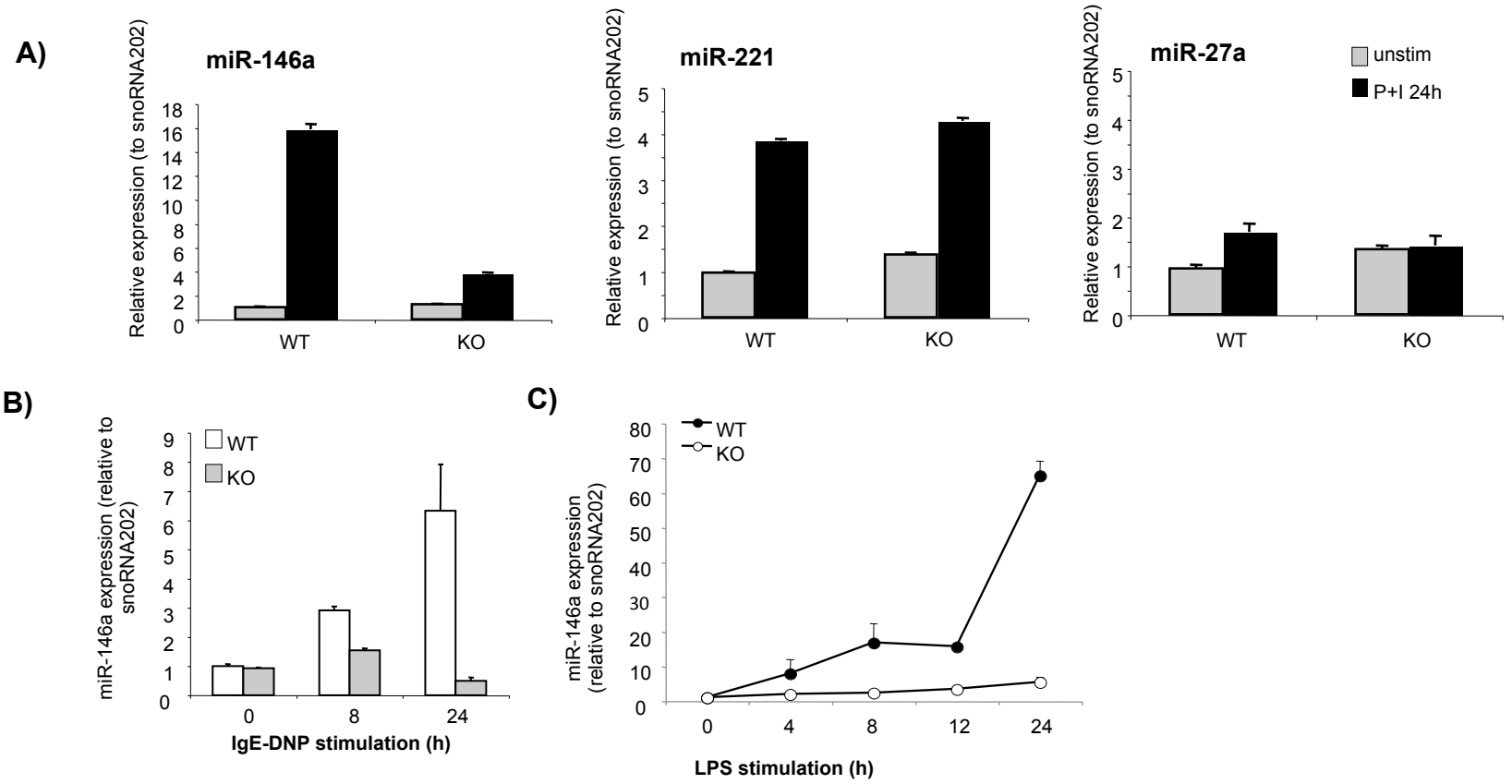


Figure 5 : miR-146a

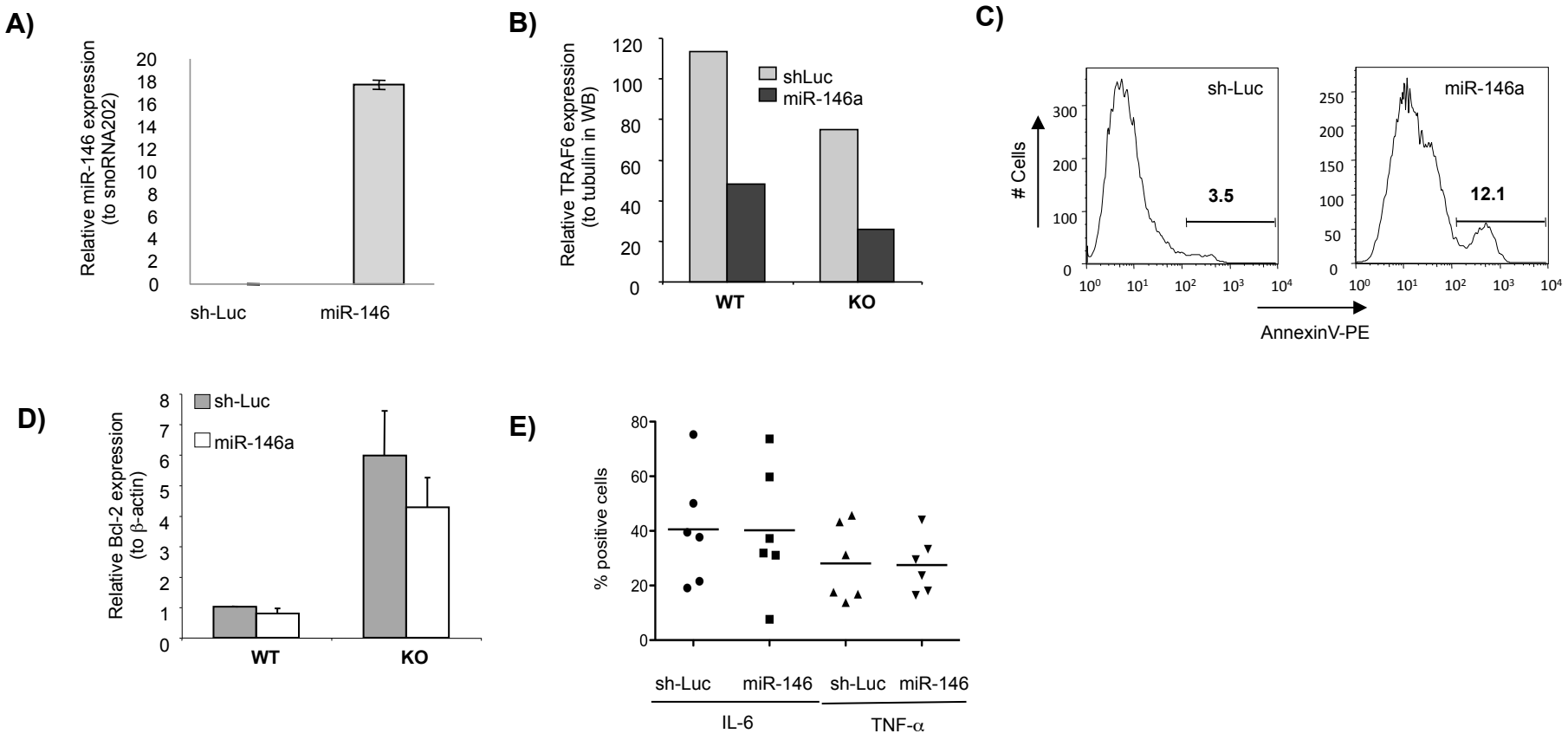
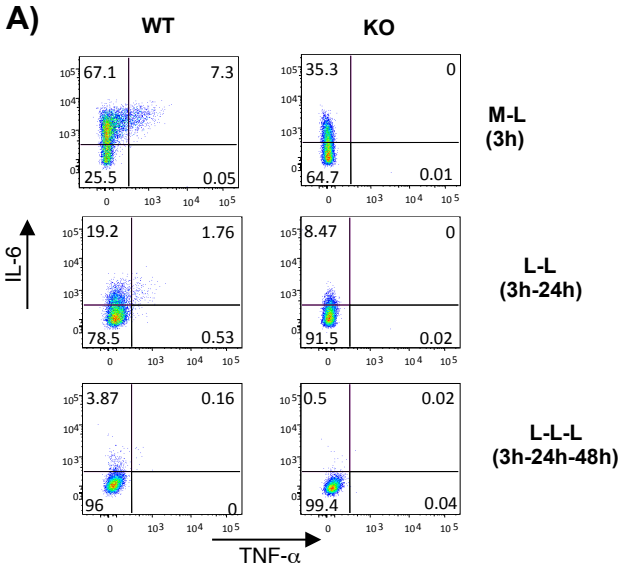


Figure 6 : miR-146a and cell death



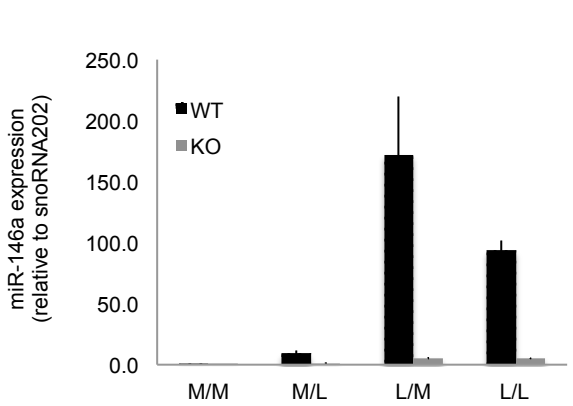


Figure 7: tolerization of BMMCs

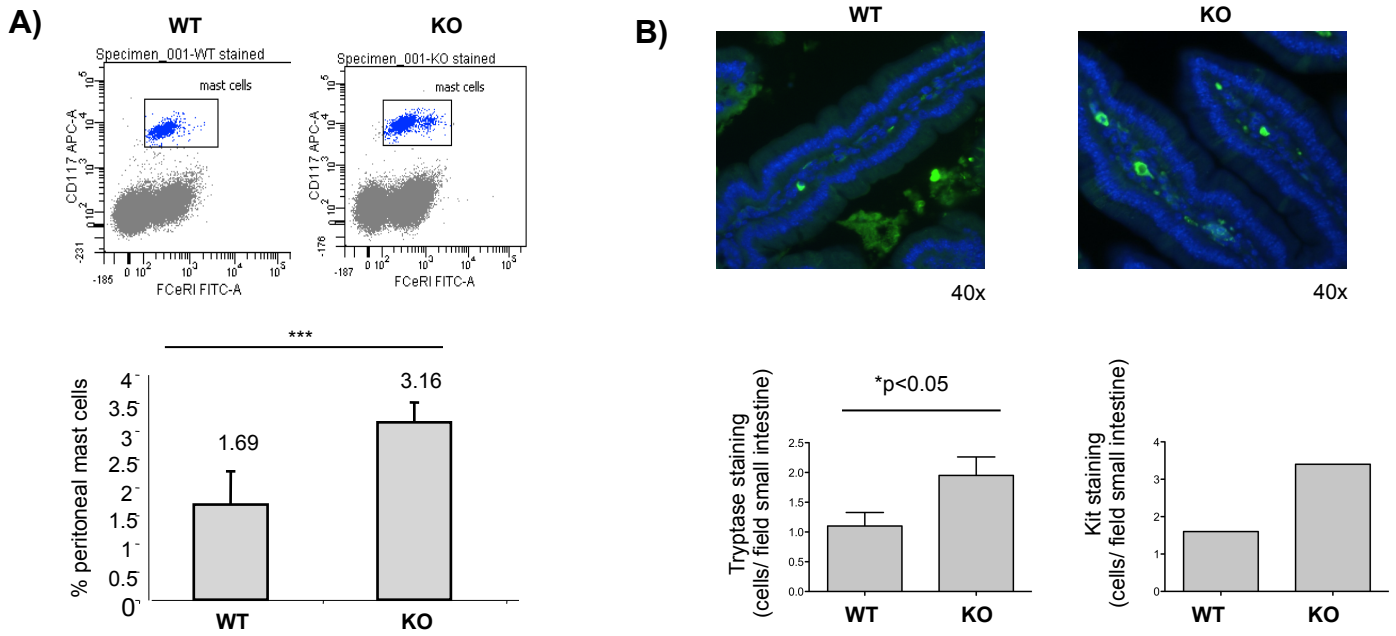


Figure 8: BMMCs in the tissues

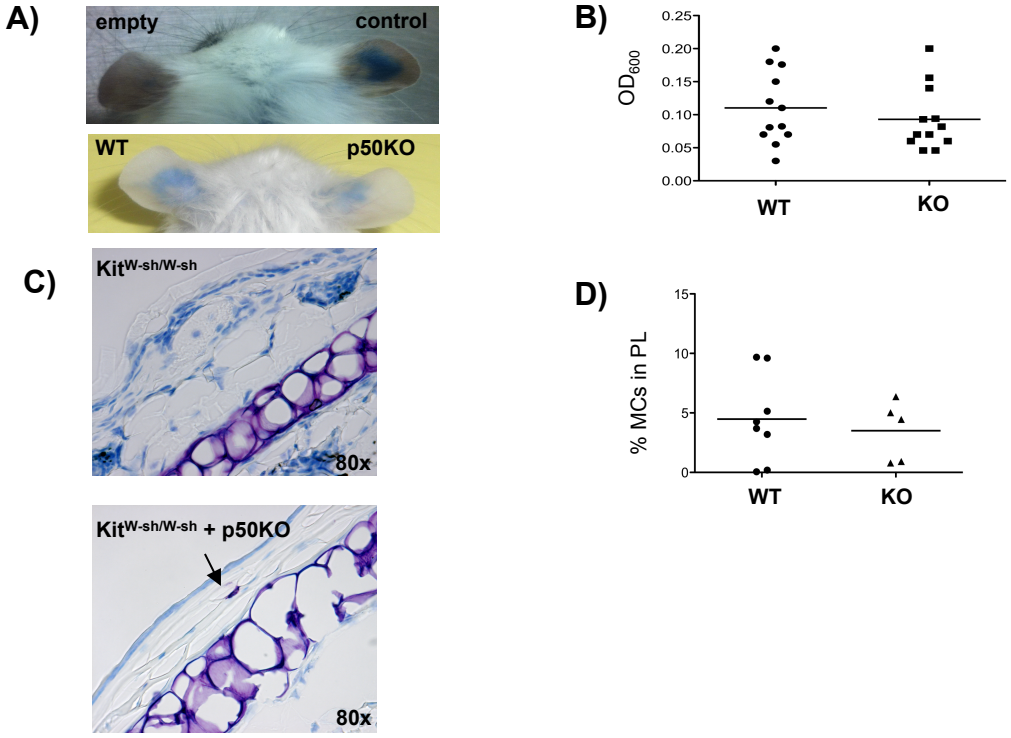


Figure 9: adoptive transfer

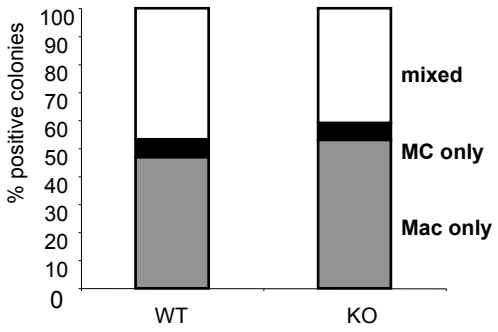


Figure 10: BMMCs hematopoiesis

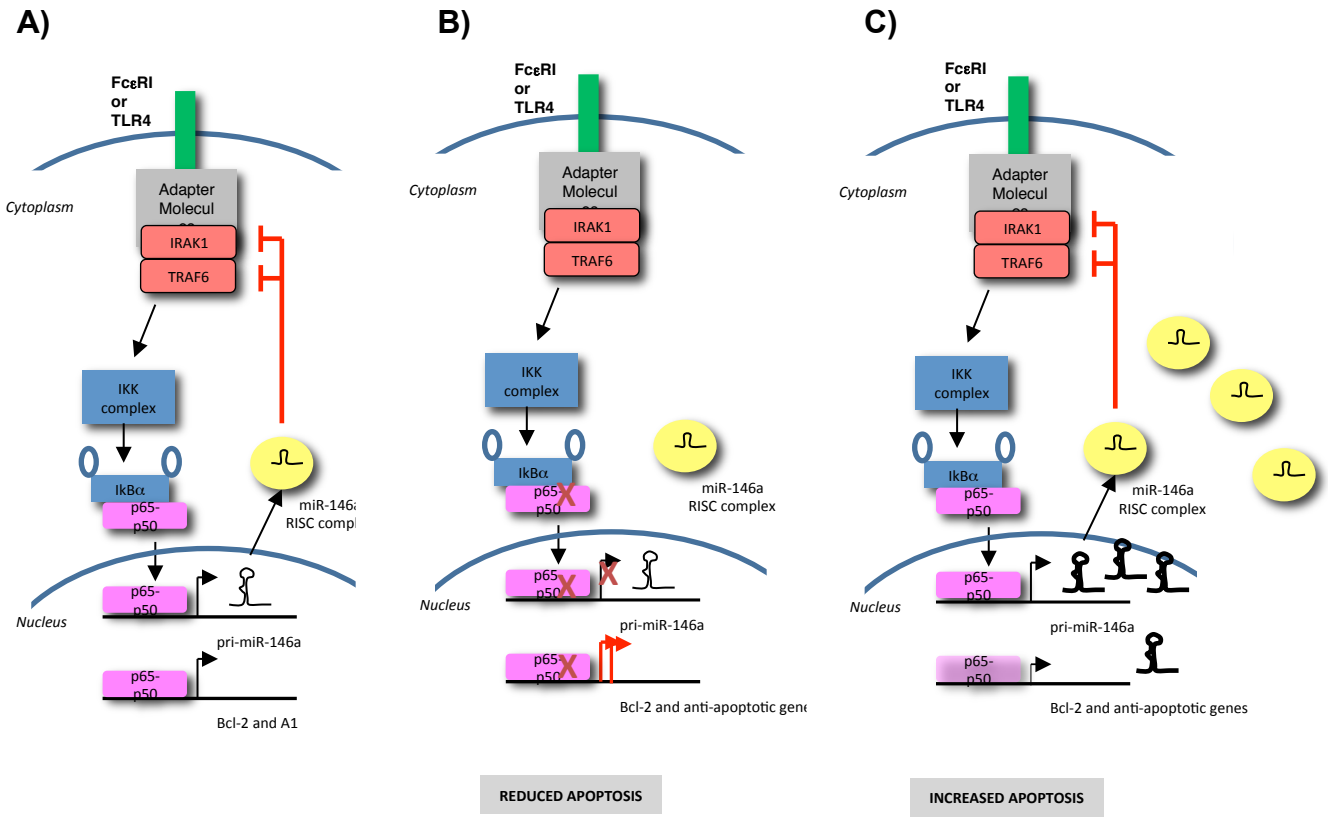


Figure 11: summary

Chapter 3

"MiR146a influences human T cell expansion and memory formation, but does not alter Th1/Th2 balance or cell death"

Summary

The development and propagation of an adaptive immune response to an invading pathogen is a highly orchestrated process that involves the precise regulation of cytokine expression, as well as the formation of an immunological memory. MiR-146a is a known regulator of the immune system, with established roles in the regulation of both innate and adaptive immunity. However, little is known about its role in primary human T cells, and the role of miRNAs in the establishment of a memory phenotype is heavily under-investigated. Here, we found that miR-146a was expressed at high levels in the memory T cell compartment in both human and mouse. Importantly, expression of miR-146a in primary human T cells favored cell expansion in response to TCR triggering and reduced CCR7 expression, while showing no significant effect on cytokine production or cell death, pointing towards a role for this miRNA in regulating memory formation.

Introduction

Memory T cells can be broadly separated into central memory (T_{CM}) that express the chemokine receptor CCR7 and recirculate through lymphoid organs, and effector memory (T_{EM}) that lack CCR7 and preferentially home to non-lymphoid tissues[50]. Despite intense investigation, the requirements for the acquisition and maintenance of the memory phenotype have yet to be completely elucidated. MiR-146a is a well-known regulator of the immune system[144]: it has an established role in regulating NF-kB activation and tolerance in innate immunity[83], it acts as an oncosuppressor, and it modulates T regulatory (Treg) cell functions[87/89]. However, the role of miR-146a (and miRNAs in general) in the establishment of immunological memory remains almost completely unexplored. Here, we found that miR146a was expressed at high levels in the human memory compartment. Although expression of miR-146a in primary human T cells determined no significant effect on cytokine production and Fas-mediated cell death, miR-146a expression lead to increased expansion in response to TCR stimulation as well as to reduced expression of CCR7, pointing towards a role for this miRNA in human T cell activation and memory formation.

Material and methods

Human T cell cultures

Primary human T cells (CD4+ CD45RA– CD25– CD8–) were purified from peripheral blood and expanded as described[145]. After initial experiments, TH1 (CCR6– CCR4– CXCR3+), TH2 (CCR6– CCR4+ CXCR3–) or TH17 (CCR6+ CCR4+ CXCR3–) subsets were used interchangeably as they gave identical results. Cells were stimulated with immobilized anti-CD3 TR66 (1-10μg/mL) and anti-CD28 (2μg/mL), and expanded in medium containing 500U/mL rhIL-2.

Mouse T cell cultures.

Naïve CD4+ or CD8+ T cells were purified using beads (Dynal or Miltenyi Biotec) from spleen and lymph nodes of OT-II or C57Bl/6 mice, followed by sorting for CD62Lhi and CD44lo naïve cells. Cells were then activated with plate-bound anti-CD3 (0.25μg/mL) and anti-CD28 (1μg/mL) for 48h in IMDM medium supplemented with 10% FBS, 1% penicillin-streptomycin, non-essential aminoacids, L-glutamine, 50 μM β- mercaptoethanol and rhIL-2 (20U/mL; Peprotech). Cells were cultured in Th1/Th2 skewing conditions exactly as described[88]. Naïve, T_{EM} and T_{CM} cells were obtained *ex-vivo* from the spleen and draining lymph nodes of C57Bl/6 mice challenged sub-cutaneously with CFA/OVA. Cells were collected two weeks after challenge and they were sorted in the different subsets based on the expression of CD4, CD8, CD62L, CD44 and CD127. All animal studies were performed in accordance with the Swiss Federal Veterinary Office guidelines and were approved by the Dipartimento della Sanita' e della Socialita'.

Plasmids

The control lentiviral vectors were previously described and expressed either a synthetic hairpin against the luciferase gene (shLuc) or a non-targeting hairpin (NT) or GFP alone[125]. The 394bp PCR fragment encompassing the pre-miR-146a hairpin plus ~150bp of surrounding genomic sequence was obtained from a BAC clone and cloned using standard cloning techniques. The TRAF6-luciferase reporter vector was generated by cloning in the pGL3-promoter vector 1439bp of the 3'UTR of murine TRAF6 (RefSeq NM_009424) encompassing 3 predicted miR-146 binding sites (as predicted by TargetScan[73/74], http://www.targetscan.org) downstream a luciferase

reporter vector, using standard cloning-techniques.

Transductions

Lentiviral particles were produced exactly as described[124] and added to primary T cell cultures with a multiplicity of infection of ~60. Jurkat T cells were transduced exactly as for primary human T cells, except that the vector used to express miR-146a contained the puromycin resistance gene (rather than GFP), and cells were therefore selected by culturing for several days in 10μg/mL puromycin.

Intracellular cytokine staining.

Cells were stimulated for 5h with 20nM PMA and 2μM ionomycin, with addition of brefeldin-A (10μg/mL, Sigma) for the last 2h prior permeabilization and staining[146].

qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). First-strand synthesis and qPCR were performed using a miRNA first-strand synthesis kit and TaqMan assays from Applied Biosystems.

Apoptosis

For death receptor-mediated apoptosis, 20ng/mL of anti-Fas antibody (BioLegend) were added to the cultures for ~18h. Levels of apoptosis were assessed by Annexin-V staining (BD Pharmingen).

Luciferase reporter assays

Jurkat cells were transiently transfected with 100ng of TK-Renilla plasmid (Promega), and 1μg luciferase reporter plasmid. Cells were transfected at room temperature by electroporation in serum-free medium with pulses at 270V, 960μF. Twenty-four hours after transfection, cells were stimulated with 2μM ionomycin and 20nM PMA. Eighteen hours after stimulation, cells were harvested, and cell extracts were assayed for luciferase and Renilla activity using an automated luminometer and a dual-luciferase reporter assay system (Promega) following manifacturer's instructions. Luciferase data were normalized to Renilla readings. Primary murine T cells were transiently transfected with the Amaxa nucleofactor with 100nM dsRNA

oligonucleotides and program X-01.

Northern blots

Northern blots were performed exactly as described[88]. Briefly, total RNA (25µg) was separated on 12–15% denaturing 8M urea-polyacrylamide gels in 0.5x Trisborate- EDTA (TBE) buffer. RNA was transferred on a Nytran SuPerCharge membrane (Schleicher & Schuell Microscience) via wet transfer in 0.5x TBE buffer at 4°C, followed by cross-linking to the membrane in a UV Stratalinker (Stratagene). Membrane hybridization was performed overnight at 39°C with radiolabeled DNA-oligo probes (complementary to the mature miRNA sequence as reported in the miRBase database[58]). Membranes were washed at 37°C, three times for 10min with 2x SSC/0.1% SDS and once for 5min with 0.1x SSC/0.1% SDS. Band intensities were quantified using a PhosphorImager and Image-Quant 5.0 software (Molecular Dynamics).

Cell cycle analysis

DNA content in cycling cells was assessed by propidium iodide staining. Briefly, 1.10^6 cells were fixed in 70% ethanol for 45min on ice, followed by incubation for 30min at 37°C with 100µg/mL RNaseA and 40µg/mL propidium iodide. Cells were analyzed at the FACS immediately after staining.

Results

- MiR-146a is consistently elevated in the CD4 and CD8 memory compartment in both human and mouse.

Despite the growing importance of miR-146a in various immune processes, its role in human T cell function and in the establishment of immunological memory is still almost completely unexplored. Here, we found that expression of miR-146a was consistently elevated in the CD4 and CD8 memory compartment in both human and mouse (respectively Figure 1A and B). Increased miR-146a expression was observed in both T_{EM} and T_{CM}, although T_{EM} cells showed consistently slightly elevated expression (**Figure 1A and B**), indicating that this miRNA may have a role in regulating T cell activation and/ or in the establishment of immunological memory. Indeed, miR-146a expression also increased upon acute stimulation of both human and mouse cells (**Figure 1A right and B right**).

- Primary human T cells expressing miR-146a did not show alteration in Fas-mediated cell death.

In accordance with published data[84], ectopic expression of miR-146a in Jurkat cells led to a mild reduction in Fas-mediated cell death (**Figure 2A**). To have this result, an artificial overexpression of miR-146a in Jurkat cells was performed with a lentiviral vector expressing both a puromycin reporter and miR-146a or a control vector. (**Figure 2B Top**). Moreover a northern blotting was assessed to proof the efficiency of the transduction, checking on the miR-146a expression levels in resting and stimulated cells. (**Figure 2B Bottom**). After a positive selection with puromycin, the levels of miR-146a were assessed (**Figure 2C**). Interestingly, we did not see any effect in proliferation in Jurkat cells (**Figure 2D**) or in T cells at early time-points after stimulation. To further investigate the role of miR-146a specifically in human lymphocytes, cells were transduced with a lentiviral vector expressing both a GFP reporter and miR-146a or a control vector (**Figure 3A**). After sorting, levels of miR-146a expression were assessed by qRT-PCR (**Figure 3B**), and resulted to be overall comparable to the physiological levels of endogenous overexpression observed in human and mouse lymphocytes upon TCR stimulation (**Figure 1A and B**). However, we found that primary human cells expressing miR-146a did not show any significant

alteration in Fas-mediated cell death (**Figure 4A**), with levels of CD95 that also remained comparable between miR-146a expressing cells and controls (**Figure 4B)**. Such discrepancy in cell death could be due to the fact that while in Jurkat cells activation-induced cell death (AICD) was shown to be completely cell autonomous, primary T cells undergo AICD only if they can make contact with their activated neighbors, resulting in 'fratricide' rather than 'suicide'[146-148]. Moreover, we observe no significant effect of miR-146a on IL-2, IFN-γ or IL-4 cytokine production (**Figure 5A and data not shown**).

- MiR-146a could have a role in regulating human T cell responses and memory formation possibly though the modulation of CCR7 and TRAF6.

Interestingly, as we showed in figure 2D, we did not see any effect in proliferation in Jurkat cells or in T cells at early time-points after stimulation but human T cells expressing miR-146a showed increased expansion upon TCR stimulation (**Figure 5B**). Specifically, cell expansion was initially similar between miR-146a-expressing and control cells (up to ~day 3-4 after stimulation), but miR-146a-expressing cells were able to sustain proliferation for longer times after TCR stimulation. This effect was especially evident in the absence of exogenous IL-2, and it was independent of anti-CD3 concentration (**Figure 5B and data not shown**). TCR stimulation of unsorted populations containing both transduced (GFP+) and un-transduced (GFP–) cells, led to a steady increase of the percentage of GFP+ cells in miR-146a-expressing cells, but not in control cells, further indicating that this miRNA could act directly in response to TCR stimulation to favor cell expansion (**Figure 6A**). Ruled out the fact that cytokine production was largely unaffected by miR-146a expression in all the conditions tested, the possibility that miR-146a-expressing cells could sustain their own proliferation through increased IL-2 production. Of note, the IL-2 signal strength has been shown to contribute to the differentiation of CD8+ effector and central memory T cells in the mouse[149]. Along the same line, in our culture conditions, the absence of IL-2 evidentiated a T_{EM}-like phenotype, with inability to sustain CCR7 expression and cell expansion for long time after TCR stimulation (**Figure 6B**). Interestingly, miR-146a expression determined a reduction of CCR7 expression in all condition tested meaning that not only miR-146a contributes to cell expansion upon stimulation, but it may also modulate the establishment of a T_{EM}-like memory phenotype (with reduced CCR7 expression) in

primary human T cells (**Figure 6C**) as well as in naïve CD4+ T cells (**Figure 6D, left panel**). Of note, it was shown that mice with a T-cell-specific deletion of TRAF6 mounted robust CD8 effector responses, but had a profound defect in their ability to generate memory cells[150]. Interestingly, Fas-mediated apoptosis in TRAF-KO T cells was normal, and cells showed increased proliferation in response to TCR stimulation[151]. Indeed, in reporter assay experiments the 3'untranslated region (UTR) of Traf6 was efficiently targeted by miR-146a in human naïve T cells **(Figure 6D middle and right panel respectively)** as well as in Jurkat cells and in primary murine Th2 cells (**Figure 7)**. These data point towards a role for miR-146a in regulating human T cell responses and memory formation possibly though the modulation of a number of targets including TRAF6.

- Human Th1 and Th2 cells express similar levels of miR-146a, meaning that it is probably not implicated in Th1/Th2 polarization.

Since we originally reported that miR-146a was differentially expressed between Th1 and Th2 murine lymphocytes[88], we evaluated a possible role for this miRNA in lymphocytes polarization by analyzing its expression in human T cell subsets. Surprisingly, human Th1 and Th2 cells expressed similar levels of this miRNA (**Figure 8A-B**), indicating that miR-146a is probably not implicated in Th1/Th2 polarization, which in fact appears to be normal in mice lacking *miR-146a*[89]. To identify new relevant miRNA candidates that may be important in human T cell polarization we performed miRNA arrays on highly purified T cell subsets from peripheral blood (Zielinski, Sallusto and Monticelli, unpublished). Confirming our own data, there was no significant difference in miR-146a expression in primary human Th1, Th2 and Th17 cells. However, this miRNA turned out to be expressed at very high levels in Tregs, as it has been already shown for the mouse[89], suggesting a possible role for miR-146a in regulating Treg functions also in human.

Discussion

Memory cells are characterized by the ability to quickly expand and respond upon secondary exposure to their cognate antigen. Two phenotypic subpopulations of T_M cells have been identified based on the expression of different surface markers: T_{EM} and T_{CM}.

MiR-146a is a known regulator of the immune system, with established roles in the regulation of both innate and adaptive immunity. Recently it has been found that NF-kB and c-ETS binding sites are essential for the induction of miR-146a transcription upon TCR stimulation in T cells[83]. Moreover, in a Jurkat T cell line, miR-146a was shown to regulate activation-induced cell death (AICD) through modulation of FADD expression[84]. In human T cells, miR-146a is expressed at low levels in naïve T lymphocytes while it is abundantly expressed in memory T cells[88] as well as in Treg[89] and it is induced upon TCR stimulation, consistent with its expression being dependent on NF-kB induction[84]. In Treg cells, miRNA ensures their suppressor function by maintaining a normal activation of transcription factors[89].

In this chapter we showed that expression of miR-146a was consistently elevated in the CD4 and CD8 memory compartment in both human and mouse, meaning that this miRNA may have a role in regulating T cell activation and/ or in the establishment of immunological memory compartment. Moreover we found that miR-146a-expressing cells were able to sustain proliferation for longer times after TCR stimulation. This effect was especially evident in the absence of exogenous IL-2. While IL-2 does not significantly contribute to the initial cycling of antigen stimulated T cells, as signals generated through TCR stimulation are sufficient for initial activation, it is nevertheless necessary for the survival of activated cells and the successful generation of effector and memory responses[152]. Of note, the IL-2 signal strength has been shown to contribute to the differentiation of CD8+ effector and central memory T cells in the mouse[149]. Interestingly, miR-146a expression determined a reduction of CCR7 expression in all condition, with or without exogenous IL-2, meaning that miR-146a contributes to cell expansion upon stimulation and also modulate the establishment of a T_{EM}-like memory phenotype in primary human T cells. Such effect on the human primary T cells is unlikely to be exerted directly on CCR7 as this molecule is not a direct target for this miRNA[73], and it is therefore probably mediated by other miR-146a targets such as TRAF6-IRAK1

and STAT1[83/89/144].

In summary, we showed that in human primary T cells (and possibly mouse T cells as well) a general role for this miRNA, independent on the T cell subset, appears to be the regulation of cell expansion upon TCR engagement, potentially also influencing the generation of the memory compartment. Although our effect was too modest to allow a full biochemical investigation of the targets, the importance of the data shown here relies on the identification of a new possible role for a miRNA in the establishment of human T cell memory, and on the identification of other miRNAs that may be relevant in human T cell polarization.

Figures Legends

Figure 1. Memory T cells express high levels of miR-146a. A) (Left panel) Primary human T cells were sorted from peripheral blood as follows: naïve: CD4+ CD8– CD25– CD45RA+ CCR7+; TEM: CD4+ CD8– CD25– CD45RA– CCR7–; TCM: CD4+ CD8– CD25–CD45RA– CCR7+. Cells were lysed in Trizol immediately after sorting and miR-146a expression was assessed by qRT-PCR. (**Right panel**) resting primary human CD4+ cells were either immediately lysed in Trizol or were stimulated with plate-bound anti-CD3 and anti- CD28 for 3 and 6 days prior analysis of miR-146a expression by qRT-PCR. **B) (Left and middle panel)** C57Bl/6 mice were challenged sub-cutaneously with CFA-OVA. Four days after challenge effector cells were sorted from peripheral lymph nodes as CD4+ (or CD8+) $CD62L^{lo}$ $CD44^{hi}$ CD127–, while memory cells were sorted from spleen and lymph nodes two weeks after challenge. T_{EM} cells were sorted as CD4+ (or CD8+) $CD62L^{lo}$ $CD44^{hi}$ CD127+, while T_{CM} cells were CD4+ (or CD8+) $CD62L^{hi}$ $CD44^{hi}$ CD127+. Naïve T cells were sorted as CD4+ (or CD8+) $CD62L^{hi}$ $CD44^{lo}$ CD127–. Cells were lysed in Trizol immediately after sorting for RNA extraction and miR-146a levels were assessed by TaqMan qRT-PCR. Each bar in the graph is a pool of two mice, and the graph is representative of four independent experiments. **(Right panel)** Naïve CD8+ T cells were isolated from the spleen and lymph nodes of C57Bl/6 mice and were stimulated for 2 days with plate-bound anti-CD3 and anti-CD28 and further expanded in the presence of 10-100U/mL IL-2. At day 5, cells were either left resting or were restimulated with PMA and ionomycin for 6h prior RNA extraction and TaqMan analysis to assess miR-146a expression.

Figure 2. MiR-146a overexpression in Jurkat T cells led to a mild reduction in cell death. A) Jurkat cells transduced with the lentiviral vectors (schematically represented on B top) were selected with puromycin prior treatment with 20ng/mL anti-Fas antibody for 24-48h. Cell death was assessed by annexin V staining. Shown is the mean of 4 independent experiments, normalized in each case to the level of cell death in the control cells. **B)** Cells as in A) were stimulated with PMA and ionomycin (P+I) for 24h and RNA was extracted. The control vector used expressed an irrelevant hairpin directed against luciferase (ctrl-shLuc). MiR-146a

expression was assessed by Northern blot. As a control, the same blot was hybridized with a probe corresponding to the mature product from the luciferase shRNA. A probe against an Arg-tRNA was used as loading control. Differently from primary T cells, unstimulated Jurkat cells expressed undetectable levels of miR-146a. **C)** Same as in A), except that the control vector used expressed a non-targeting hairpin (NT) and that miR-146a expression was assessed by qRT-PCR. **D**) Cells as in figure A) were stained with propidium iodide for DNA content and cell cycle analysis.

Figure 3. Forced miR-146a expression does not influence Fas-mediated cell death or CD95 expression. A) Top: schematic of the lentiviral vectors used throughout this study. **Bottom**: Primary human T cells transduced with the indicated vector were sorted for GFP expression 2-5 days after transduction. Shown are cells obtained after sorting in one representative experiment. **B**) Primary human T cells transduced with the indicated vectors were lysed in TRIzol to determine miR-146a expression by qRT-PCR. Shown is one representative experiment.

Figure 4: Primary human cells expressing miR-146a did not show alteration in Fas-mediated cell death. A) Primary human T cells of different subsets (mostly Th1 and Th2) were transduced with the indicated lentiviral particles and were either left untreated of were treated over-night with 20ng/mL of an anti-Fas antibody. Shown is the mean of 10 independent experiments. **B)** Primary human T cells were transduced with the indicated vectors, and CD95 expression was assessed by surface staining at different days after transduction. Shown is one representative experiment out of three.

Figure 5: MiR-146a expression enhances human T cell expansion A) Primary human T cells transduced with either a miR-146a- or control-expressing vector were stimulated with PMA and ionomycin for 5h prior intracellular cytokine staining to assess expression of IL-2 and IFN-γ. The staining is representative of tens of experiments. **B)** Primary human T cells transduced with either a miR-146a- or control-expressing vector were stimulated for 48h on plate-bound anti-CD3 and anti-CD28 in the presence or absence of 500U/mL IL-2. Cell number was assessed daily and plotted as fold expansion. The left panel shows one representative experiment while the right panel shows the mean result of four experiments (fold expansion at

day 6 only).

Figure 6: MiR-146a expression determined a reduction of CCR7 expression. **A)** Cells were treated as in figure 5A except that they were not sorted after transduction and were left as a mixture of GFP+ and GFP– (untransduced) throughout the experiment. **B)** Primary human T cells were either left resting or were stimulated with plate-bound anti-CD3 and anti-CD28 for 48h in the presence or absence of exogenous IL-2, after which the expression of CD45, CD25, CD62L and CCR7 was assessed daily. Stimulated cells were homogenously CD45RA– CD25+. Shown is one representative FACS-plot of resting or day 3 and day 5-stimulated cells. **C)** Primary human T cells transduced with either a miR-146a- or control-expressing vector were sorted and lysed in Trizol prior analysis of CCR7 mRNA expression by qRT-PCR. Shown is one representative experiment out of three. **D)** Naïve T cells were sorted from peripheral blood as follows: CD4+ CD8– CD25– CD45RA+ CCR7+ and transduced as figure 3A. After 48h from the transduction, cells were stimulated with plate-bound anti-CD3 and anti-CD28 for 48h in the presence or absence of exogenous IL-2, and after 5 days of stimulation, the expression of CCR7 (**left panel**), TRAF 6 (**middle panel**) and miR-146a (**right panel**) was assessed by q-RT –PCR.

Figure 7: TRAF6 is a target of miR-146a in JKT cells and in murine Th2 cells. Top: Jurkat cells were transduced with the lentiviral vectors. After puromycin selection, cells were electroporated with a Traf6-UTR luciferase reporter vector, and luciferase levels were assessed 36h later. Shown is the mean of three independent experiments. **Bottom**: Primary murine Th2 cells were differentiated *in vitro* for 5 days prior transfection with Amaxa. $2.5x10^6$ cells were transfected with 1-3μg Traf6-UTR reporter plasmid, 0.5μg TK Renilla, 100nM double-stranded oligonucleotides (miR-21 was used as an irrelevant miRNA control). Luciferase assay was performed 24h after transfection. Shown is the mean of two independent experiments, with levels of 'mock' transfected cells (no double-stranded (ds) RNA) set to 100 and used as a reference.

Figure 8: Human Th1 and Th2 cells express similar levels of miR-146a. A) Naïve CD4+ T cells were isolated from the spleen and lymph nodes of C57Bl/6 mice and were differentiated *in vitro* to Th1 and Th2 T cell subset by stimulating them for 2 days with plate-bound anti-CD3 and anti-CD28 in the presence of IL-12 and anti-IL-4 antibody (Th1 conditions), or IL-4 and anti-IL-12 and anti-IFNg antibodies (Th2 conditions). Cells were then expanded until day 5 in media containing 20U/mL IL-2. At day 5, cells were either left resting or were restimulated with PMA and ionomycin for 6h prior RNA extraction and TaqMan analysis to assess miR-146a expression (**top**) and intracellular cytokine staining to verify polarization (**bottom**). SnoRNA202 was used as endogenous control. **B)** MiR-146a expression in human Th1 and Th2 clones was assessed by TaqMan qRT-PCR (**top**). RNU48 was used as endogenous control. Clones were characterized by the expression of surface markers and by the expression of IFN-g and IL-4 (**bottom**). Th1 cells were CD4+ CD8– CD45RA– CD25– CCR5+ CRTh2–, and Th2 cells were CD4+ CD8– CD45RA– CD25– CCR5– CRTh2+. In some experiments, Th1 cells were also sorted as CD4+ CD8– CD45RA– CD25– CXCR3+ CCR6– CCR4–, and Th2 cells as CD4+ CD8– CD45RA– CD25– CXCR3– CCR6– CCR4+. Both of these sorting protocols gave similar results in terms of enrichment of specific cytokine-producing subsets.

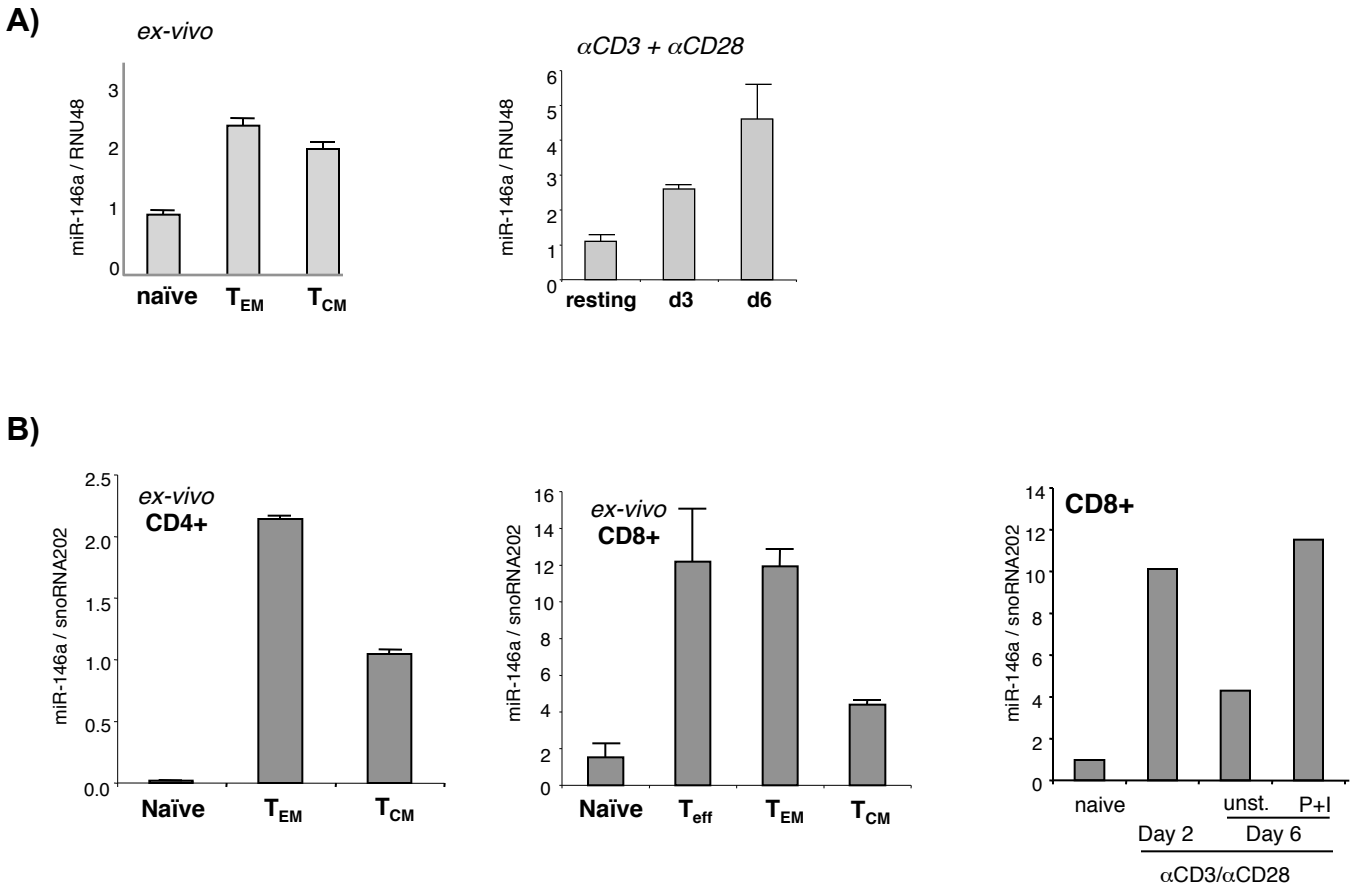
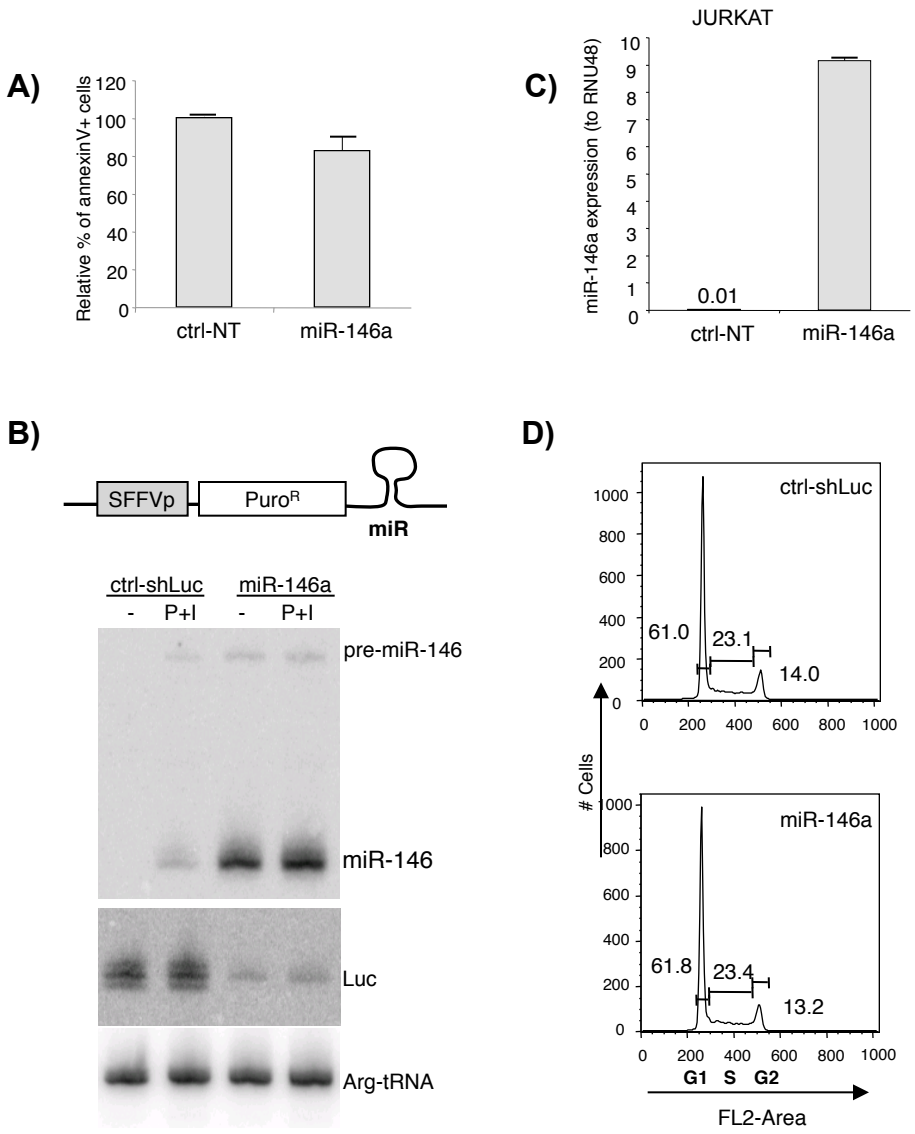


Figure 1: miR-146a in human and mouse T cells



<u>Figure 2</u>: miR-146a overexpression in JKT cells and cell death

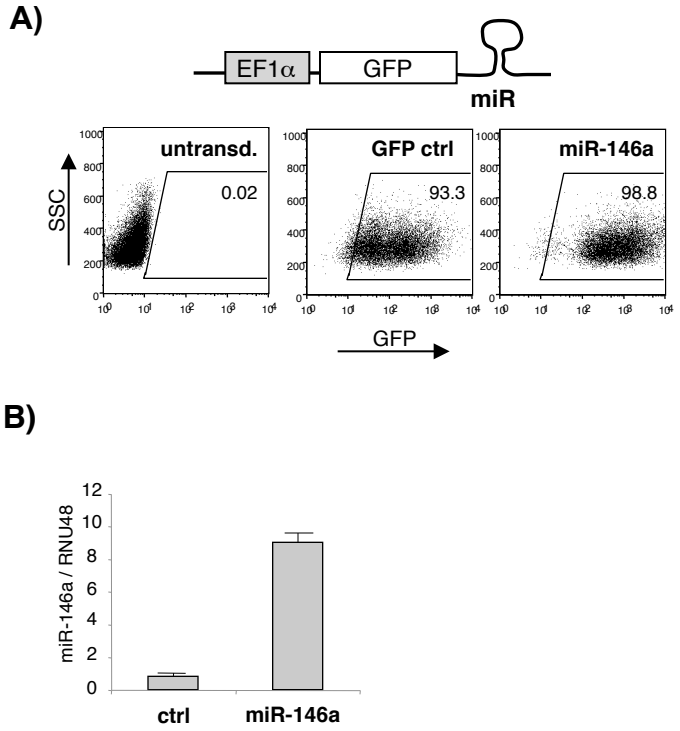
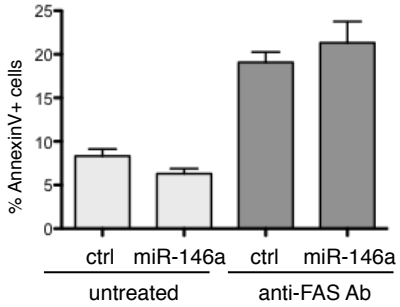
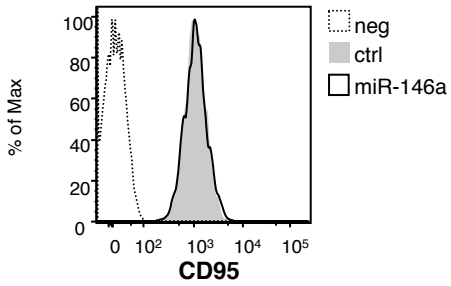


Figure 3: miR-146a overexpression in Human T cells

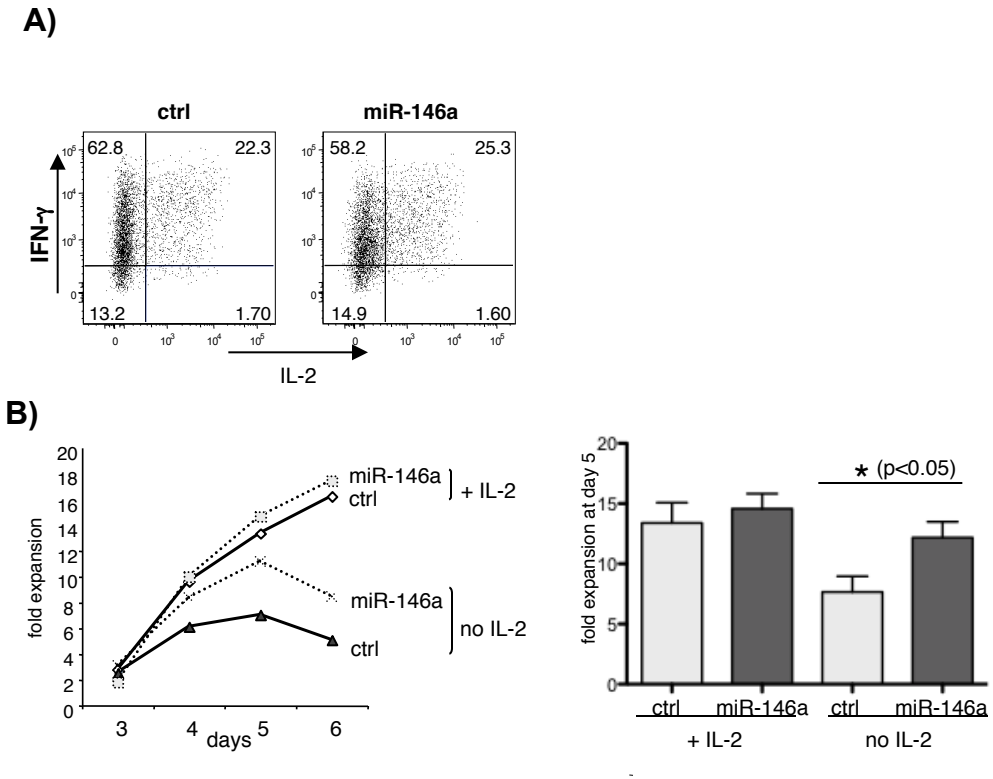
A)



B)



<u>Figure 4</u>: miR-146a and Fas-mediated cell death



<u>Figure 5:</u> MiR-146a favors human T cell expansion

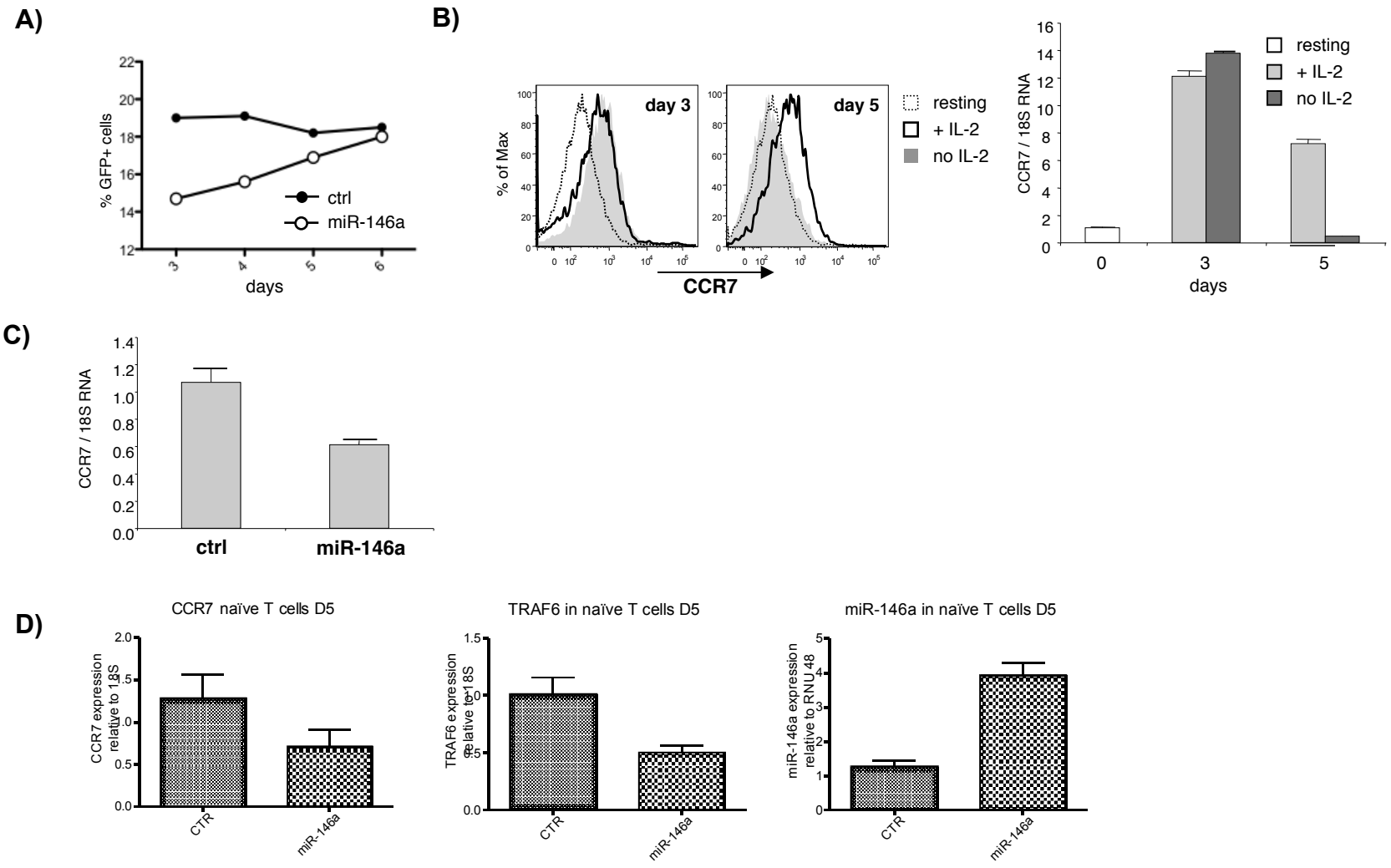
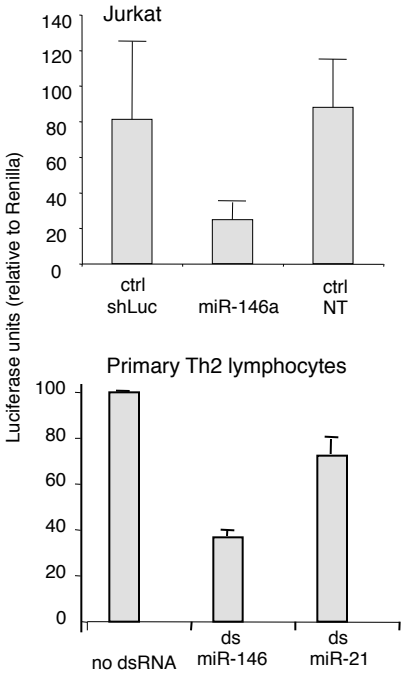


Figure 6: CCR7 expression



<u>Figure 7</u>: TRAF6 is a target of miR-146a in JTK and primary murine Th2 cells

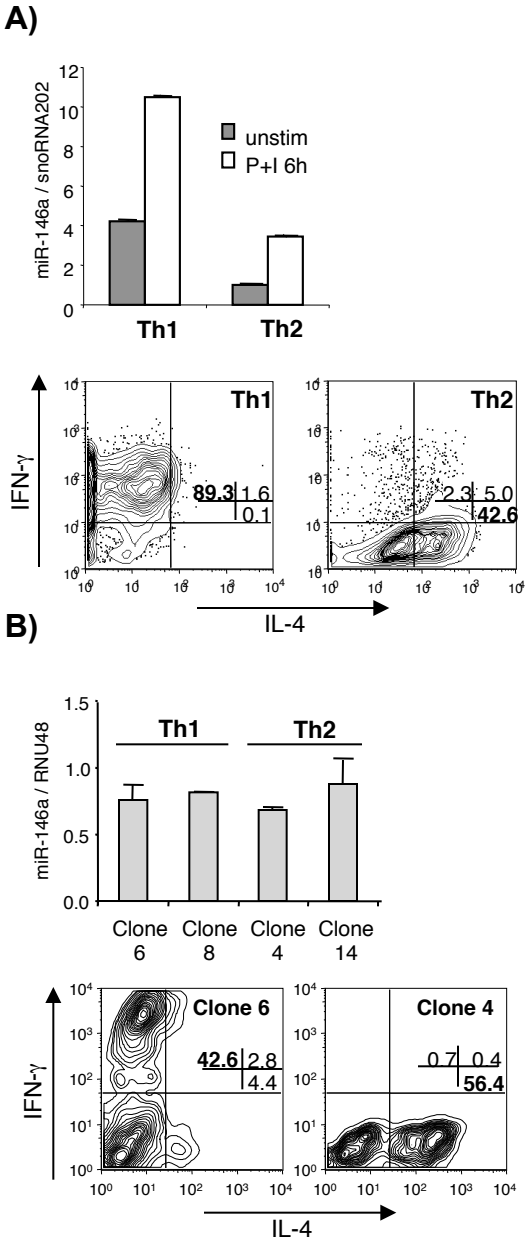


Figure 8: miRNA in Human T cells

Chapter 4

"MiR146a in immunity and disease"

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Review Article

MiR-146a in Immunity and Disease

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MicroRNAs (miRNAs) are regulatory molecules able to influence all aspects of the biology of a cell. They have been associated with diseases such as cancer, viral infections, and autoimmune diseases, and in recent years, they also emerged as important regulators of immune responses. MiR-146a in particular is rapidly gaining importance as a modulator of differentiation and function of cells of the innate as well as adaptive immunity. Given its importance in regulating key cellular functions, it is not surprising that miR-146a expression was also found dysregulated in different types of tumors. In this paper, we summarize recent progress in understanding the role of miR-146a in innate and adaptive immune responses, as well as in disease.

1. Introduction

MicroRNAs (miRNAs) represent a pervasive feature of all cells, as they regulate large fractions of the cell's transcriptome. So far, 672 mouse miRNAs and 1048 human miRNAs have been described in the miRBase database (http://www.mirbase.org/, release Sept. 2010) with each miRNA potentially regulating the expression of hundreds of target genes, highlighting the extent of this form of regulation [1]. Whereas some miRNAs are widely expressed, others exhibit only limited developmental stage-, tissue-, or cell type-specific patterns [2]. Similar to any other mammalian cell type, cells of the immune system rely on miRNAs to regulate lineage commitment, proliferation, migration, and differentiation. In most cases, these activities are orchestrated by both ubiquitously expressed and cell type-specific miRNA species [3–7]. The importance of miRNAs in regulating differentiation and function of immune cells is underlined by the phenotypical perturbations that occur when miRNA expression is altered. Given the emerging roles of miRNAs in modulating immune responses, it is likely that any dysregulation of miRNA expression may contribute to the pathogenesis of autoimmune diseases, chronic inflammation, and malignancies. Indeed, several human diseases have now been associated with dysregulated miRNA expression, and miRNAs have been shown to function both as oncogenes and tumor suppressor genes [8, 9]. MiR-146a has been recently shown to be an important modulator of differentiation and function of cells of innate as well as adaptive immunity. Here, we summarize recent progress in understanding the role of miR-146a in immune responses and in disease (see also Table 1).

2. What Are MicroRNAs?

MiRNAs are small (20–25 nucleotides), noncoding RNA molecules involved in posttranscriptional gene regulation. They derive from primary transcripts (pri-miRNA) that are processed into hairpin precursors (pre-miRNAs) within the nucleus of the cell by the Microprocessor complex, which includes the RNAseIII enzyme Drosha. Pre-miRNAs are translocated into the cytoplasm and processed by Dicer into their mature form (for a recent review see [25]). An exception to this rule is represented by the less abundant "mirtrons", that bypass Drosha and are processed only by Dicer [26]. Mature miRNAs loaded onto the RNA-induced silencing complex (RISC) recognize sites located mostly in the 3′ untranslated region (3′ UTR) of target mRNAs through canonical base-pairing between the seed sequence of the miRNA (nucleotides 2–8 at its 5′ end) and its complementary sequence in the target mRNA. This leads to a block in translation with or without destabilization and degradation of the targeted mRNA. MiRNAs modulate a

Table 1: Summary of the described roles for miR-146a in immune responses and disease, with indicated references and known targets.

Ref.	Expression/Function	Targets
	Mouse	
[5]	Differential expression in Th1/Th2 cells	
[10]	Impaired Treg function in mice lacking miR-146a	STAT1
[11]	Provided protection from bacteria induced epithelial damage in neonates	IRAK1
	Human	
[12]	Attenuated TLR4 signaling in monocytes	IRAK1, TRAF6
[13]	Regulated activation induced cell death and IL-2 expression in Jurkat T cells	FADD
[14]	Contributed to the establishment of endotoxin tolerance in monocytes	IRAK1, TRAF6
[15]	Desensitized Langerhans cells to inappropriate TLR signaling	
[16]	Negatively regulated inflammatory response in lung epithelial cells	
[17]	Controlled megakaryopoiesis	CXCR4
[18]	Reduced migration and invasion capacity of breast cancer cells	IRAK1, TRAF6
[19]	Promoted cell proliferation in cervical cancer	
[20]	Tumor suppressor in hormone-refractory prostate cancer	ROCK1
[21]	Suppressed invasion of pancreatic cancer cells	EGFR, IRAK1
[22]	SNP in pre-miR-146a predisposes to papillary thyroid carcinoma	
	Viruses	
[23]	Promoted VSV replication in macrophages	IRAK1, IRAK2, TRAF6
[24]	EBV-encoded LMP1 induced cellular miR-146a expression	

broad range of gene expression patterns during development and homeostasis, as well as in pathogenesis of disease. Most miRNAs are transcribed by RNA polymerase II, and their upstream regulatory regions contain canonical core promoters and enhancers, regulated by transcription factors. The importance of miRNA biogenesis as a whole in the immune system is clearly highlighted by the fact that conditional ablation of Dicer or other miRNA processing factors resulted in a profound block of both B- and T-cell development [27–30]. Moreover, deletion or overexpression of certain individual miRNAs also led to a severe impairment of the development and/or function of cells of the immune system [31–38]. Some miRNAs, like miR-146, are expressed as a family that shares the same seed sequence, but is encoded by different loci in the genome. Indeed, the miR-146a gene is located on mouse chromosome 11, while miR-146b is located on chromosome 19 (chromosomes 5 and 10, resp., in human). The mature sequences for miR-146a and miR-146b differ by only two nucleotides. Nevertheless, since they share the same seed sequence they should in principle recognize the same targets [39]. While in some cell types (like monocytes) these miRNAs appear to have similar functions [12], in other cases, like in regulatory T (Treg) cells [10], only miR-146a, but not miR-146b, was shown to be highly expressed. While it is still unclear if these two miRNAs have redundant and/or separate functions, the majority of the published work focuses mostly on miR-146a, which is therefore the miRNA we will be mainly referring to in this paper.

3. MiR-146a in Adaptive Immune Responses

Adaptive immune responses are vital for the efficient eradication of infectious agents, although dysregulated responses might also lead to autoimmune and chronic inflammatory diseases. The development and propagation of an adaptive immune response specific for an invading pathogen is a highly orchestrated process that involves the activation and proliferation of immune cells and their subsequent migration to sites of inflammation. The first indication that miRNAs were involved in regulating differentiation of cells in the immune system came from a study from Chen and colleagues that identified miR-181 as a miRNA specifically expressed in hematopoietic cells [31]. Its ectopic expression in hematopoietic progenitors led to an increased fraction of B-lineage cells in both *in vitro* differentiation assays and mouse models. Following this pioneering work, many studies identified miRNAs as crucial components of the molecular circuitry that controls differentiation and functions of cells of the immune system.

Upon encounter with the antigen, naïve CD4+ T-cells give rise to T-cell subsets (Th1, Th2, Th17, Tregs, T follicular helper and probably others) with functions that are tailored to their respective roles in host defence [40]. Initial expression profiling studies identified miRNAs specifically expressed in different T-cell subsets and stages of differentiation [5–7]. T-cell-specific deletions of Dicer revealed a requirement for the miRNA pathway in the development of T cells [27, 29], as well as for differentiation of effector

T-cell subsets. Indeed, T cells lacking Dicer showed increased differentiation to the Th1 subset with a correspondingly reduced polarization to Th2 [29]. Adding to the complexity of gene regulatory networks, proliferating T cells express genes with shorter 3′ UTRs than those expressed in resting T cells, making these mRNAs less susceptible to regulation by miRNAs due to the loss of miRNA binding sites [41]. Finally, individual miRNAs were also shown to play important roles in T-cell differentiation and function. For example, miR-181a, which is upregulated during T-cell development, was shown to enhance T-cell receptor (TCR) signalling strength by directly targeting a number of protein phosphatases [32], while mice lacking miR-155 showed an altered Th1/Th2 polarization with a bias towards Th2, indicating that miR-155 promotes differentiation towards Th1 cells [35].

As for the role of miR-146a in T cells, by analyzing the expression of miRNAs in highly purified subsets of cells of the immune system, we showed that miR-146a is one of the very few miRNAs differentially expressed between Th1 and Th2 cells in the mouse, suggesting that it might be involved in fate determination of these cells [5]. Recent work performed in miR-146a-deficient mice showed an increase in the percentage of INFγ-producing T-cell subset in the absence of miR-146a [10]. In human T cells, miR-146a is expressed at low levels in naïve T lymphocytes while it is abundantly expressed in memory T cells and it is induced upon TCR stimulation, consistent with its expression being dependent on NF-κB induction [12, 13]. Indeed, NF-κB and c-ETS-binding sites were shown to be required for the induction of miR-146a transcription in human T cells, and such induction potentially modulated cell death in these cells by targeting FADD and by impairing both AP-1 activity and IL-2 production [13]. Treg cells constitute a specialized T-cell subset able to maintain immune homeostasis by limiting the inflammatory responses, and their suppressive function is indispensable for immune homeostasis and survival of higher organisms. Recently, Lu and colleagues reported that miR-146a is among the miRNAs prevalently expressed in Treg cells and showed that it is critical for Treg functions. Indeed, deficiency of miR-146a resulted in increased numbers but impaired function of Treg cells and as a consequence, breakdown of immunological tolerance with massive lymphocyte activation, and tissue infiltration in several organs [10]. The immune-mediated lesions induced by the lack of miR-146a in Tregs were dependent on INFγ and Stat1.

4. MiR-146 in Innate Immunity and Nonimmune Systems

Cells of the innate immune system, such as granulocytes, natural killer (NK) cells, monocytes, and macrophages, provide an important first line of defense for the organism against invading pathogens. MiRNAs have been implicated in both the development and functions of innate immune cells. For example, the macrophage inflammatory response to infection involves the upregulation of several miRNAs, such as miR-155, miR-146, miR-147, miR-21, and miR-9 [12, 43–46]. Several studies linked miR-146a expression to NF-κB signaling within the innate immune system (Figure 1) and were initiated by a study showing that miR-146a is quickly induced upon activation of human monocytes [12]. In this study, miR-146a was found to be inducible upon stimulation with LPS in a NF-κB-dependent manner, and to target the TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) genes. These genes encode two key adapter molecules downstream of cytokine and Toll-like receptors (TLR), pointing towards a role for miR-146a in controlling signaling from these receptors through a negative feedback regulatory loop involving downregulation of TRAF6 and IRAK1 [12]. It was also suggested that miR-146a contributes to the establishment of endotoxin tolerance in monocytes and to the regulation of TNFα production [14]. In this context, miR-146a would therefore act as a tuning mechanism to prevent an overstimulated inflammatory state. In human Langerhans cells (LCs), miR-146a was found to be constitutively expressed at high levels, as compared to interstitial dendritic cells (intDCs) [15]. In these cells, high miR-146a expression was induced by the transcription factor PU.1 in response to TGF-β1, a key

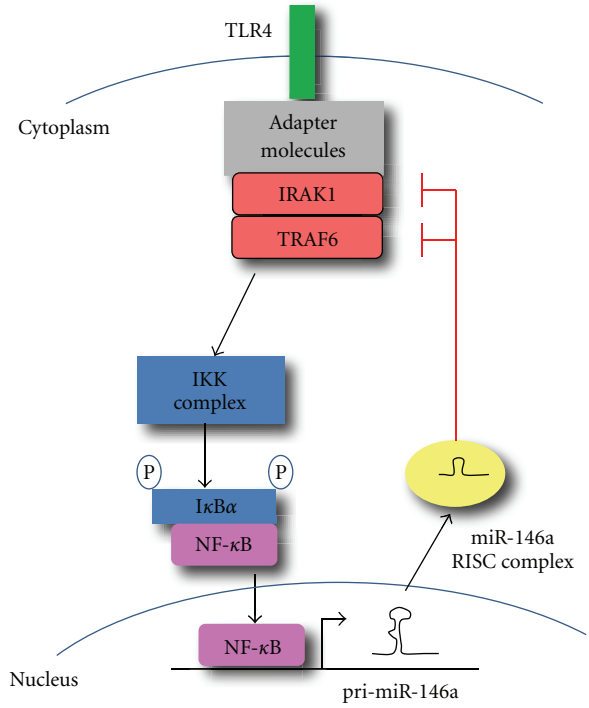

Figure 1: MiR-146a negatively regulates signal transduction pathways leading to NF-κB activation. Upon activation of a cell surface receptor such as TLR4, a molecular cascade including TRAF6 and IRAK1 leads to IκBα phosphorylation and degradation and to NF-κB activation and nuclear translocation [12, 42]. NF-κB activation induces transcription of many genes, including pri-miR-146a. Once translocated to the cytoplasm and loaded onto the RISC complex, mature miR-146a contributes to attenuate receptor signaling through the downmodulation of IRAK1 and TRAF6.

signal for epidermal LC differentiation, and while it did not influence myelopoiesis or DC subset differentiation, the authors suggested that constitutively high miR-146a expression may represent a novel mechanism to desensitize LCs to inappropriate TLR signaling at epithelial surfaces through decreased NF-κB signal strength downstream of the receptor [15].

Somewhat differently from the studies mentioned above, a study performed in human lung alveolar epithelial cells showed a rapid, time- and concentration-dependent increase in miR-146a upon stimulation with IL-1β [16]. Such increased miR-146a expression negatively regulated the release of the proinflammatory chemokines IL-8 and RANTES in a way that did not seem to involve IRAK1 and TRAF6, highlighting how the role of miRNAs can be exquisitely cell type-specific. MiR-146a was also shown to have an important role in normal epithelial functions: specifically, miR-146a-mediated downregulation of IRAK1 was sufficient to induce innate immune tolerance and provide protection from bacteria-induced epithelial damage in neonates [11].

A molecular cascade involving miR-146a, the miR-146a negative regulator PLZF, and the miR-146a target CXCR4 was also shown to be active during megakaryopoiesis [17]. This regulatory pathway involved enhanced expression of PLZF, which in turn inhibited miR-146a transcription. The resulting downmodulation of miR-146a caused an increase in CXCR4 expression, which is necessary for megakaryocytes differentiation and maturation. As a result, miR-146a overexpression, as well as PLZF or CXCR4 silencing, impaired megakaryocytic proliferation, differentiation, and maturation, as well as colony formation [17].

5. MiR-146a and Viruses

Various layers of negative regulators are used by immune cells to avoid uncontrolled immune responses when facing viral invasion, and such regulatory mechanisms can also be used by viruses in order to escape immune surveillance. A role for miR-146a was discovered in the regulation of vesicular stomatitis virus (VSV) infection [23]. In macrophages, VSV infection upregulated miR-146a expression in a RIG-I/NF-κB-dependent manner. Elevated miR-146a expression led to negative regulation of the production of VSV-triggered type I IFN through downregulation of TRAF6, IRAK1, and IRAK2, thus promoting VSV replication in macrophages [23]. The authors proposed a model in which VSV infection is first sensed by RIG-I, which in turn initiates type I IFN production against VSV infection. At the same time, VSV infection upregulates miR-146a expression, which inhibits innate antiviral immune response by impairing RIG-I signaling.

The Epstein-Barr virus (EBV) infects over 90% of the human population worldwide. EBV infection can result in a number of malignancies, including Burkitt's and Hodgkin's lymphomas. LMP1 (latent membrane protein 1) is the major oncoprotein of EBV, able to activate transcription factors such as NF-κB and AP-1, and thus to manipulate host cellular processes that regulate cell proliferation, migration, and apoptosis. Through its ability to activate transcription factors, LMP1 also induces expression of cellular miRNAs, most notably miR-146a, which therefore could contribute to cellular immortalization and tumorigenesis in the context of EBV infection [24].

6. MiR-146 and Cancer

Cancer is the result of a complex multistep process that involves the accumulation of sequential alterations in several genes, including those encoding miRNAs [8, 9]. Since miRNAs participate in keeping the balance of gene regulatory networks that determine the fate of a cell, their dysregulation potentially weakens this balance, thereby contributing to oncogenesis and cancer progression. Indeed, miRNA profiling has uncovered significantly altered miRNA expression in many types of cancer [8].

Initial evidences on the possible involvement of miR-146a in cancer came from a study showing that miR-146a was upregulated in papillary thyroid carcinoma (PTC) samples compared with unaffected thyroid tissue. Interestingly, a set of five miRNAs, including miR-221, miR-222, and miR-146, was sufficient to distinguish unequivocally between PTC and normal thyroid [47]. Similarly to the observations performed in immunologic settings, overexpression of miR-146a/b in the highly metastatic human breast cancer cell line MDA-MB-231 significantly downregulated expression of IRAK1 and TRAF6, negatively regulating NF-κB activity [18]. Functionally, this resulted in markedly impaired invasion and migration capacity relative to control cells. These findings implicated miR-146 not only as a negative regulator of constitutive NF-κB activity in breast cancer cells, but also suggested that modulating miR-146 levels might have therapeutic potential to suppress breast cancer metastases. Along the same line, miR-146a was among the miRNAs found upregulated in cervical cancer tissues compared to normal cervix [19]. When introduced into cell lines, miR-146a promoted cell proliferation. Although the molecular mechanism underlying such increased proliferation remains to be investigated, these observations potentially implicate miR-146a in cervical carcinogenesis. In another type of cancer, the hormone-refractory prostate carcinoma (HRPC), miR-146a levels were diminished compared to androgen-sensitive noncancerous epithelium [20]. In this context, miR-146a acted as a tumor suppressor, reducing levels of its target ROCK1, one of the key kinases involved in HRPC transformation. Accordingly, forced miR-146a expression reduced ROCK1 protein levels, cell proliferation, invasion, and metastasis to human bone marrow endothelial cell monolayers. Similarly, miR-146a was lower in pancreatic cancer cells compared with normal human pancreatic cells [21]. Re-expression of miR146a inhibited the invasive capacity of pancreatic cancer cells with downregulation of EGFR (epidermal growth factor receptor) and IRAK-1. Finally, a recent study showed that the treatment of bone marrow-derived mesenchymal stem cells (MSCs) with

diazoxide (DZ) markedly increased the expression of miR-146a and promoted cell survival. Moreover the down-regulation of miR-146a expression by antisense inhibitors eliminated the DZ-induced cytoprotective effects. This result suggested a critical role of miR-146a in MSC survival [48].

7. Polymorphisms and Posttranscriptional Modifications

Polymorphisms affecting miRNA expression, maturation, or mRNA recognition may also become important determinants for increased tumor risk. Indeed, a genetic variant in the 3′ UTR of the KIT oncogene was recently described, that resulted in a mismatch in the seed region of miR-221 and correlated with increased risk of melanoma [49]. As for miR-146a, a single nucleotide polymorphism (rs2910164; G/C) was found on the passenger strand of pre-miR-146a [22]. The rarer C allele decreased nuclear pri-miR-146a processing, reducing levels of pre-miR-146a and mature miR-146a and unblocking expression of its target genes, including TRAF6 and IRAK1. In an association study of PTC patients, the germ-line GC heterozygous state was associated with an increased risk of acquiring PTC, while both homozygous states (GG and CC) were protective. Importantly, this polymorphism was also found to undergo somatic mutation in PTC tumor tissue [22], underlying the need for more studies on polymorphisms, both on the miRNAs themselves as well as on the binding sites in their targets as, based on the studies mentioned above, they can clearly contribute to cancer progression.

Along the same line, point mutations in either a miRNA or its targets may dramatically alter miRNA expression and/or functionality, respectively. Even in normal, nondiseased conditions, specific adenosine residues of certain miRNA precursors can be edited by adenosine deaminase acting on RNA enzymes (ADAR) [50]. The resulting A → I conversions replace A-U Watson-Crick pairs with I : U wobble pairs in the double-stranded RNA, altering miRNA processing. For example, editing of pri-miR-142, expressed in hematopoietic tissues, resulted in suppression of its processing by Drosha and consequently in its degradation [50]. Interestingly, interferons induce the upregulation of ADAR1 [51], thus raising the possibility that mutations introduced by ADAR in the pri-miRNAs might lead to alteration of miRNA expression and/or target recognition during inflammation [52].

8. miR-146: A Role as Biomarker?

A biomarker is a measurable indicator of a biological state, either normal or pathological, with or without pharmacological treatments [53]. Ideal biomarkers should be detectable with high sensitivity and specificity, should have high predictive power, and should be accessible in a noninvasive manner. The data accumulated on miRNA expression in tumors demonstrate that miRNAs are indeed promising candidates to distinguish between different tumors and different subtypes of tumors, as well as to predict their clinical behavior [53]. Large miRNA expression studies have supported the role of miRNAs as either prognostic and/or diagnostic markers in various types of cancer (for a detailed review see [8]). These studies generally showed that the miRNA profiles reflected the developmental lineage and the differentiation status of tumors, and that such miRNA signatures enabled the tumor samples to be grouped on the basis of their tissue of origin. Such profiling studies will become a useful tool to identify miRNA signatures that are associated with a particular diagnosis or probable outcome of a disease.

As mentioned above, a good biomarker should also be easily accessible; the ability to detect clinically relevant miRNAs in the plasma or serum of patients raises therefore a lot of interest, particularly since serum miRNAs appear to exist in a stable and protected form, possibly within exosome-like particles [53, 54]. Moreover, the levels of miRNAs in serum were shown to be stable, reproducible, and consistent among individuals of the same species [55]. While more studies need to be performed on the role that miR-146a specifically might have as a biomarker, there is little doubt that miRNA detection in serum or PBMCs could provide a convenient and noninvasive measure for diagnosis and monitoring of many different types of disease.

9. Concluding Remarks

The discovery of miRNAs has revealed a new layer of regulation of gene expression with a profound impact on many biological systems. Studies in recent years have shown that miRNAs have a unique expression profile in cells of the innate and adaptive immune system and have crucial roles in the regulation of both cell development and function. Moreover, it is becoming widely accepted that miRNAs can function both as oncogenes or tumor suppressors in an expanding number of tumors and cell types. There is now increasing evidence to suggest that miR-146a is involved in the regulation of the adaptive as well as innate immune response, and that miR-146a can be an important player in regulating tumor progression. However, more work remains to be done to fully understand its role and mechanism of action in normal and pathologic conditions, so that expression of this miRNA can potentially be exploited as a new point of entry for therapy. With the identification of a vast number of miRNAs each carrying a long list of putative targets, the challenge is now to understand the details of their biological functions.

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Conclusions

While it is clear that miRNAs regulate many different cellular processes, understanding the details of the functions of individual miRNAs remains challenging.
A difficult aspect of dealing with miRNAs is that each miRNA has many potential targets with disparate functions, with no means to decide a priori which is the most meaningful and therefore worthy of experimental validation. Moreover, the degree of target down-regulation is typically less than 50%[123/165] and understanding which fraction of the miRNA-target interaction is actually relevant for a biological response remains a challenging task.

Here we found that miR-221 and miR-146a are activation-induced miRNAs important in regulating MCs activation and survival. Specifically miR-221 regulates basic cellular functions such as the actin cytoskeleton, as weel as stimulation dependent mechanisms, such as cytokine production and degranulation.

Moreover miR_146a regulated MC survival in a cell-intrinsic manner in a regulatory network with NF-kB p50.

Finally we showed that miR-146a had also an important role in regulating human T lymphocytes activation and expansion and possibly memory formation.

SPECIFIC CONTRIBUTIONS

The vast majority of the experiments presented in this thesis were performed by me, with the following specific exceptions for which I received help from other IRB members:

Chapter 2:

Lorenzo Dehò from my laboratory performed the thymidine incorporation assay in **Figure 2A**, the immunofluorescence in **Figure 8B**, as well as the adoptive transfer experiments shown in **Figure 9**.

Chapter 3:

Lorenzo Dehò performed the adoptive transfer assay with CD8 T cells (**Figure 1B**). Moreover Federico Mele and Christina Zielinski performed the separation of human T cell subsets from peripheral blood.

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