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MiR-146a and NF-kB1 regulate mast cell survival and T lymphocyte differentiation

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Abstract

The transcription factor NF-kB regulates the expression of a broad number of genes central to immune and inflammatory responses. We identified a new molecular network that comprises specifically the NF-kB family member NF-kB1 (p50) and miR-146a, and we show that in mast cells it contributes to the regulation of cell homeostasis and survival, while in T lymphocytes it modulates T cell memory formation. Increased mast cell survival was due to an unbalanced expression of pro- and anti-apoptotic factors, and particularly to the complete inability of p50-deleted mast cells to induce expression of miR-146a, which in the context of mast cell survival acted as a pro-apoptotic factor. Interestingly, in a different cellular context, namely human and mouse primary T lymphocytes, miR-146a and NF-kB p50 did not influence cell survival or cytokine production, but rather T cell expansion and activation in response to TCR engagement. Our data identify a new molecular network important in modulating adaptive and innate immune responses and shows how the same activation-induced miRNA can be similarly regulated in different cell types even in response to different stimuli, but still determine very different outcomes, likely depending on the specific transcriptome.

Introduction

The NF-kB family of transcription factors comprises five related proteins (c-Rel, RelA, RelB, NF-kB1 (p50) and NF-kB2), which are critical regulators of immunity, stress response, apoptosis and differentiation, and bind as dimers to kB sites in promoters and enhancers of a variety of genes to induce or repress transcription (reviewed in (25)). The crucial role played by this transcription factor in orchestrating immune responses is highlighted by the number of stimuli that can elicit NF-kB activation, including bacterial and viral infections, inflammatory cytokines and engagement of antigen receptors. As a consequence, dysregulation of NF-kB activity is linked to inflammatory disorders, autoimmune diseases, as well as cancer (25). Given the wide range of cellular responses regulated by NF-kB, it is not surprising that its activity must be tightly controlled at multiple levels by positive and negative regulatory elements. MicroRNAs (miRNAs) are now widely established modulators of many aspects of the immune responses (13). MiR-146a in particular is a well-studied modulator of the immune system (31), known to regulate NF-kB activation and tolerance in innate immunity (36), to act as an oncosuppressor, and to modulate T regulatory (Treg) cell functions (17, 42).

Mast cells are key effector cells in immediate hypersensitivity reactions and allergic disorders. Mice lacking the transcription factor p50 are unable to mount airway eosinophilic inflammation in the lung due to the inability to produce IL-4, IL-5 and IL-13, and to a defect in the polarization of Th2 lymphocytes (5, 29, 41). Despite the important role of mast cells in allergy and asthma and as a source of Th2-type cytokines, mast cell responses were never specifically evaluated in these mice. Here, we investigated whether p50 may have a role in regulating mast cell differentiation, homeostasis and function, as it could improve our understanding of the molecular mechanisms at the basis of mast cell-related diseases such as asthma, allergy and even mastocytosis. Specifically, we identified a role for p50, but also

for miR-146a, whose transcription was completely dependent on p50, in regulating mast cell homeostasis and cell survival. Interestingly, the same molecular network involving p50 and miR-146a acted also at the level of T lymphocytes to modulate immunological memory. 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 nonlymphoid tissues (33). Specifically, we found that the absence of p50 (and as a consequence, of miR-146a), led preferentially to a T_{CM} phenotype and accordingly, both human and mouse T cells forced to express higher levels of miR-146a preferentially differentiated towards a T_{EM} -like phenotype.

Overall, we provide evidence that in the absence of p50, mast cells showed altered tissue homeostasis and survival due to increased expression of pro-survival factors such as Bcl-2 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 signaling by targeting TRAF6 and reducing mast cell survival. Interestingly, in T cells miR-146a had no role in regulating T cell survival or cytokine production, but it emerged as an important regulator of T cell expansion and memory formation.

Material and Methods

Cell cultures and cell stimulation. Bone marrow-derived mast cells (BMMCs) from C57Bl/6 mice and p50-deleted mice (34) were differentiated *in vitro* by culturing total bone marrow cells for at least three weeks in IMDM medium containing 10% FBS, 2mM L-glutamine, 0.1mM non-essential amino acids, 50µM β-mercaptoethanol, antibiotics and 50% WEHI-3 conditioned supernatant as a source of IL-3. WEHI-3 conditioned supernatant was prepared exactly as described (19). When indicated, SCF (10ng/ml, Peprotech) was also added to the cultures during mast cell differentiation. Cell differentiation was assessed by surface staining for FcεRI and Kit receptor and by toluidine blue staining as described (19). Mast cells were acutely stimulated with either 1.5µg/mL IgE-anti-DNP (clone SPE7, Sigma) and 0.2µg/ml DNP-HSA (Sigma), or with 20-100µg/mL LPS, depending of the experimental conditions. In some cases, cells were stimulated with 20nM PMA and 2µM ionomycin. Primary human T lymphocytes were purified from peripheral blood and expanded as described (21). T cells were stimulated with immobilized anti-CD3 (1-10µg/mL) and anti-CD28 (2µg/mL), and expanded in presence of 500U/mL rhIL-2. Blood from healthy donors was obtained from the Swiss Blood Donation Center of Basel and Lugano, and used in compliance with the Federal Office of Public Health. Mouse naïve CD8 and CD4 T cells were purified using beads (Dynal or Miltenyi Biotec) from spleen and lymph nodes of OT-I, OT-II or C57Bl/6 mice, and FACS-sorted as CD62L+ CD44–. Naïve CD4 T cells were cultured in Th1/Th2 skewing conditions exactly as described (1, 22). 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' (approval numbers 17/2010, 18/2010 and 03/2012).

Plasmids, lentiviral and retroviral transductions. The control lentiviral vectors were previously described and expressed either an shRNA against luciferase (shLuc), a non-targeting hairpin (NT) or GFP alone (18). The 394bp PCR fragment encompassing the pre-miR-146a genomic sequence was cloned using standard cloning techniques. Depending on the experimental conditions, transduced cells were either selected with puromycin (2µg/mL for two days) or FACS-sorted for GFP expression. To optimize transgene expression, the same miR-146a or control insert was driven by the spleen focus-forming virus promoter (SFFVp) for transductions of mast cells, and by the EF1alpha promoter for transductions of human T lymphocytes. Lentiviral particles were produced exactly as described and used at a multiplicity of infection of ~60 (19, 44). Retroviral particle were generated by transient transfection of Phoenix cells as previously described (24).

qRT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen). To analyze 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 analyze Bcl-2 family member expression, total RNA (1µg) was reverse transcribed using the iScript kit (Bio-Rad), before PCR amplification with the following primers: *bcl2* FW: 5'-TTCGCAGCGATGTCCAGTCAGCT; *bcl2* RV: 5'-TGAAGAGTTCTTCCACCACCGT; *A1* FW: 5'-GATTGCCCTGGATGTATGTGCTTA; *A1* RV: AGCCATCTTCCCAACCTCCATTC; *bcl-X_L* FW: CAGTGCCATCAATGGCAACCCATC; *bcl-X_L* RV: 5'-CGCAGTTCAAACTCATCGCCTGC; *bax* FW: 5'-ACTGGACAGCAATATGGAGCTG; *bax* RV: 5'-CCCAGTTGAAGTTGCCATCAG. *β-actin* was used as an endogenous control.

Apoptosis and proliferation. For cell death analysis, mast 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 using the

AnnexinV-PE apoptosis kit (BD Pharmingen) following manufacturer's instructions. For thymidine incorporation assays, $1x10^5$ mast cells were stimulated for 24h, and in the last 16h of incubation, 1µCi/mL of [^{3}H]thymidine (GE Healthcare) was added to the cultures. Cells were then collected and levels of thymidine incorporation were evaluated with a scintillation beta-counter.

Degranulation assay. Mast cell degranulation was assessed as described (18). Briefly, $5x10^4$ cells were resuspended in 50µL OptiMEM, 1% FBS and stimulated for 1h with either PMA and ionomycin or IgE-antigen complexes. 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.

Intracellular cytokine staining. Cells were stimulated with either PMA and ionomycin, IgE and antigen complexes, or LPS for 3-5h, with addition of 10µg/mL brefeldin-A in the last 2h of stimulation. The cells were then fixed with 4% paraformaldehyde and permeabilized in 0.5% saponin/ 1% BSA prior staining with fluorescent anti-cytokine antibodies (eBioscience) and FACS analysis.

Western Blots and Immunofluorescence staining. For Western blots, total protein extracts were prepared by direct lysis of the cells in Laemmli sample buffer. Samples were separated on 12% SDS-polyacrylamide gels and immune-detection was performed with antibodies against NF-kB p50 (NLS), TRAF6 (H-274) and, as a loading control, β-Tubulin (H-235) (all from Santa Cruz Biotechnologies). Quantification was performed with a

biomolecular imager (ImageQuant LAS 4000). For immunofluorescence staining, tissue slides of paraffin-embedded organs from wild-type and p50-deleted mice were de-paraffinized, rehydrated and stained with 2µg anti-mast cell tryptase antibody (FL-275, Santa Cruz Biotechnologies), followed by an anti-rabbit AlexaFluor-594 secondary antibody (Invitrogen). Nuclei were counter-stained with DAPI. Images were captured with a Nixon Eclipse E800 microscope and analyzed with the Openlab software (Improvision).

Passive cutaneous anaphylaxis (PCA) and peritoneal lavages. For passive cutaneous anaphylaxis experiments, $1x10^6$ differentiated BMMCs were injected intradermally (i.d.) in the ear pinna of mice lacking mast cells ($Kit^{W\text{-}sh/W\text{-}sh}$, Jackson Laboratory) (8). Four weeks after reconstitution, transferred mast cells were sensitized by i.d. injections of IgE-anti-DNP (1.5µg/mL), and challenged 24h later with intravenous (i.v.) injections of 250µg/mL DNP-HSA together with 5mg/mL Evans-blue dye to assess extravasation. Mice were sacrificed 30min after challenge and the blue dye was extracted from the tissues by incubation in formamide at 63°C O/N. Intensity of the blue dye (correlating with the extent of extravasation and therefore mast cell activation) was measured spectrophotometrically (OD_{600}). Presence of mast cells in the reconstituted ears was assessed by toluidine-blue staining of paraffin-embedded ears. For peritoneal lavages, the total number of recovered cells was assessed by manual counting, while the percentage of mast cells was evaluated by surface staining for Kit and FcεRIα and FACS analysis.

Transfection of naïve T cells. Sorted naïve (CD4+ CD8– $CD62L^{hi}$ $CD44^{lo}$) T cells were transfected with Amaxa mouse T cells nucleofector kit following manufacture's instructions, using program X-01. Cells were rested for at least 3h after transfection and prior stimulation with plate-bound anti-CD3 and anti-CD28 for 48h.

Adoptive transfer of mouse T cells and immunization. Sorted CD8 or CD4 naïve T cells were obtained from spleen and lymph nodes of C57Bl/6, OT-I or OT-II mice

and injected ($1x10^6$ cells/ mouse) in C57Bl/6 recipients. Mice were then challenged sub-cutaneously with CFA-OVA and naïve, effector and memory cells were obtained from the spleen and draining lymph nodes 4 days (effector) or two weeks (memory) after challenge, and they were sorted in the different subsets based on the expression of CD4, CD8, CD62L, CD44 and CD127.

CFSE labeling. CFSE labeling was performed using a CellTrace cell proliferation kit (Invitrogen). Briefly, $1x10^6$-10^7 naïve T cells (human or mouse) were labeled with 5µM CFSE for 8min at 37°C prior extensive washing and antigenic stimulation.

Statistical analysis. Results are expressed as a mean ± s.d. or s.e.m. Comparisons were made using the unpaired t-test and the GraphPad Prism Software.

Results

Elevated numbers of mast cells in the absence of NF-kB p50. Mice lacking p50 (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 (5, 29, 41). Since mast cells are master effector cells in asthmatic and allergic responses, we evaluated whether such asthma-resistant phenotype could be partially due to a defect in mast cell development or function. First, we evaluated the numbers of mast cells in the tissues of p50ko animals. In the peritoneal lavages of control and p50ko mice, the total number of cells and the percentage of mast cells were assessed respectively by manual counting and by surface staining for FcεRIα and Kit. Surprisingly, the percentage of mast cells recovered from the peritoneal cavity in the absence of p50 was significantly augmented ($p<0.0002$) compared to control animals (Figure 1A). The percentage of Gr-1+ cells was also significantly increased in the peritoneal lavage of p50ko mice relative to controls. A similar kind of increase in the presence of mast cells was also observed in the small intestine of p50ko animals compared to C57Bl/6 mice, as assessed by immunofluorescence staining using two different mast cell markers (Tryptase or Kit) (Figure 1B). These results showed an overall increase in mast cells in the tissues of p50ko animals, without however revealing any alteration in the homing properties of these cells. Indeed, mast cells were found in all tissues and compartments where they are normally present, but not in organs, like the spleen or liver, where they are usually not found (not shown). Having ruled out that the asthma-resistant phenotype of p50ko animals does not correlate with the number of mast cells in these mice, and considering that mast cells are able to produce very high amounts especially of IL-13, we assessed the ability of these cells to respond to a variety of stimuli.

Reduced cytokine production in mast cells lacking p50. Similarly to controls, *in vitro* differentiated, bone marrow-derived p50ko mast cells were homogenously FcεRIα+ Kit+, expressed granzyme B and mMCP5 (not shown), and looked phenotypically normal as assessed by toluidine blue staining (Supplementary Figure 1A). Since the percentage of mast cells was increased in the tissues of p50ko animals relative to C57Bl/6 mice, we asked whether the absence of p50 could favor cell proliferation. As assessed by thymidine incorporation assay, p50ko cells proliferated similarly to the controls in all condition tested (Figure 1C). Next, we evaluated whether the impaired Th2 responses observed in these mice could be partially explained by an altered ability of mast cells to perform their effector functions (degranulation, cytokine production) in response to acute stimulation. The extent of mast cell degranulation was assessed both *in vitro*, by measuring release of β-hexosaminidase from cytoplasmic granules upon stimulation (18), and *in vivo*, by transferring mast cells into mast cell-deficient recipient mice ($Kit^{W\text{-}sh/W\text{-}sh}$) and performing passive cutaneous anaphylaxis (PCA) experiments. Even in the absence of p50 mast cells degranulated normally in response to IgE crosslinking or PMA and ionomycin stimulation, both *in vitro* and *in vivo* (Figure 1D-E). In PCA experiments, efficient mast cell reconstitution of the ear pinna was confirmed by toluidine blue staining of paraffin-embedded tissue sections (Figure 1F).

Next, we evaluated the ability of p50ko mast cells to produce cytokines, and we focused mostly on cytokines, such as IL-13, that are highly expressed by mast cells and have essential and non-redundant roles in allergy and asthmatic responses (10, 23, 38). Control and p50ko cells were stimulated with either LPS or IgE crosslinking, and expression of IL-6, TNF-α and IL-13 was assessed by intracellular cytokine staining (Figure 2A-C). Interestingly, mast cells lacking p50 showed reduced cytokine production in response to both LPS and IgE-Ag complexes, which was not due to altered expression of the surface receptors

FcεRI or TLR4 (Figure 2D). Reduced cytokine expression was particularly evident for IL-13, which is the cytokine necessary and sufficient for asthmatic responses in models of experimental asthma (10, 38) (Figure 2B-C). Despite the increased number of mast cells in the tissues of p50ko animals, reduced IL-13 production from p50ko mast cells may therefore contribute to the asthma-resistant phenotype and overall lack of eosinophilia and Th2 responses observed in these mice. However, our data so far could not explain the increased mast cell numbers observed in the tissues of p50ko mice, which is what we set out to investigate next.

Increased survival in the absence of p50 correlates with increased expression of anti-apoptotic genes. Given the increased number of tissue mast cells despite the normal proliferation capacity, we investigated whether p50ko mast cell were significantly affected in their ability to survive in response to IgE crosslinking and/ or withdrawal of essential cytokines. The essential survival factors IL-3 and/ or SCF were removed from the culture medium, with or without concomitant stimulation with IgE, which are known to provide a survival signal for mast cells, by inducing anti-apoptotic factors (14, 39). In all conditions tested p50ko cells showed a consistently increased ability to survive compared to the controls (Figure 3A-C). As expected, stimulation with IgE partially rescued control cells from apoptosis, but this effect was much more pronounced in the absence of p50 (Figure 3C). To get an insight in the molecular mechanism that could explain such enhanced survival, we evaluated the expression of candidate pro- and anti-apoptotic genes. Indeed, NF-kB binding sites have been identified in promoters and enhancers of a number of inducible genes involved in cell death, including Bcl-2, A1 and Bcl-X_L (14, 39). In particular, Bcl-2 is a known regulator of IL-3 withdrawal-dependent apoptosis, while A1 is a specific regulator of IgE-dependent survival in mast cells (14, 39). We therefore investigated whether these factors

may be involved in regulating survival in the absence of p50. To this end, p50ko and control cells were either left resting or were stimulated with either IgE crosslinking or LPS, after which expression of pro- and anti-apoptotic gene candidates was assessed by qRT-PCR (Figure 3D-G). Interestingly, both *bcl2* and *A1* were upregulated in cells lacking p50. Such upregulation was already evident at basal levels, but it became even more pronounced upon stimulation with IgE-DNP or LPS (Figure 3D-E). Expression of another pro-survival factor, *bcl-*X_L, was slightly diminished in p50ko cells (Figure 3F), as well as the expression of the pro-apoptotic gene *bax* (Figure 3G). Since Bcl-2 and especially A1 were already shown to be involved in regulating mast cell survival, 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 mast cells exhibit increased survival 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 favored.

MiR-146a regulates mast cells survival, but not cytokine production or LPS desensitization. Since miRNAs are known to be involved in the regulation of a variety of cell functions, we assessed whether they might also be involved in regulating the enhanced survival and reduced cytokine production observed in p50ko mast cells. We assessed in particular expression of two miRNAs (miR-146a and miR-221) that we found to be inducible in mast cells and to be dependent on NF-kB (18, 20, 36). P50ko and control mast cells were either left resting or were stimulated with PMA and ionomycin for 24h, after which expression of miR-146a and miR-221 was assessed by qRT-PCR. MiR-27a expression was also measured as a control since it should not change upon stimulation of mast cells. Strikingly, p50ko mast cells showed specifically impaired expression of miR-146a in response to stimulation in all conditions tested, including PMA and ionomycin, IgE

crosslinking or LPS stimulation (Figure 4A-B), indicating that p50 is indeed absolutely required for the expression of this miRNA. Vice versa, expression of miR-221, also known to be inducible upon mast cell stimulation in a NF-kB-dependent manner (20), 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 (36), we asked whether at least part of the phenotype observed in mast cells in the absence of p50 could be due to the inability of these cells to induce miR-146a expression. We therefore transduced mast cells with either a control lentiviral vector (shLuc, expressing an irrelevant hairpin), or a vector expressing miR-146a, and we asked whether miR-146a expression led to altered survival in mast cells. Indeed, mast cells transduced with miR-146a showed consistently increased cell death compared to control cells, even when cultured in medium supplemented with all survival factors (Figure 4C-D). As a control, to confirm that our lentivirally-derived miR-146a was indeed properly functional, we evaluated levels of expression of the known miR-146a target, Traf6 (36). Western blot analysis showed a significant decrease in Traf6 expression whenever miR-146a was expressed, in both p50ko and control cells (Figure 4E). Interestingly, forced miR-146a expression led to an only modest reduction of *bcl2* 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 4F). Along the same line, we did not observe any particular effect of miR-146a on IL-6 and TNFα expression (Figure 4G).

Repeated stimulation of cells can result in loss of responses. MiR-146a is known to regulate tolerance to LPS in macrophages (36), and given the inability of p50ko mast cells to induce miR-146a expression in response to LPS, we assessed whether these cells

might be impaired in their responses to sequential stimulations with LPS. However, mast cells lacking p50 showed a reduction in cytokine production in response to acute LPS stimulation, but responded similarly to control cells, becoming unresponsive to subsequent stimulations with LPS (Supplementary Figure 1B-C), indicating that such LPS-dependent unresponsiveness in mast cells is not strictly dependent on miR-146a.

MiR-146a is highly expressed in the T cell memory compartment from both human and mouse. Since we previously reported that miR-146a is differentially expressed between Th1 and Th2 T cell subsets in the mouse (22), we asked whether the novel molecular network we identified in mast cells, involving miR-146a and p50, could also be at play in regulating T lymphocyte polarization, which was also shown to be altered in mice in the absence of p50 (5). In mouse CD4 T cells we found that miR-146a was indeed expressed at higher levels in Th1 cells compared to Th2 (Figure 5A), however, it remained inducible in both T cell subsets upon restimulation, with high strength of TCR stimulation correlating with high levels of miR-146a expression and also with high and sustained number of cell cycles, as assessed by CFSE dilution (Figure 5B). We therefore hypothesized that miR-146a could have a role in regulating T cell activation and expansion rather than T cell polarization. In line with this hypothesis, we found that miR-146a expression was consistently elevated in the effector and memory compartment (T_{EM} and T_{CM}) in both CD4 and CD8 murine T cells (Figures 5C-D). Similarly to CD4 T cells, naïve CD8 T cells activated *in vitro* with anti-CD3 and anti-CD28 showed increased expression of miR-146a (Figure 5D, left panel). Next, we reasoned that if miR-146a is an important regulator of T cell polarization and/ or activation, such process should be conserved also in human T cells. Human TH1 and TH2 cells, either differentiated *in vitro* or separated *ex vivo* from peripheral blood, did not show any differential expression of this miRNA (Figure 5E and Supplementary Figure 2A). However,

similarly to mouse T cells, resting human CD4 lymphoctyes retained the ability to activate expression of miR-146a once restimulated and even more importantly, miR-146a was expressed at high levels in memory T cells isolated *ex vivo* from peripheral blood (Figure 5F-G). These data indicate that miR-146a is unlikely to play a major role in T cell polarization in both human and mouse (except probably Treg cells in which it is expressed at high levels ((17) and Supplementary Figure 2A), but point towards a role in regulating lymphocyte activation and/or in the establishment of immunological memory.

MiR-146a influences cell expansion, but not cell death of primary human lymphocytes. To further investigate the role of miR-146a specifically in human lymphocytes, and to assess whether it could modulate cell survival, similarly to mast cells, human CD4 T cells were lentivirally transduced to express GFP, alone or in combination with miR-146a (Figure 6A). Levels of miR-146a in transduced cells were overall lower or comparable to the physiological levels of endogenous expression observed in lymphocytes upon TCR stimulation (Supplementary Figure 2B). However, we found that primary human T cells expressing miR-146a did not show any significant alteration in Fas-mediated cell death or in CD95 expression or in the ability to survive in response to withdrawal of IL-2 (Supplementary Figure 2C-D and data not shown). Moreover, we observed no significant effect of miR-146a on IL-2, IFN-γ or IL-4 cytokine production (Supplementary Figure 2E and data not shown).

While we did not observe any effect on cell death, memory human T cells expressing miR-146a showed increased expansion after restimulation with anti-CD3 and anti-CD28 (Figure 6B). Specifically, cells expanded similarly up to ~day 4-5 after stimulation, regardless of miR-146a expression, but miR-146a-expressing cells continued to expand more vigorously then control cells in the subsequent days, an effect that was especially evident in

387 the absence of exogenous IL-2, indicating that this miRNA could act directly in response to
388 TCR stimulation to favor cell expansion (Figure 6B). The fact that cytokine production was
389 largely unaffected by miR-146a in all conditions tested, ruled out the possibility that miR-
390 146a-expressing cells could sustain their own proliferation through increased IL-2 production.
391 We also performed adoptive transfer experiments in which mouse OT-II naïve T cells
392 transfected with either miR-146a mimic or non-targeting control were transferred into a
393 recipient mouse. A few days after challenge with OVA, both the draining lymph node and the
394 spleen showed a slightly higher percentage of transferred cells whenever miR-146a was
395 present, suggesting increased expansion abilities following challenge (Supplementary Figure
396 3A-B). Finally, in a different experimental setting, human naïve T cells were transiently
397 transfected with a miR-146a mimic and then stimulated with allogeneic PBMC, after which
398 the percentage of activated T cells was assessed. Consistently with the results obtained using
399 lentiviral vectors, we observed a reproducible, albeit small, increase in the percentage of
400 activated cells whenever miR-146a was present (Supplementary Figure 3C).

401

402 **MiR-146a regulates CCR7 expression.** While IL-2 does not significantly contribute
403 to the initial cycling of antigen-stimulated T cells, it is nevertheless necessary for the
404 successful generation of memory responses (12). Indeed, the IL-2 signal strength has been
405 shown to contribute to the differentiation of murine CD8 T_{EM} and T_{CM} (28). In our culture
406 system, the absence of IL-2 uncovered a T_{EM}-like phenotype, with inability to sustain CCR7
407 expression and expansion after TCR stimulation (Figure 6C-D). Interestingly, miR-146a
408 expression determined a reduction of CCR7 expression in all conditions tested, indicating that
409 not only miR-146a contributed to T cell expansion upon TCR stimulation, but it may also
410 modulate the establishment of a T_{EM}-like phenotype (with reduced CCR7 expression) in
411 primary human lymphocytes (Figure 6E). Since most of our experiments so far were

performed on memory TH1, TH2 and TH17 cells subsets isolated from peripheral blood and restimulated *in vitro*, to better assess the effect of miR-146a during the first antigenic stimulation of naïve T cells, we lentivirally transduced human naïve T cells freshly isolated from peripheral blood to express miR-146a. Three days after transduction GFP+ cells were sorted and stimulated with plate-bound anti-CD3 and anti-CD28, and five days after stimulation total RNA was extracted and levels of expression of miR-146a and *CCR7* were assessed by qRT-PCR. As a control, we also measured expression of the known miR-146a target *TRAF6*. Similarly to memory cells, naïve T cell expressing miR-146a showed reduced *CCR7* and *TRAF6* expression (Figure 6F). However, the reduction in CCR7 expression was very selective, as we could not observe any alteration in the expression levels of CD25, CD45RA, CD45RO, CD62L or CD127 (data not shown). Moreover, such effect of miR-146a is unlikely to be exerted directly on CCR7, which is not a predicted target for this miRNA (16).

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 (26). To further demonstrate that TRAF6 is indeed a target for miR-146a in primary T lymphocytes we also performed reporter assay experiments using the 3'untranslated region (3'UTR) of *TRAF6*, and found that it was efficiently targeted by miR-146a in both Jurkat cells and primary murine Th2 cells (Supplementary Figure 3D). Our data point towards a role for miR-146a in regulating human T cell responses and memory formation possibly through the modulation of TRAF6 expression.

Dysregulated T cell memory formation in the absence of the p50/ miR-146a molecular circuitry. Since we showed that miR-146a dysregulation could influence T cell memory formation, leading to a $CCR7^{lo}$, T_{EM}-like phenotype, and we also found that in mast

cells miR-146a expression was fully dependent on p50 expression, we looked into T cell activation and memory formation in the absence of p50 (and therefore in the absence also of miR-146a). First, we assessed whether miR-146a expression was indeed impaired in the absence of p50 also in T lymphocytes. Naïve CD8 and CD4 T cells were isolated from p50-deleted animals and were stimulated *in vitro* with plate-bound anti-CD3 and anti-CD28 for 5 days, after which they were either left resting or were restimulated with PMA and ionomycin (P+I) for 6h. Similarly to mast cells stimulated with either IgE or LPS, T cells (both CD4 and CD8) lacking p50 were completely unable to induce miR-146a expression, neither upon TCR engagement of naïve T cells, nor upon restimulation (Figure 7A-B). Moreover, there was no significant difference between p50-deleted T cells and controls in IFNγ, IL-4 and IL-2 cytokine production, as assessed by intracellular cytokine staining (data not shown). We therefore analyzed the memory compartment of p50-deleted mice *ex vivo*. As shown in Figure 7C, in the absence of p50, both the CD4 and especially the CD8 T cell compartment showed a striking increase in the T_{CM} compartment, and a reduction of effector cells, even in the absence of any challenge. Importantly, sorted p50ko naïve T cells differentiated *in vitro* for 5 days also showed a T_{CM}-like phenotype, with high expression of CD44 and CD62L and intermediate expression of CD25 (Figure 7D), indicating that the absence of p50 and miR-146a intrinsically favors naïve T cell differentiation to a T_{CM} phenotype, independently of thymic development. However, forced expression of miR-146a in p50ko CD4 T cells by either retroviral transduction or transient transfection could not rescue the phenotype observed in the absence of p50 (Figure 7E-G), indicating once again that miR-146a alone is not able to fully compensate for the lack of a transcription factor, at least in our *in vitro* experimental settings.

462

463 **Discussion**

464 NF-kB is involved in regulating many aspects of cellular activity during an
465 immune response, and activation of this transcription factor by receptors of the innate and
466 adaptive immune response is essential for host defense (reviewed in (25)). Almost all danger-
467 sensing receptors activate NF-kB to mediate effector functions, however, NF-kB-driven
468 responses must be promptly terminated once danger is eliminated, as aberrant NF-kB activity
469 can directly lead to uncontrolled tissue damage and disease (reviewed in (30)).

470 Here, we found that the absence of one specific NF-kB family member, p50,
471 improved mast cell survival in response to a variety of signals. The underlying mechanism for
472 such enhanced survival involved increased expression of pro-survival factors such as Bcl-2
473 and A1, as well as reduced expression of pro-apoptotic factors such as Bax and miR-146a,
474 which in this context acted as a pro-apoptotic factor and as a modulator of NF-kB signaling
475 by targeting Traf6. In our current working model NF-kB activation in mast cells can occur as
476 a result of FcεRI crosslinking or TLR4 engagement leading to nuclear translocation of p65-
477 p50 heterodimers that activate transcription of both anti-apoptotic factors (such as *bcl2* and
478 *A1*) as well as of *pri-miR-146a*, which acts as a negative regulator of NF-kB activation
479 (Supplementary Figure 4A-C). In the absence of p50, not only some survival factors are
480 strongly upregulated, but the negative feedback on NF-kB activation is lost, as miR-146a
481 cannot be expressed, reinforcing the positive survival signal. The fact that in the context of
482 mast cell survival miR-146a acts as a pro-apoptotic factor is also highlighted by the fact that
483 forced expression of miR-146a leads to increased cell death.

484 What remains to be investigated is the mechanism by which NF-kB p50 may
485 act as a positive regulator of some genes (namely 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 in the absence of p50). One possible explanation concerns the formation of compensatory heterodimers in the absence of p50 with an altered pattern of binding and activation. Indeed, the unique DNA-binding properties of distinct NF-kB dimers influence the selective regulation of NF-kB target genes. NF-kB dimers can be separated in three distinct DNA-binding classes based on their DNA-binding preferences: p50 or p52 homodimers, heterodimers, c-Rel or RelA homodimers (35). Clearly, sites that are normally preferentially bound by p50 homodimers could become 'free' to be bound by other NF-kB family members in the absence of p50, altering regulation of transcription at these specific sites. Our initial data in this direction show that p65 nuclear translocation is normal in mast cells in the absence of p50, however its overall expression is slightly reduced (Supplementary Figure 1D). We have yet to uncover specific differences in promoter binding, which will be the topic of future work. Another possibility is that by lacking a transactivating domain, p50 homodimers may negatively regulate expression of *bcl2* and *A1* in resting conditions, which is overcome by other activating heterodimers upon activation. For example, negative regulation by p50 homodimers has been reported to correlate with repression of NF-kB-driven transcription in tolerant T cells (9), and tolerance to LPS in monocytes has been shown to involve LPS-dependent mobilization of NF-kB with a predominance of p50 homodimers (43). P50 homodimers are also known to be nuclear even in the absence of stimulation, which may explain the increased expression of Bcl-2 and A1 at basal levels in the absence of p50 (2, 32). On the other hand, miR-146a expression remained exquisitely dependent on p50 in all conditions tested. Further experiments will elucidate the exact molecular mechanism responsible for the enhanced survival observed in mast cells in the absence of p50, and this will be the topic of future work.

As for the observed increase of mast cells in the tissues of p50ko animals, the possibility remains that such alteration may arise from an increased ability of hematopoietic stem cells (HSC) in the bone marrow to differentiate more promptly to mast cells and/ or in general to the myeloid lineage. The increased number of mast cells could therefore be a composite effect of increased differentiation from stem cells, combined with a cell-intrinsic ability of the cells to survive in response to a variety of stimuli. A detailed analysis of HSC differentiation in the absence of p50 will surely provide new insights on the role of this transcription factor in hematopoietic differentiation, particularly to the myeloid lineage.

As opposed to mast cells, in T lymphocytes miR-146a did not regulate cell death, but rather influenced T cell activation upon TCR engagement. It is interesting to notice that in accordance with published data (4), we found that ectopic expression of miR-146a in Jurkat cells led to a mild reduction in apoptosis (data not shown). However, primary cells expressing miR-146a did not show any significant alteration in Fas-mediated cell death or in CD95 expression. Such discrepancy may be due to the fact that while in Jurkat cells activation-induced cell death (AICD) can be completely cell autonomous, primary T lymphocytes undergo AICD by making contact with their activated neighbors, resulting in 'fratricide' rather than 'suicide' (3, 6, 7). Alternatively, miR-146a may have roles that vary in the different cell types depending on the type and relative abundance of mRNA targets that constitute the transcriptome of that specific cell, as we have shown for mast cells and T lymphocytes. We therefore propose a model in which miR-146a upregulation upon TCR stimulation contributes to the overall strength of signal arising from the TCR, by favoring cell activation and cell expansion, and by modulating the establishment of immunological memory, in particular by favoring a $CCR7^{lo}$, T_{EM}-like phenotype (Supplementary Figure 4D).

Importantly, it has recently been shown that mice lacking p50 have altered negative selection in the thymus and develop a population of single-positive CD8 thymocytes

with memory T cell-like properties that populate peripheral immune organs (11). Here, we were able to show that in the absence of p50 even highly purified naïve CD4 and CD8 T cells stimulated *in vitro* preferentially acquired a T_{CM}-like phenotype, with high CD62L and moderate expression of CD25. While this effect is clearly dependent on the absence of p50, we speculate that it might also be due, at least in part, to the inability of these cells to express miR-146a in response to TCR stimulation. It would be interesting to estimate the proportion of the effect of a given transcription factor, such as NF-kB p50, that goes through the altered expression of a specific miRNA, like miR-146a. However, is has to be noted that miR-146a-deleted mice showed normal proportions of CD4 and CD8 cells both in the thymus and in the periphery (17), and even in our hands in mast cells, miR-146a was only partially able to compensate for the lack of p50, indicating that at least in this case, the effect of a transcription factor remains predominant to that of the miRNA. Moreover, some of our data are also in line with a very recent publication showing that miR-146a also controls the resolution of T cell responses in mice and its absence leads to increase survival through modulation of *bcl2* expression (40).

As for targets of miR-146a, we found that TRAF6 was clearly targeted by this miRNA both in mast cells and primary T lymphocytes from human and mouse, similarly to what has been extensively shown for macrophages and other cell types (36). However, other targets for miR-146a have been suggested (17, 31), and TRAF6 may not be the only or the most relevant target for this miRNA in these particular cell context: for example, we showed for miR-221 that although some specific genes are targeted by this miRNA in mast cells, the effect of miR-221 does not go predominantly through these targets, and bioinformatics analysis clearly showed that miR-221 affected a few hundred primary and secondary targets (18). Nevertheless, in an attempt to phenocopy the effects of miR-146a, we attempted knock-down experiments for TRAF6 in primary human T cells using siRNAs. While unfortunately

we were so far unable to draw definite conclusions from these experiments, it remains to be noted 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 (26), therefore resembling the increased T_{EM}-like phenotype of our miR-146a-expressing cells. Moreover, Fas-mediated apoptosis in the absence of Traf6 was normal, and cells showed increased proliferation in response to TCR stimulation, again similarly to what we observed in miR-146a-expressing cells (15).

Finally, as for the fact that miR-146a is expressed at higher level in Th1 murine lymphocytes compared to Th2 (22), we think that such differential expression might be related to the strength of signal that favors one or the other phenotype, with stronger stimulation favoring Th1 responses (37). Further supporting this possibility, it has been suggested that strong stimulation is needed for commitment to T_{EM} cells, whereas weaker stimulation favors the generation of less-committed T_{CM} cells (27), indicating that miR-146a might play an important role in lowering the strength of signal required for full T cell activation and therefore mimic stronger TCR engagement resulting in increased activation and generation of T_{EM}. Alternatively, IL-4 and/ or IL-12 signaling may also contribute to modulate expression of miR-146a in conjunction with TCR stimulation, a possibility that remains to be investigated. However, it has to be noted that we found no significant difference in miR-146a expression in human TH1, TH2 and TH17 cells, indicating that cytokine signaling may not be at play (Supplementary Figure 2A). Similarly to the mouse (17), this miRNA was instead expressed at very high levels in human Tregs.

Overall, our findings indicate that NF-kB p50 acts in a cell-autonomous manner in differentiated mast cells to favor survival in response to withdrawal of essential cytokines or antigenic stimulation, while in T lymphocytes it enhances TCR-dependent activation and modulates memory formation. This novel molecular network comprising

specifically p50 and miR-146a and regulating cell survival, tissue homeostasis, and T cell activation may have important implications for our understanding of the physiologic responses occurring for example during infections with helminthes parasites or allergic reactions, and possibly even in mast cell disorders such as systemic mastocytosis.

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Conflict of Interest

The authors have no conflict of interest to declare.

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Figure Legends

Figure 1. Increased tissue mast cells in mice lacking p50. A) Peritoneal lavage was performed on control and p50ko mice, and total cell numbers and percentages of mast cells (Kit+ FcεRI+), Mac-1+ and Gr-1+ cells were assessed by manual counting and FACS staining, respectively. Each dot represents one animal. **B)** Immunofluorescence staining of small intestine sections of control and p50ko mice. Mast cells were identified by staining with an anti-Tryptase antibody (shown, top), or an anti-Kit-antibody. At least 10 fields per tissue slide were observed and counted blindly (the operator counting the cells was not aware of the type of sample, which was coded) and the observed percentage of Tryptase + or Kit+ cells are plotted (bottom). **C)** Mast cell proliferation was assessed by thymidine incorporation assay. Control and p50ko mast cells 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. **D)** To assess mast cell degranulation in response to acute stimulation *in vitro*, mast cells 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. **E)** Mast cells were differentiated from C57Bl/6 and p50ko mice and injected intradermally (i.d.) in the ear pinna of $Kit^{W\text{-}sh/W\text{-}sh}$ mice. Four weeks after injection, mast cells were sensitized by i.d. injection of IgE-anti-DNP and then challenged 24h later intravenously with DNP-HSA and Evans-blue to assess extravasation. Blue ear intensity was analyzed spectrophotometrically (OD_{600}) after extraction with formamide. Pictures on the left show one representative experiment, and the graph shows the quantification of several experiments. Each dot represents one mouse. **F)** Effective mast cell reconstitution of the ear pinna performed as in E) was assessed by fixation and embedding in paraffin of the ear tissue followed by toluidine blue staining.

Figure 2. Reduced cytokine production in the absence of p50. A) Cells were either left untreated or were stimulated with IgE-Ag complexes or LPS prior analysis of IL-6 and TNFα expression by intracellular cytokine staining. **B)** Same as in A), except that IL-13 production is shown. **C)** Same as in A) except that the percentage of cells positive for the indicated cytokine is provided. Each dot represents one independent experiment. **D)** (Top) Surface expression of FcεRIα in control and p50ko mast cells, unstimulated or stimulated with IgE and antigen for 24h to assess cell ability to upregulate FcεRIα expression. (Bottom) Expression of TLR4 on the surface of control and p50ko mast cells, unstimulated or stimulated with LPS for 3h.

Figure 3. **Enhanced survival and increased expression of anti-apoptotic factors in the absence of p50.** Mast cells were cultured with IL-3 alone **(A)** or IL-3+SCF **(B)** prior removal of IL-3 or SCF for ~4 days. Analysis of cell death was performed by AnnexinV 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 as in (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 two. **D)** P50ko and control cells were either left resting or were stimulated for 24h with IgE-Ag complexes (top) or LPS (bottom). Total RNA was extracted and *A1* mRNA expression was assessed by qRT-PCR. Shown is one representative experiment out of four. **E)** Same as in D) except that expression of *bcl2* was assessed. Shown is one representative experiment out four. **F-G)** Expression levels of *bcl-*X_L (F) and *bax* (G) were analyzed by qRT-PCT in unstimulated control and p50-deleted mast cells. Shown is one representative experiment out four for *bax*, and out of three for *bcl-*X_L.

Figure 4. **MiR-146a is not expressed in mast cells in the absence of p50. A)** Differentiated mast cells were either left resting or were stimulated with PMA and ionomycin

(P+I) for 24h prior analysis of the expression of miR-146a, miR-221 and miR27a by qRT-PCR. SnoRNA202 was used as endogenous control. Shown is one representative experiment out of three. **B)** Same as in (A), except that cells were stimulated with either IgE and antigen (top) or LPS (bottom) for the indicated times. Shown is one representative experiment out of three. **C)** Mast cells were lentivirally (LV) transduced to express either miR-146a or an irrelevant hairpin as a control (shLuc). MiR-146a expression was assessed by qRT-PCR. **D)** Cells treated as in (C) were analyzed for apoptosis by AnnexinV staining. **E)** Cells treated as in (C) were lysed in Laemmli sample buffer and expression of Traf6 was analyzed by Western blot. β-tubulin was used as loading control and quantification was performed using an image reader. Shown is the ratio between the Traf6 and β-tubulin signals in one out of two independent experiments with similar results. **F)** Same as in (C), except that levels of expression of *bcl2* were analyzed by qRT-PCR. **G)** Cells lentivirally transduced as in (C) were stimulated with IgE and antigen prior intracellular cytokine staining to assess IL-6 and TNFα expression. Each dot represents one independent experiment.

Figure 5. Memory T cells express high levels of miR-146a, which is induced by TCR stimulation. A) Naïve CD4 T cells were isolated from the spleen and lymph nodes of C57Bl/6 or OT-II mice and differentiated to either Th1 or Th2. At day 5, cells were either left resting or were restimulated with PMA and ionomycin for 6h prior RNA extraction and qRT-PCR for miR-146a (left) and intracellular cytokine staining to verify polarization (right). SnoRNA202 was used as endogenous control. **B)** Naïve CD4 T cells were isolated from the spleen and lymph nodes of C57Bl/6 or OT-II mice, were labeled with CFSE and stimulated with the indicated concentrations of anti-CD3 in the presence of anti-CD28 for 48h. The number of cycles was counted based on CFSE dilution (left) and miR-146a expression was evaluated in the same samples by qRT-PCR (right). **C)** Naïve CD4 T cells were isolated from the spleen and lymph nodes of OT-II mice, and were transferred i.v. into recipient C57Bl/6

807 mice prior challenge with OVA. Five and fifteen days after transfer T_{EM} and T_{CM} were FACS-
808 sorted (T_{EM}: CD62L^{lo} CD44^{hi} CD127^{hi}; T_{CM}: CD62L^{hi} CD44^{hi}, CD127^{hi}) and miR-146a
809 expression was measured by qRT-PCR. **D)** (Left) Same as in (C) except that OT-I naïve T
810 cells were used and mice were challenged with either OVA or SIINFKL peptide. (Right)
811 Purified naïve CD8 cells were stimulated *in vitro* with plate-bound anti-CD3 and anti-CD28
812 for two days and further expanded up to day 6 with 10-100 U/mL of rIL-2, after which they
813 were either left resting or were restimulated for 6h with PMA and ionomycin (P+I), prior
814 analysis of miR-146a expression. **E)** MiR-146a expression in human TH1 and TH2 clones
815 was assessed by qRT-PCR (left). RNU48 was used as endogenous control. Clones were
816 characterized by the expression of surface markers and by the expression of IFNγ and IL-4
817 (right). **F)** Resting primary human CD4 cells were either immediately lysed in Trizol or were
818 stimulated with plate-bound anti-CD3 and anti-CD28 for 3 and 6 days prior analysis of miR-
819 146a expression by qRT-PCR. **G)** Primary human CD4 T cells were sorted from peripheral
820 blood as follows: naïve: CD4+ CD8– CD25– CD45RA+ CCR7+; T_{EM}: CD4+ CD8– CD25–
821 CD45RA– CCR7–; T_{CM}: CD4+ CD8– CD25–CD45RA– CCR7+. Cells were lysed in Trizol
822 immediately after sorting and miR-146a expression was assessed by qRT-PCR.

823 **Figure 6. MiR-146a expression in T cells led to enhanced expansion and reduced**
824 **CCR7 expression upon TCR stimulation. A)** (Top) Schematic of the lentiviral vector used.
825 The control vectors expressed GFP alone or in combination with a non-targeting hairpin or an
826 shRNA against luciferase. (Bottom) Primary human T cells (CD4+ CD45RA– CD25– CD8–)
827 transduced with the indicated vector were sorted for GFP expression 2-5 days after
828 transduction. Shown are cells obtained after sorting in one representative experiment. After
829 initial experiments, TH1 (CXCR3+), TH2 (CCR4+) or TH17 (CCR6+ CCR4+) subsets were
830 used interchangeably as provided identical results. **B)** Primary human T cells transduced with
831 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, and then expanded for 6 days with or without addition of exogenous 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 5 only). **C)** CCR7 surface expression was assessed on cells treated as in (B). Stimulated cells were homogenously CD45RA– CD25+. **D)** *CCR7* mRNA expression was assessed by qRT-PCR in cells as in (C). **E)** Primary human T cells were transduced with the indicated lentiviral vectors, and *CCR7* mRNA expression was assessed 3-5 days after transduction. Representative of 4 independent experiments performed in various conditions (i.e. with or without exogenous IL-2, at resting state or upon restimulation with anti-CD3 and anti-CD28 for 3 days), all with comparable results. **F**) Primary naïve human T cells transduced with the indicated vectors were stimulated with plate-bound anti-CD3 and anti-CD28 for 5 days prior qRT-PCR to determine expression of miR-146a, *CCR7*, and *TRAF6*. Shown is one experiment out of two.

Figure 7. T_{CM} differentiation in the absence of p50 and miR-146a. A) CD8+ or **B)** CD4+ naïve T cells were sorted from the spleen and lymph nodes of p50ko and control mice and were either immediately lysed in Trizol or were stimulated for 2-5 days with plate-bound anti-CD3 and anti-CD28, after which they were either left resting or were restimulated with PMA and ionomycin (P+I) for 6h. RNA was extracted and expression of miR-146a assessed by qRT-PCR. **C)** Spleen and lymph nodes were collected from p50ko and control mice (3 mice per group) and the percentage of naïve (CD62L+ CD44–), effector (CD62L– CD44+) and memory (CD62L+ CD44+) cells was evaluated in the CD4 and CD8 compartments. **D)** Naïve CD4 and CD8 T cells were FACS-sorted from the spleen and lymph nodes of p50ko and control mice (3 mice per group) and were stimulated for 5 days with plate-bound anti-CD3 and anti-CD28, after which expression of CD44, CD62L and CD25 was assessed by FACS staining. **E)** Top: schematic representation of the retroviral vector used for transduction

of primary murine T cells, expressing either GFP alone or GFP and miR-146a. The insert was verified by sequencing and expression of vector-derived miR-146a evaluated in a separate transduction experiments of total wild-type CD4+ T cells (qRT-PCR graph at the bottom). Indicated below each bar are the percentages of GFP+, transduced cells. Untr.=untransduced control. **F)** Sorted naïve CD4+ T cells ($CD62L^{hi}$ $CD44^{lo}$) from p50ko mice were transduced with the indicated retroviruses 48h after initial activation with plate-bound anti-CD3 and anti-CD28, and expression of CD62L and CD44 was assessed at day 5 after stimulation (day 3 after transduction). Cells shown in the FACS-plots on the right were gated on the GFP+ cells. **G)** To assess whether changes in miR-146a expression during the first two days of T cell stimulation could be essential for the final outcome on the phenotype, which we could not investigate by using retroviruses, sorted naïve p50ko CD4+ T cells were transiently trasfected with Amaxa prior anti-CD3/ anti-CD28 stimulation. Transfection efficiency was assessed by using a non-targeting fluorescent oligo (siGLO, left panel). Expression of CD44, CD62L and CD25 was assessed at day 2, 3 and 4 after transfection. Shown is day 3 of one representative experiment out of two.

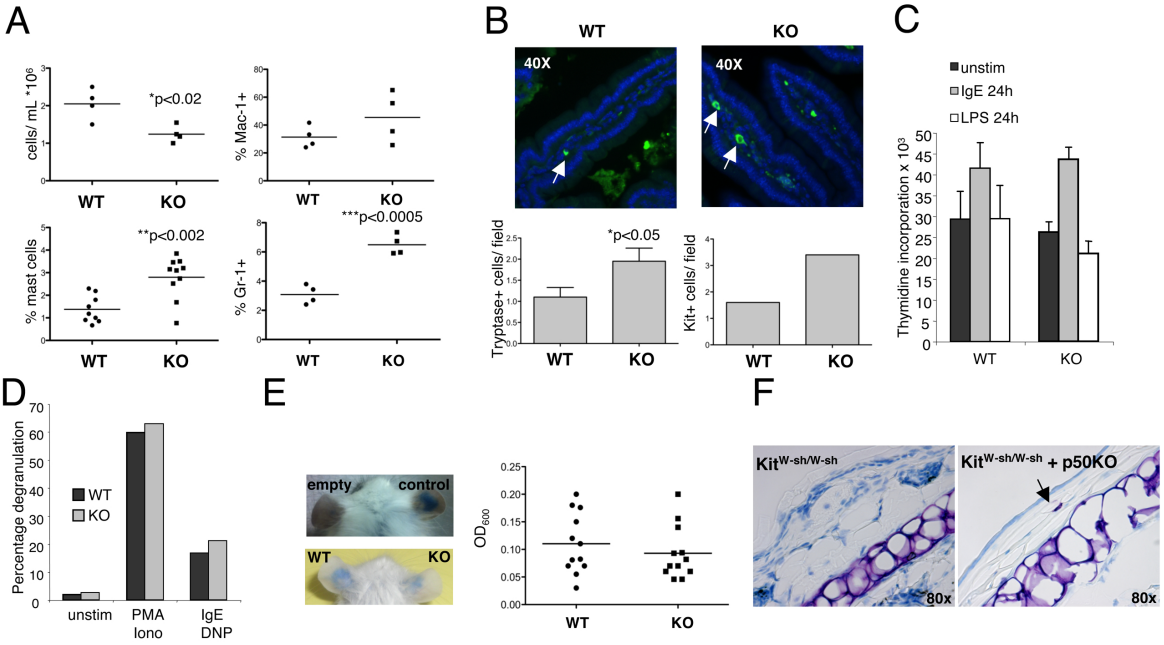

Figure 1

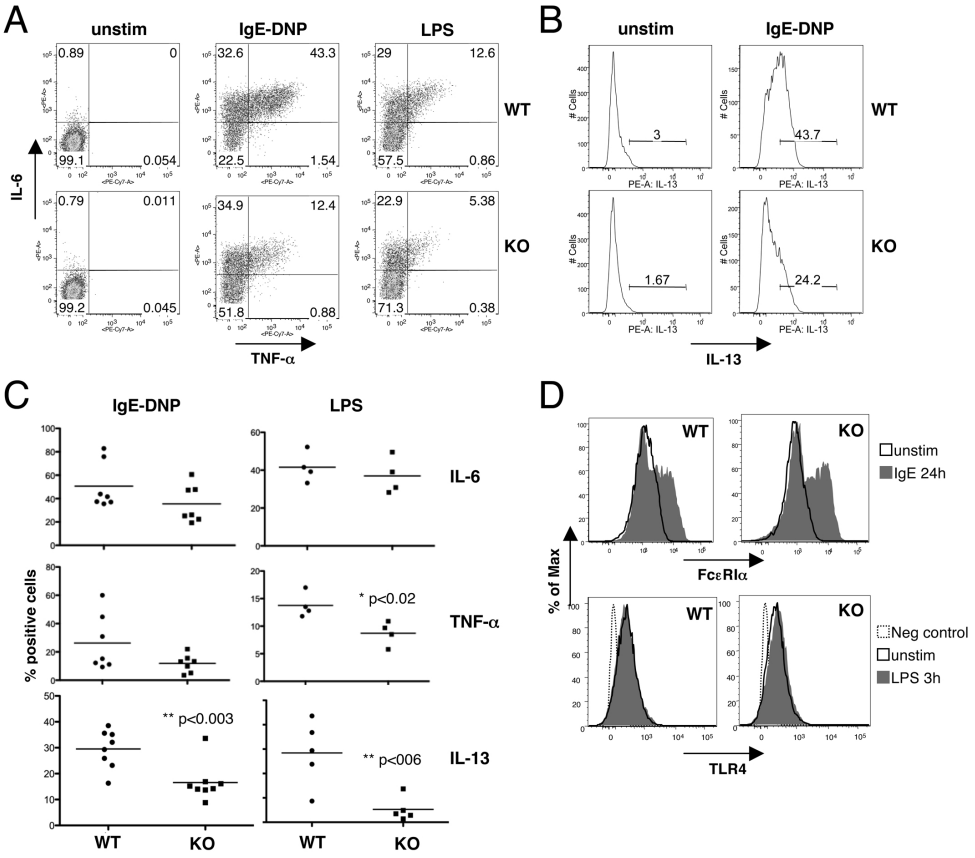

Figure 2

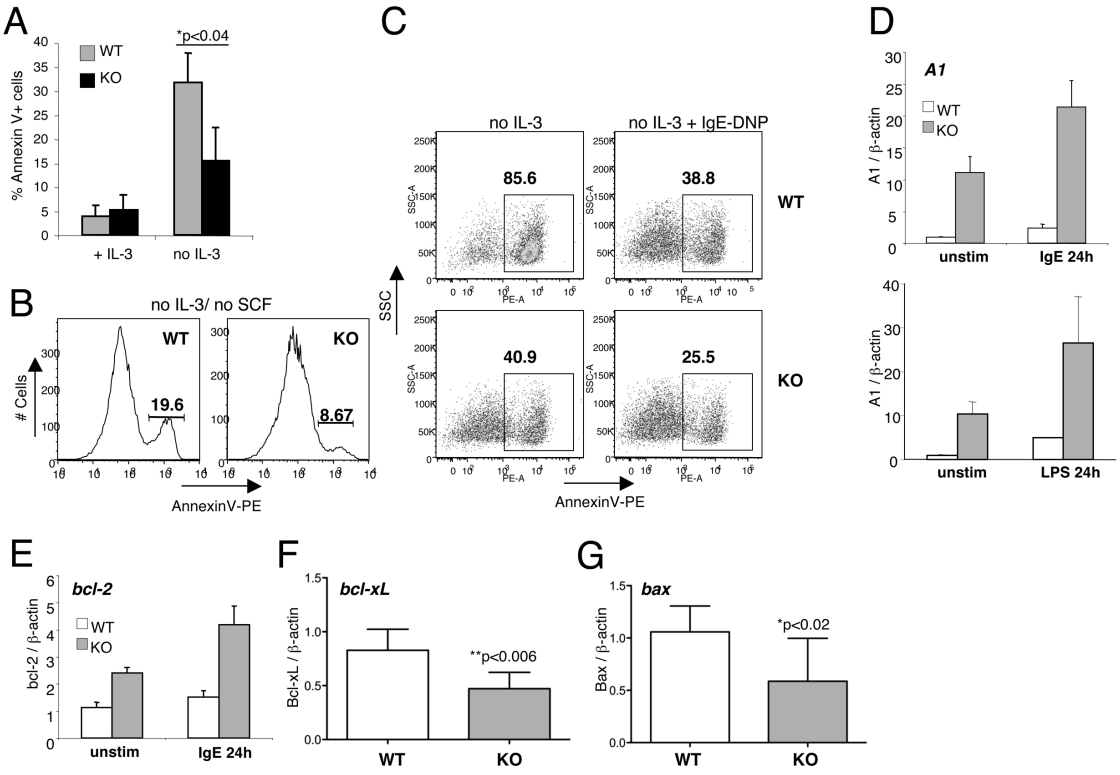

Figure 3

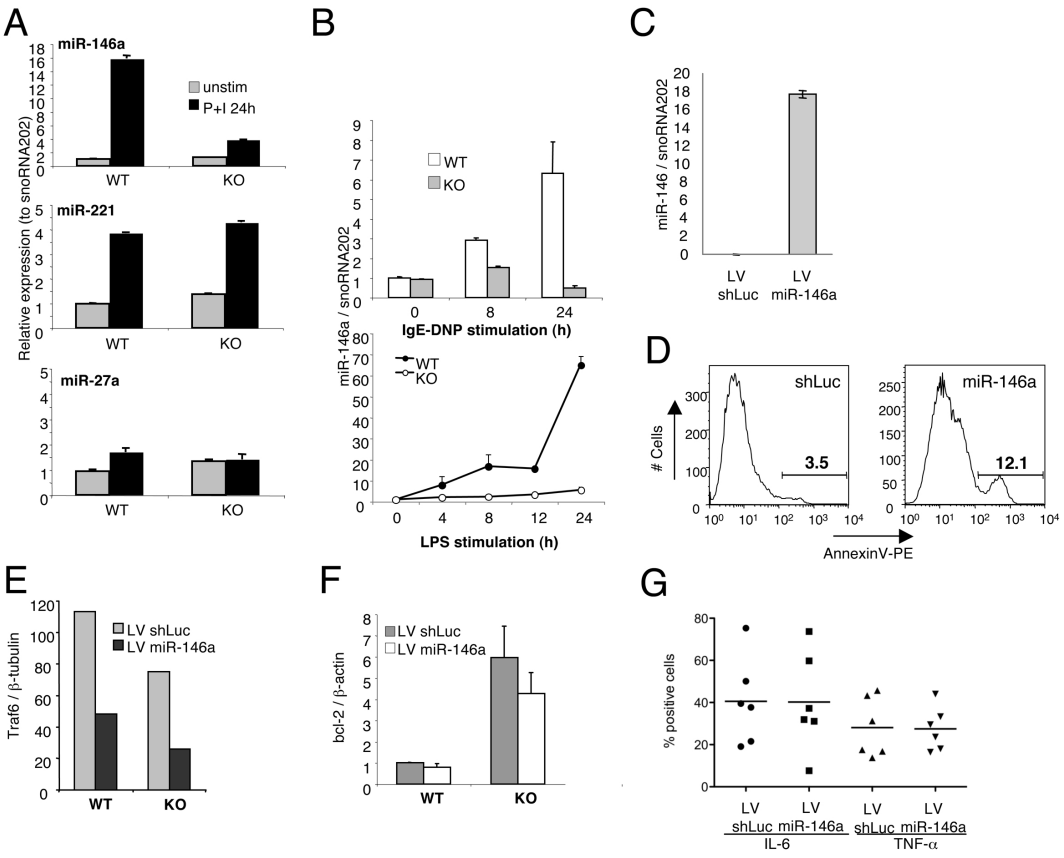

Figure 4

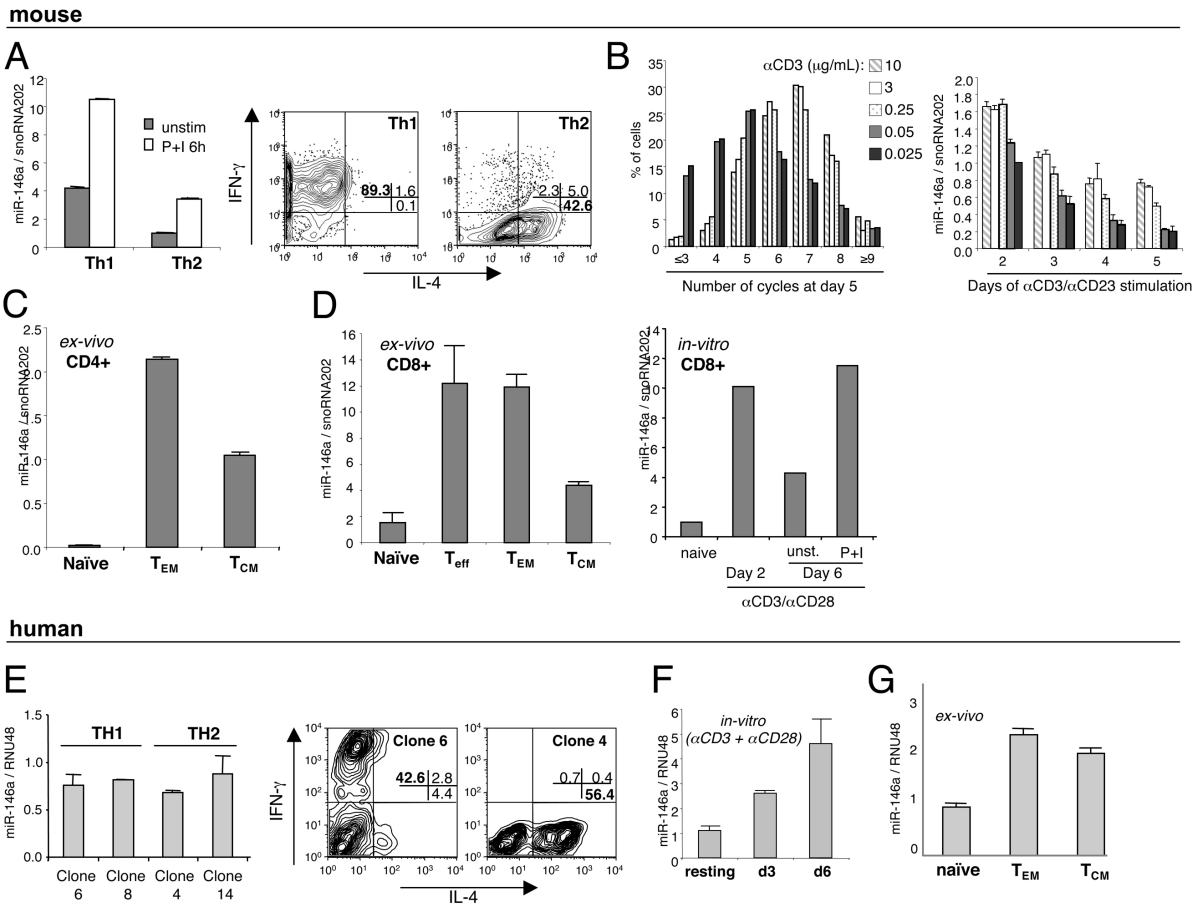

Figure 5

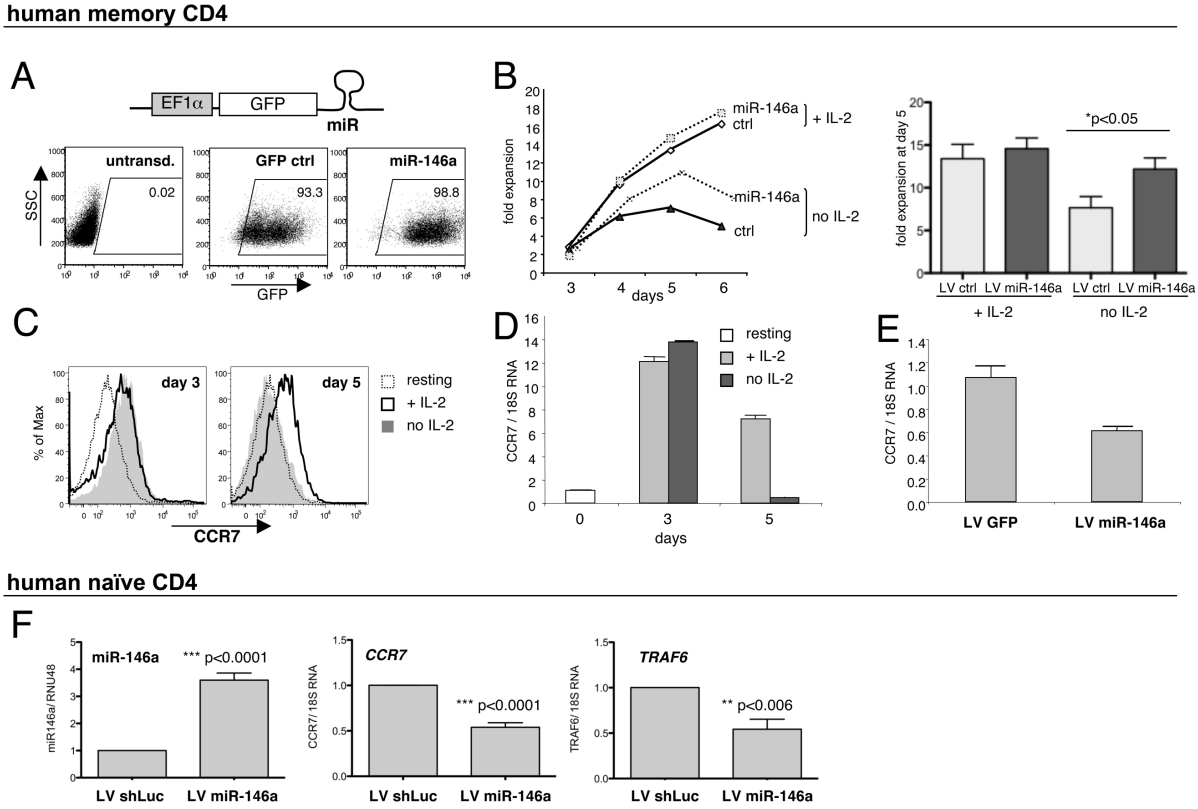

Figure 6

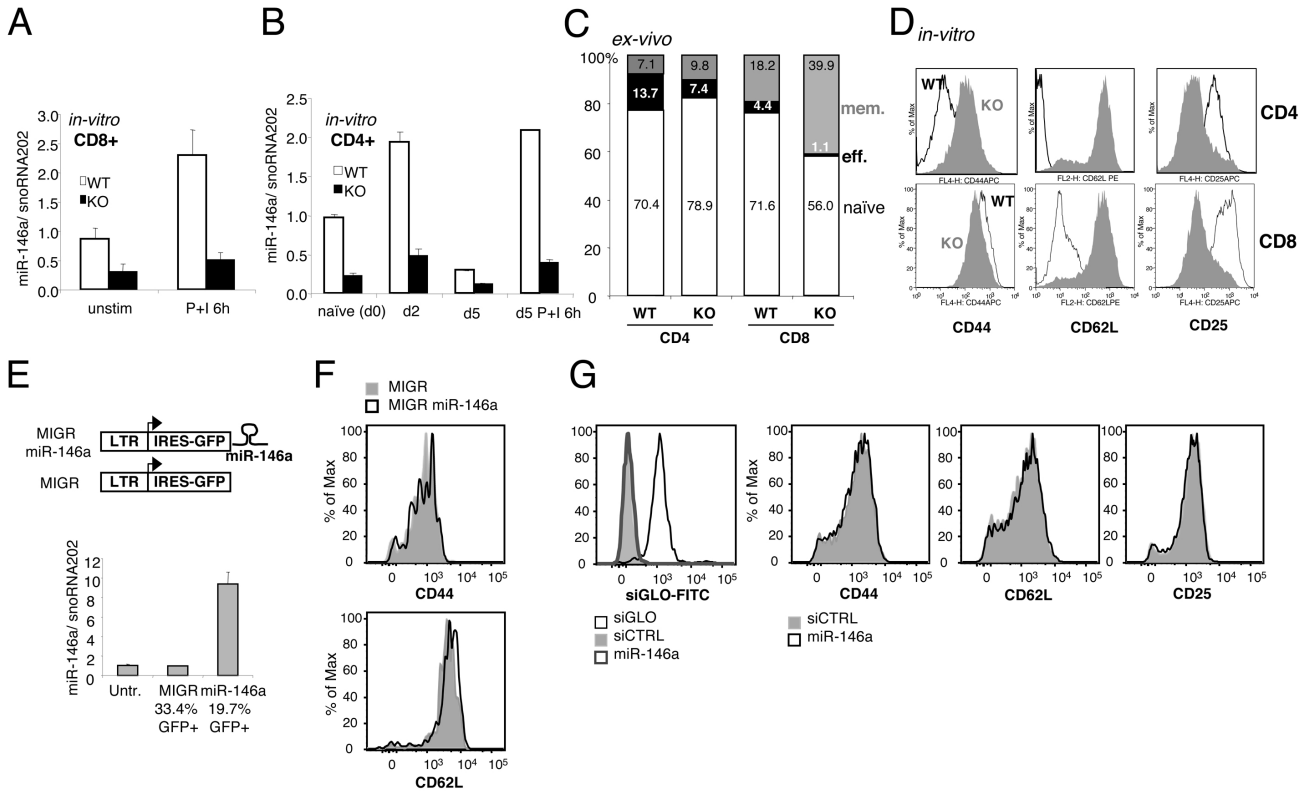

Figure 7