Enhancing anti-tumor immunity to MHC class I-deficient tumors: role of regulatory T cells and type I IFN

Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University Of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by
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born in Acharnes, Greece
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Oral examination: 20.06.2008
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1. Referee: Prof. Dr. G. Hämmerling
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**Conference presentations:**


## CONTENTS

1  ZUSAMMENFASSUNG .................................................................................................................................. 1

2  SUMMARY ........................................................................................................................................... 3

3  INTRODUCTION ..................................................................................................................................... 5

3.1  THE IMMUNE SYSTEM ............................................................................................................................ 5

3.1.1  The immune response to tumors ........................................................................................................ 5

3.1.2  The innate immune system .................................................................................................................. 7

3.1.2.1  Natural Killer cells ........................................................................................................................ 7

3.1.2.1.1  NK cells and tumor ................................................................................................................... 11

3.1.2.1.2  NK cells as regulatory cells ...................................................................................................... 13

3.1.2.2  Macrophages ..................................................................................................................................... 14

3.1.2.2.1  Tumor associated macrophages .............................................................................................. 15

3.1.2.2.2  Myeloid-Derived Suppressor Cells .......................................................................................... 16

3.1.3  The adaptive immune system ........................................................................................................... 17

3.1.3.1  Mechanisms of T cell tolerance ..................................................................................................... 18

3.1.3.2  Regulatory T cells .......................................................................................................................... 20

3.1.3.2.1  Phenotypic characterization of Treg .......................................................................................... 20

3.1.3.2.2  Treg and autoimmunity .......................................................................................................... 22

3.1.3.2.3  Treg and tumor immunity ....................................................................................................... 22

3.1.3.2.4  Treg and innate immunity ........................................................................................................ 23

3.1.3.2.5  Manipulation of the suppressive function of Treg .................................................................... 24

3.2  TYPE I IFN .......................................................................................................................................... 25

3.2.1  Regulatory effects of type I IFN ........................................................................................................... 26

3.2.2  Type I IFN and tumor ....................................................................................................................... 27

4  AIM OF THE STUDY .................................................................................................................................. 29

5  MATERIAL AND METHODS .................................................................................................................... 30

5.1  MATERIALS ......................................................................................................................................... 30

5.1.1  Laboratory equipment ....................................................................................................................... 30
5.1.2 Cell culture products ............................................................................................31
5.1.3 Cell culture media ................................................................................................32
5.1.4 Solutions...............................................................................................................33
5.1.5 Chemicals.............................................................................................................34
5.1.6 Antibodies ............................................................................................................35
  5.1.6.1 Antibodies for FACS analysis .......................................................................35
  5.1.6.2 Antibodies for in vitro activation.................................................................35
  5.1.6.3 In vivo administered antibodies .....................................................................35
5.1.7 Cell lines...............................................................................................................38
5.1.8 Magnetic Cell Sorting (MACS) beads and columns ............................................38
5.1.9 Kits .....................................................................................................................39
5.2 MICE .............................................................................................................................40
5.3 METHODS .....................................................................................................................40
  5.3.1 Cell culture methods.............................................................................................40
      5.3.1.1 Determination of cell number .................................................................40
      5.3.1.2 Freezing and thawing of cells .................................................................40
      5.3.1.3 Splitting of adherent cells ........................................................................41
      5.3.1.4 Splitting of suspension cells .....................................................................41
  5.3.2 Mouse tumor model .............................................................................................41
      5.3.2.1 Isolation of organs and preparation of single cell suspensions .................42
          5.3.2.1.1 Blood .....................................................................................................42
          5.3.2.1.2 Spleen and LN .......................................................................................42
          5.3.2.1.3 Tumor infiltrating leukocytes ...............................................................42
          5.3.2.1.4 Bone marrow (BM) cells .................................................................42
      5.3.2.2 In vivo depletion and neutralization experiments ........................................43
      5.3.2.3 Bone marrow transplantation ....................................................................43
      5.3.2.4 Memory experiments ................................................................................44
          5.3.2.4.1 In vivo memory experiments ...............................................................44
          5.3.2.4.2 Adoptive transfer memory experiments ..............................................44
  5.3.3 Antibody purification ...........................................................................................44
      5.3.3.1 Purification of the anti-CD25 mAb ............................................................44
      5.3.3.2 Purification of the anti-IFN-β mAb ..........................................................45
  5.3.4 Fluorescence-activated cell sorting (FACS).........................................................45
      5.3.4.1 FACS staining and analysis ......................................................................45
      5.3.4.2 FACS sorting .............................................................................................46
5.3.5 MACS sorting ......................................................................................................46
5.3.6 Determination of cytokines ..................................................................................46
5.3.6.1 Intracellular FACS staining ...........................................................................46
5.3.6.2 Enzyme-Linked ImmunoSorbent Assay (ELISA) ..............................................46
5.3.6.3 \textit{In vivo} IFN-\(\gamma\) capture assay .................................................................47
5.3.6.4 Bio-plex protein array assay for cytokine measurement .................................47
5.3.7 Proliferation and suppression assays .................................................................47
5.3.7.1 Treg suppression assay ..................................................................................47
5.3.7.2 Tumor cell growth inhibition assay ...............................................................48
5.3.7.3 Documentation of proliferation by CFSE dilution ............................................49
5.3.8 Killing assays .......................................................................................................49
5.3.8.1 \(^{51}\text{Cr} \) release cytotoxicity assay ..............................................................49
5.3.8.2 \textit{In vitro} induction of apoptosis by death ligands .........................................49
5.3.9 Visualization of angiogenesis by confocal microscopy .......................................50
5.3.10 Statistical analysis ...........................................................................................50

6 \hspace{1em} RESULTS .....................................................................................................51

6.1 REGULATORY T CELLS SUPPRESS ANTI-TUMOR IMMUNITY AGAINST A MHC CLASS I DEFICIENT LYMPHOMA ..................................................................................51

6.1.1 Regulatory T cells suppress IFN-\(\gamma\) dependent leukocyte accumulation in lymphoma ..................................................................................................................51
6.1.1.1 Accumulation of Treg in RMA-S tumor-bearing mice ........................................51
6.1.1.2 Depletion of Treg abrogates RMA-S tumor growth .........................................52
6.1.1.3 NK1.1\(^{+}\) cells and CD4\(^{+}\)CD25\(^{-}\) T cells mediate the rejection of RMA-S tumors in the absence of Treg .................................................................53
6.1.1.4 Depletion of Treg leads to the generation of anti-tumor memory responses against RMA-S tumor cells .................................................................55
6.1.1.5 Neutralization of IFN-\(\gamma\) abrogates RMA-S tumor rejection mediated by Treg depletion \textit{in vivo} .................................................................................................58
6.1.1.6 Perforin, FasL and inhibition of angiogenesis are not involved in the rejection of RMA-S tumor in the absence of Treg .........................................................60
6.1.1.7 In the absence of Treg, increased numbers of leukocytes accumulate in the RMA-S tumor tissue in an IFN-\(\gamma\) dependent manner ...........................................62
6.1.1.8 Increased numbers of NK cells, conventional CD4+ T cells and macrophages are detected in the RMA-S tumors in the absence of Treg..........................63

6.1.1.9 Treg suppress RMA-S anti-tumor immunity independently of TGF-β or IL-10 .......................................................................................................................65

6.1.2 Regulatory T cells suppress macrophage activation in lymphoma ............66

6.1.2.1 Increased amounts of MHC class II on macrophages in the absence of Treg .......................................................................................................................66

6.1.2.2 Enhanced macrophage activity in the absence of Treg............................68

6.1.2.3 Macrophages kill RMA-S cells independently of the enzymatic activity of iNOS, PGE2 and IDO....................................................................................70

6.1.3 Regulatory T cells suppress CD8+ T cell recognition of TAP-deficient tumors ..74

6.1.3.1 CD8+ T cells contribute to RMA-S tumor rejection in the absence of Treg by direct recognition of RMA-S cells.................................................................74

6.1.4 Immune intervention in established RMA-S tumors..................................78

6.1.4.1 Depletion of Treg in established RMA-S tumors .....................................78

6.1.4.2 Depletion of NK cells in established RMA-S tumors..............................78

6.2 CONTROL OF ANTI-TUMOR IMMUNITY BY TYPE I IFN ...............................................81

6.2.1 Enhanced RMA-S tumor growth in the absence of type I IFN signaling ..81

6.2.2 Requirement of hematopoietic and non-hematopoietic compartment for responsiveness to type I IFN ..................................................................................................................82

6.2.3 Contribution of the hematopoietic compartment for responsiveness to type I IFN ..................................................................................................................83

6.2.3.1 NK cell cytotoxicity is impaired in IFNAR1-/- mice......................................83

6.2.3.2 Macrophage tumorstatic activity is not impaired in IFNAR1-/- mice ..........83

6.2.3.3 Intratumoral leukocyte accumulation is not impaired in IFNAR1-/- mice ....84

6.2.4 Contribution of the non-hematopoietic compartment for responsiveness to type I IFN ..................................................................................................................85

6.2.4.1 Angiogenesis is impaired in IFNAR1-/- mice.............................................85

7 DISCUSSION.........................................................................................................87

7.1 SUPPRESSION OF INNATE IMMUNE CELLS BY TREG ..................................................87

7.2 THE ROLE OF ENDOGENOUS TYPE I IFN IN THE ANTI-TUMOR RESPONSE ..........92

8 REFERENCES......................................................................................................96
9 ABBREVIATIONS........................................................................................................112

10 ACKNOWLEDGEMENTS..........................................................................................116
1 Zusammenfassung


2 SUMMARY

Naturally occurring CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg) have been shown to suppress immune responses, including anti-tumor immunity. Strategies of manipulating Treg in cancer patients are currently evaluated in clinical trials with the aim of enhancing the efficiency of vaccinations, targeting the adaptive arm of immunity. Many tumors, however, lose expression of MHC class I and thus become protected from CD8⁺ T cell-mediated recognition and elimination. Such types of tumors can still be efficiently eliminated by cells of the innate immune system, in particular NK cells, through antigen- and MHC class I-independent mechanisms. The role of Treg in the rejection of tumors, which are predominantly under the control of innate immune cells has been poorly addressed so far.

In this study, we investigated the influence of Treg on the immune response against the MHC class I-deficient mouse lymphoma RMA-S after subcutaneous injection. We showed that Treg accumulate in the tumor tissue and lymphoid organs of tumor-bearing animals. Treg depletion upon application of an anti-CD25 monoclonal antibody led to the rejection of high tumor cell numbers, which in contrast grew progressively in untreated mice. Our experiments demonstrated that NK1.1⁺ cells, CD8⁺ and CD4⁺CD25⁻ T cells are recruited in high cell numbers to the tumor site in the absence of Treg and that all of these three cell populations contribute to RMA-S tumor rejection. Primary immune responses elicited during Treg depletion led to the generation of protective immunological memory; rechallenge of mice that had rejected the initial tumor with either RMA-S or MHC class I-sufficient RMA tumor cells resulted in immediate tumor rejection. Furthermore, we showed that IFN-γ is produced in higher amounts by the tumor-infiltrating lymphocytes in the absence of Treg. Neutralization of IFN-γ completely abrogated the tumor rejection observed after Treg depletion, which correlated with the inhibition of accumulation of leukocytes at the tumor site. Among the tumor-infiltrating leukocytes, macrophages constituted the major cell population infiltrating the RMA-S tumor tissue in the absence of Treg. Tumor-infiltrating macrophages from Treg depleted mice expressed increased amounts of MHC class II and produced highly enhanced levels of chemokines and pro-inflammatory cytokines as compared to control mice. Macrophages isolated from the tumors also inhibited tumor cell proliferation through a mechanism independent of iNOS, PGE2 and IDO.
In conclusion, this study supports a role for Treg in blunting the immune response to a MHC class I-deficient tumor target, by interfering with leukocyte accumulation at the tumor site. In addition, high numbers of activated tumor-infiltrating macrophages correlated with tumor rejection in the absence of Treg. These data identify macrophages as novel potential targets for Treg mediated immune suppression in cancer.

In the second part of this study, we aimed at defining further mechanisms controlling the immune response against the MHC class I-deficient RMA-S tumor. For this purpose, we focused on the role of endogenously produced type I IFN. Although exogenously administered type I IFN have been used to treat various types of cancer, their endogenous production and function during an anti-tumor response has not been extensively studied. We studied the growth of RMA-S tumor in mice that cannot respond to type I IFN (IFNAR1⁻/⁻ mice) and observed an acceleration in the tumor growth. In addition, we prepared bone marrow chimeras in which either the hematopoietic or the non-hematopoietic cells cannot respond to type I IFN and we found that type I IFN responsiveness is required in both compartments. Namely, IFNAR1 was important for NK cell cytotoxicity and proper development of the vessel network in the tumor tissue. In contrast, its absence did neither affect macrophage effector functions nor accumulation of leukocytes within the tumor tissue.

In summary, endogenously produced type I IFN contribute to the control of the MHC class I-deficient RMA-S tumor growth, via targeting both the hematopoietic and non-hematopoietic compartments, and regulate NK cell activity and tumor vessel formation.
3 INTRODUCTION

3.1 The immune system

The immune system is a complex and highly developed system, which has evolved to protect the host against the attack of foreign pathogens as well as tumors. In vertebrates, it consists of the innate and the adaptive immunity. The non-specific component of the immune system – innate immunity – is a set of mechanisms that are not specialized for a particular pathogen. Adaptive immunity, on the contrary, displays a higher degree of specificity in recognizing foreign antigens than innate immunity as well as the property of memory. Typically, an adaptive immune response against an antigen is raised five or six days after the initial exposure to that antigen. Subsequent exposure to the same antigen results in a memory response, which is faster, stronger, and often more effective in neutralizing or clearing the pathogen than the first one. Due to this attribute, the immune system can confer life-long immunity to many infectious agents after an initial encounter. Adaptive and innate immunity, however, do not operate independently of each other but rather function as a highly interactive and cooperative system.

The cells of the immune system originate in the bone marrow (BM), where many of them also mature, from the same progenitor, the hematopoietic stem cells. Initially, they give rise to stem cells of more limited potential, i.e. the common myeloid progenitor and the common lymphoid progenitor. The first one is the precursor of granulocytes, macrophages, dendritic cells (DC) and mast cells, while the second one gives rise to the lymphocytes.

3.1.1 The immune response to tumors

In order to fulfill its role, the immune system has developed mechanisms for the discrimination of self and non-self antigens, preventing the host from suffering autoimmune diseases via tolerance to self-antigens, while recognizing and eliminating the foreign pathogens. Cancer cells can be viewed as altered self-cells that have escaped normal growth-regulating mechanisms.

The theory of immune surveillance suggests that cancer cells arise frequently in the body, are recognized as foreign and are eliminated by the immune system; when tumor cells escape immune surveillance, tumors form that grow too large for the immune system to control. A more elaborate theory on how the immune system responds to a tumor is cancer immunoediting $^{1-5}$, which is comprised of three phases: elimination, equilibrium and escape. The elimination phase
basically refers to the immune surveillance. In the equilibrium phase, the tumor cell variants that have survived the elimination phase and the immune system enter into a dynamic equilibrium leading to a population of tumor clones with reduced immunogenicity. Evidence for the equilibrium phase was provided by a clinical case where metastatic melanoma occurred 1-2 years post-transplant in the two recipients who each received a kidney from the same donor. In the escape phase, finally, the tumor cell variants surviving the equilibrium phase grow and become clinically detectable (Figure 3.1).

The immune system can detect the tumor cells by the recognition of the so called tumor-specific antigens (TSA) or tumor-associated antigens (TAA). TSA are unique to tumor cells and not present on normal cells, while TAA are not unique to tumor cells but quite often proteins either expressed at low levels by normal cells or expressed on normal cells during fetal development when the immune system is immature to respond. However, the tumor can impede the immune response directly e.g. by the shedding of recognition structures or indirectly e.g. by the production of immunosuppressive cytokines (TGF-β, IL-10) or the induction of immunosuppressive cell populations.

The immune system itself can form tumors that are classified as lymphomas or leukemias. Lymphomas are solid tumors which form within a lymphoid tissue, such as the bone marrow,
lymph nodes or thymus, while leukemias – either of lymphoid or myeloid lineage – proliferate as single cells.

### 3.1.2 The innate immune system

The innate immune system is a collection of distinct subsystems that appeared at different stages of evolution including anatomic, physiologic, phagocytic and inflammatory barriers that prevent the entrance and establishment of infectious agents. A summary of the main subsystems found in mammals and their function in innate host defence can be found in Table 3.1.

Innate immune recognition is also known as pattern recognition and is based on the detection of molecular structures unique to microorganisms. The innate immune system senses pathogens through pattern-recognition receptors (PRR), which trigger the activation of antimicrobial defenses. The targets of PRR are named pathogen-associated molecular patterns (PAMP), although they are present on both pathogenic and non-pathogenic microorganisms. Bacterial PAMP are often components of the cell wall, such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids and cell-wall lipoproteins. β-glycan is an important fungal PAMP, which is present on the fungal cell wall. The detection of these structures by the innate immune system can signal the presence of microorganisms. Moreover, the recognition of viruses also follows this principle; however, since viral components are synthesized within the host cells, the main targets of innate immune recognition in this case are the viral nucleic acids. In addition, the innate immune system is able to discriminate between self and viral nucleic acids based on specific chemical modifications unique to viral RNA and DNA, as well as on the cellular compartments where viral, but not host-derived, nucleic acids are found. There are several classes of PRR, the best characterized being Toll-like receptors (TLR), which can elicit inflammatory and antimicrobial responses after activation by their microbial ligands.

The innate immune response depends on the coordinate activity of several effector cells, like natural killer cells, monocytes/macrophages, DC and granulocytes.

#### 3.1.2.1 Natural Killer cells

Natural Killer (NK) cells constitute one of the major lymphocyte populations, representing 5-10% of cells among peripheral blood lymphocytes (PBL). They differ from the
other lymphocytes by the absence of clonally distributed receptors derived via somatic gene rearrangement. NK cells were first described in the early 1970’s by R. Kiessling and R.B. Herberman and defined based on their functional entity, i.e. cells that are capable of recognizing and killing tumor cells without a prior exposure. Afterwards, they were described to

<table>
<thead>
<tr>
<th>Innate subsystem</th>
<th>Primary sensors</th>
<th>Prototypical responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal epithelia</td>
<td>TLR and NOD proteins</td>
<td>Production of antimicrobial peptides</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Production of mucins</td>
</tr>
<tr>
<td>Phagocytes</td>
<td>TLR, dectins and NOD proteins</td>
<td>Production of antimicrobial proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Production of cytokines: IL-1β, IL-6 and TNF</td>
</tr>
<tr>
<td>Acute-phase proteins and complement system</td>
<td>Collectins, pentraxins and ficolins</td>
<td>Lysis or opsonization of pathogens</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chemotactic attraction of leukocytes</td>
</tr>
<tr>
<td>Inflammasomes</td>
<td>NALP and NAIP</td>
<td>Production of IL-1-family members</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Apoptosis of infected host cells</td>
</tr>
<tr>
<td>NK cells</td>
<td>ND</td>
<td>Apoptosis of infected host cells</td>
</tr>
<tr>
<td>Type-I-IFN-induced antiviral proteins</td>
<td>RIG-I, MDA5, DAI and TLR</td>
<td>Induction of an antiviral state</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Apoptosis of infected host cells</td>
</tr>
<tr>
<td>Eosinophils and basophils</td>
<td>ND</td>
<td>Contraction of smooth muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Production of mucins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peristalsis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Production of biogenic amines</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Production of cytokines: IL-4, IL-5, IL-9, IL-13 and TNF</td>
</tr>
<tr>
<td>Mast cells</td>
<td>ND</td>
<td>Contraction of smooth muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Production of mucins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peristalsis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Production of biogenic amines</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Production of cytokines: IL-4, IL-5, IL-9, IL-13 and TNF</td>
</tr>
</tbody>
</table>

ND, not determined

Table 3.1. Subsystems of the innate immune system. (Adapted from R. Medzhitov, Nature, 2007)
have a large granular lymphocyte morphology\textsuperscript{13}. Phenotypically, they are defined as CD3\textsuperscript{-}CD56\textsuperscript{+} cells in humans\textsuperscript{14}, and can be further divided into two subpopulations, the CD56\textsuperscript{bright}CD16\textsuperscript{-} subset, which can be found mainly in the LN and produces high amounts of cytokines, and the CD56\textsuperscript{dim}CD16\textsuperscript{+} subset which is found predominantly in the blood and is cytolytic. There are indications that CD56\textsuperscript{bright} NK cells could be precursors of the CD56\textsuperscript{dim} subset\textsuperscript{15}. In mice, depending on the strain, the combination of CD3\textsuperscript{-}NK1.1\textsuperscript{+} or CD3\textsuperscript{-}DX5\textsuperscript{+} is commonly used to identify NK cells. Recently, NKp46 (CD335) has been described to be uniquely expressed on NK cells of various species, including human and mice\textsuperscript{16}. Importantly, NKp46 is not expressed on NKT cells, a population that shares many common markers with NK cells, such as NK1.1. NKp46 appears early during NK cell development, so it can be used to characterize NK cells of different maturation stages.

Stages of NK cell development have been described based on phenotypical and functional analysis\textsuperscript{17}. Committed NK cell progenitors express CD122 and no other lineage-specific markers (stage I). Subsequently, immature NK cells express NK1.1 – only on the C57BL/6 strain – (stage II) followed by the acquisition of the whole repertoire of activating and inhibitory receptors of the CD94-NKG2 and Ly49 families (stage III). DX5 appears on stage IV and NK cell maturation is completed with the expression of the markers CD11b and CD43, which accompanies acquisition of cytotoxicity and the ability to produce cytokines (stage V). In mice, it has been reported that the mature CD11b\textsuperscript{high} NK cell population can be further distinguished into two phenotypically and functionally distinct subsets based on the expression of the marker CD27\textsuperscript{18}. The CD11b\textsuperscript{high}CD27\textsuperscript{high} NK cell population exhibited enhanced effector functions, i.e. higher cytotoxic ability and enhanced IFN-\gamma production upon stimulation with IL-12 and IL-18 \textit{in vitro}, when compared to the CD11b\textsuperscript{high}CD27\textsuperscript{low} NK cell subset. In addition, CD11b\textsuperscript{high}CD27\textsuperscript{high} NK cells specifically expressed the chemokine receptor CXCR3 and possessed higher proliferating activity\textsuperscript{18}. Similar subdivision was also proposed for human NK cells\textsuperscript{19}. Stimulation of NK cells with CD27 ligand (CD70) artificially expressed on tumor cells could enhance proliferation and IFN-\gamma production of freshly isolated NK cells and potentiated the rejection of MHC class I-deficient tumor cells via perforin- and IFN-\gamma- dependent mechanisms\textsuperscript{20}.

NK cells require interaction with bone marrow stromal cells for functional maturation\textsuperscript{21,22}. Their development is mainly independent of the thymus, since they appear in normal numbers and function in athymic (nude), SCID and, RAG1\textsuperscript{-/-} and RAG2\textsuperscript{-/-} mice. Nevertheless,
there is accumulative evidence that not all of the NK cells derive from a unique peripheral pool. ‘Bipotent’ NK cell – T cell progenitors have been described in the thymus in both humans and mice\textsuperscript{23-25}. Mouse thymic NK cells were shown to be different from BM NK cells, with regards to higher expression of GATA-3 and CD127 (IL-7R\(\alpha\)) and to their preferential homing to the lymph nodes. They exhibited low cytotoxic ability and high IFN-\(\gamma\) production, resembling the CD56\textsuperscript{high}CD16\textsuperscript{−} human NK cell subset\textsuperscript{26}. Mouse liver contains a NK cell subset, which does not express the DX5 marker and constitutively expresses TRAIL. This effector molecule may be involved in the immunosurveillance of liver tumors\textsuperscript{27}. In addition, a CD34\textsuperscript{+} subset which can differentiate into the CD56\textsuperscript{bright} NK cell subset was identified in human LN\textsuperscript{28}.

There are various mutations described causing impairment of NK cell numbers and/or function. Lists of genes coding for different transcription factors, surface receptors or cytokines leading to such impairments are shown in Tables 3.2-3.4.

<table>
<thead>
<tr>
<th>Gene deleted</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ikaros</td>
<td>NK cells absent</td>
<td>29, 30</td>
</tr>
<tr>
<td>PU.1</td>
<td>NK cell number decreased, normal lytic function</td>
<td>31</td>
</tr>
<tr>
<td>Ets-1</td>
<td>NK cell number decreased, decreased lytic function</td>
<td>32</td>
</tr>
<tr>
<td>Id2</td>
<td>NK cell number decreased or absent, reduced lytic function</td>
<td>33, 34</td>
</tr>
<tr>
<td>TCF-1</td>
<td>Altered acquisition of Ly49s</td>
<td>34, 35</td>
</tr>
<tr>
<td>IRF-1</td>
<td>NK cell number decreased, lytic function impaired</td>
<td>36, 37</td>
</tr>
<tr>
<td>IRF-2</td>
<td>NK cell number decreased, lytic function impaired</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 3.2. Transcription factor deficiencies leading to NK cell impairments.

<table>
<thead>
<tr>
<th>Gene deleted</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT(\beta)r</td>
<td>NK cells severely decreased</td>
<td>39</td>
</tr>
<tr>
<td>LT(\alpha)(\beta)(_2)</td>
<td>NK cells severely decreased, reduced lytic function</td>
<td>40, 41, 42</td>
</tr>
<tr>
<td>IL-15R(\alpha)</td>
<td>NK cells severely decreased</td>
<td>43</td>
</tr>
</tbody>
</table>
Table 3.3. Surface receptor deficiencies leading to NK cell impairments.

<table>
<thead>
<tr>
<th>Gene deleted</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2/15Rβ</td>
<td>NK cells absent</td>
<td>[44, 45]</td>
</tr>
<tr>
<td>c-kit</td>
<td>NK cells decreased, impaired lytic function</td>
<td>[46]</td>
</tr>
</tbody>
</table>

Table 3.4. Cytokine deficiencies leading to NK cell impairments.

<table>
<thead>
<tr>
<th>Gene deleted</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-15</td>
<td>NK cells absent, no lytic function</td>
<td>[47, 48]</td>
</tr>
<tr>
<td>Flt3-ligand</td>
<td>NK cells severely decreased, impaired lytic function</td>
<td>[49]</td>
</tr>
</tbody>
</table>

3.1.2.1.1 NK cells and tumor

NK cells were the first cells shown to effectively eliminate tumor cells from the circulation of mice [50, 51]. Mice with an autosomal mutation called beige lack NK cells, and are more susceptible than normal mice to tumor growth following injection with live tumor cells [52]. Likewise, humans suffering from the Chediak-Higashi syndrome, an autosomal recessive disorder associated with impairment in neutrophils, macrophages and NK cells, have an increased incidence of lymphomas [53].

NK cells, unlike CD8+ T cells, do not require expression of MHC class I determinants for recognition of target cells. In fact, there is an inverse relationship between expression of MHC class I and susceptibility to lysis by NK cells. This observation is the milestone for the ‘missing self hypothesis’ [54], describing that cells which have normal expression of MHC class I molecules are not lysed by NK cells, while if self is absent, NK cells are activated to lyse the abnormal cells. Reduced MHC class I expression is often observed in virus-infected cells and tumor cells, which downregulate MHC class I expression as a mechanism to escape CD8+ T cell-mediated lysis. Thus, NK cells can kill virus-infected or transformed cells [55] and are especially effective at killing cells with low MHC class I expression [54, 56]. More recent studies confirmed the importance of NK cell cytotoxicity – mediated via perforin – against MHC class I-deficient tumors in vivo [57-59]. Besides perforin, NK cells can express death ligands, i.e. CD95L, TNF-α and TNF-related apoptosis-inducing ligand (TRAIL), via which they can induce apoptosis to their targets [60-62].

The more recent concept about NK cell recognition of targets involves a set of activating receptors which stimulate NK cell functions, in addition to the inhibitory receptors, which sense
the absence of MHC class I \(^{63,64}\). The dynamic balance between these activating and inhibitory signals controls NK cell activation (Figure 3.2).

**Figure 3.2.** Control of NK cell functions by the balance of activating and inhibitory signals (Smyth MJ *et al.* *Nature Reviews Cancer* **2**, 850-861).

NKG2D is an example of an activating receptor expressed on human and mouse NK cells that recognize a diversity of ligands, including the RAE-1 family (\(\alpha\), \(\beta\), \(\gamma\), \(\delta\) and \(\epsilon\)), H60 and MULT-1 in the mouse and the MICA and ULBP families in humans \(^{65,66}\). NKG2D ligands are not expressed on the surface of normal cells, but their expression is induced in virally infected, stressed and DNA damaged cells \(^{65-68}\). In tumor cell lines that fail to express NKG2D ligands, ectopic expression of RAE-1 leads to their elimination *in vivo* \(^{69,70}\). Moreover, NKG2D neutralization led to increased incidence of methylcholanthrene (MCA)-induced fibrosarcomas \(^{71}\). Finally, increased incidence of tumors was observed in NKG2D-deficient mice \(^{72}\).

Although tumors quite often bear recognition structures for NK cells, they might escape NK cell killing by desensitizing the receptor via soluble ligands released by the tumor cells, as in the case of the NKG2D pathway; in individuals with epithelial derived tumors, soluble, surface-shed MICA was detected in the sera and resulted in down-regulation of NKG2D and subsequent impairment of NK cell cytotoxicity \(^{73}\). In cancer patients, NK cell function was shown to be
impaired, as determined by reduced proliferation, response to IFN and cytotoxicity after ex vivo isolation of the cells. Nevertheless, in a 11-year follow-up study of a Japanese population, medium and high cytolytic function of peripheral blood NK cells was associated with reduced cancer risk, while low cytolytic function was associated with increased cancer risk.

Several studies aim at improving the anti-tumor effect of NK cells for cancer immunotherapy. These approaches include endogenous activation of NK cells via the administration of cytokines or adoptive transfer of ex vivo expanded and activated NK cells. Unfortunately, the application of IL-2 for the activation of NK cells has been controversial because of its accompanying toxicity in vivo. In addition, adoptive immunotherapy of cancer with systemic administration of autologous NK cells has also proven to be not so successful, as the cells are difficult to expand and localize poorly to the tumors. Based on this observation, it is necessary to develop alternative approaches to enhance anti-tumor immunity.

3.1.2.1.2 NK cells as regulatory cells

Besides their ability to lyse target cells, an important function of NK cells is the production of cytokines, such as IFN-γ, TNF-α and GM-CSF. In the past few years, evidence for a role for NK cells in promoting adaptive immune responses via their interaction with DC has been reported. These data have provided a new appreciation for the interrelated nature of innate and adaptive immunity. NK cells were shown to kill immature DC, despite high expression of MHC class I. It has also been described that NK cells with an immature phenotype were found in increased numbers in a leukemia mouse model and these immature NK cells could suppress DC functions, rather than killing them, by down-regulating the expression of I-A^d or inhibiting allo-T cell stimulatory activity. During early pregnancy in humans and rodents, c-kit^CD25^CD122^CD16^CD56^bright NK cells and Thy-1^NK1.1^asialo-GM1^+ cells, respectively, were reported to accumulate in the uterine deciduas. These uterine NK cells produce TGF-β1 and are thought to have immunoregulatory functions. Finally, NK cells are thought to play an immunoregulatory role in the prevention of autoimmune diseases, as autoimmune condition is typically deteriorated when there are NK cell defects.
3.1.2.2 Macrophages

Macrophages originate from the common myeloid progenitor in the bone marrow. They are then released in the blood where they differentiate into mature monocytes, comprising ~5% of peripheral blood leukocytes. When monocytes migrate into the tissues, they eventually differentiate into tissue-specific macrophages. This process is accompanied by several changes: increase in size, increase in number and complexity of organelles, acquisition of phagocytic ability and secretion of various soluble factors. Macrophages serve different functions in various tissues, such as alveolar macrophages in the lung, histiocytes in connective tissues, Kupffer cells in the liver, mesangial cells in the kidney, microglial cells in the brain and osteoclasts in the bones.

Monocytes/macrophages are identified by the expression of CD14 marker in humans and F4/80 marker in mice. In addition, macrosialin or CD68, a lysosomal marker, can be used to identify all tissue macrophages of both mouse and human origin. Nevertheless, peripheral blood monocytes are not a homogeneous population and they can be divided into two functional subsets in mice; a CX3CR1\textsuperscript{low}CCR2\textsuperscript{+}Gr-1\textsuperscript{+} ‘inflammatory subset’ which has a short half-life and is actively recruited to inflamed tissues, and a CX3CR1\textsuperscript{high}CCR2\textsuperscript{Gr-1} ‘resident subset’ which has a longer half-life and is recruited to non-inflammed tissues in a CX3CR1-dependent manner. Both subsets have the potential to differentiate \textit{in vivo} into DC. In addition, the level of expression of CX3CR1 correlates with the two described monocyte subpopulations in humans, the CD14\textsuperscript{+}CD16\textsuperscript{−} and CD14\textsuperscript{−}CD16\textsuperscript{+} subsets, resembling the murine inflammatory and resident subsets, respectively.

Macrophages can be activated via various stimuli during an immune response, which determine their subsequent phenotype. Macrophage activation can be triggered via TLR pathways (see Table 1.1) and/or cytokines secreted by lymphocytes, the most potent of which is IFN-γ. Activation of macrophages via LPS, a ligand for TLR4, and IFN-γ leads to the ‘classically’ activated macrophages, also known as M1, which are linked to the response to intracellular pathogens and promote Th1 responses. They are characterized by their ability to secrete significant amount of proinflammatory cytokines, such as IL-1β, IL-15, IL-18, TNFα and IL-12, and the enhanced expression of MHC class II and co-stimulatory molecules CD80 and CD86. By contrast, an ‘alternative’ activation pathway has also been described. Such macrophages are associated with parasitic infections, allergic and humoral responses. More recently, the alternatively activated macrophages or M2 have been further subdivided into 3
categories depending on the activation trigger: M2a macrophages arise after exposure to IL-4 or IL-13, M2b macrophages after engagement of Fcγ receptors in combination with IL-1β or LPS, and M2c macrophages are induced by IL-10, TGF-β or glucocorticoids. The activation pattern of macrophage populations in tumors is not so well defined, but in some cases it bears similarities with the alternative activation phenotype.

Activated macrophages can produce cytotoxic substances which can be divided into oxygen-dependent and oxygen-independent. Oxygen-dependent substances include the reactive oxygen intermediates, such as hydrogen peroxide (H2O2) and the reactive nitrogen intermediates, such as nitric oxide (NO). NO is produced by the enzyme inducible nitric oxide synthase (iNOS) via the following reaction:

\[ \text{L-arginine} + \text{O}_2 + \text{NADPH} \rightarrow \text{NO} + \text{L-citrulline} + \text{NADP} \]

The oxygen-independent substances include various hydrolytic enzymes, such as lysozyme, various antimicrobial and cytotoxic peptides, known as defensins, and cytokines, such as TNF-α.

Only a limited number of mutations which lead to macrophage deficiency have been described. In the osteopetrotic op/op mouse, a naturally occurring mutation of the gene coding for colony stimulating factor (CSF)-1 leads to differential deficiency of various macrophage subpopulations, dividing the macrophage lineage into CSF-1-dependent and CSF-1-independent. The PU.1 knockout mouse, in contrast, was artificially generated. PU.1 is a transcription factor specifically expressed in hematopoietic cells. Homozygosity for the mutant gene leads to lethality in mice in the prenatal stage; nevertheless, analysis of embryos up to day 18 of gestation revealed that no macrophages could be detected in these mice. The development of liposome-encapsulated clodronate (dichloromethylene diphosphonate) is the most commonly used method to eliminate macrophages in vivo.

### 3.1.2.2.1 Tumor associated macrophages

The tumor microenvironment has many factors that can recruit and differentiate the infiltrating macrophages, which are defined as tumor-associated macrophages (TAM). TAM quite often represent the major infiltrating cell population of various tumors, and can exert tumor promoting or tumor suppressing functions.

TAM isolated from mice bearing mammary tumors were shown to be poor producers of NO and exhibited low cytotoxic ability. In addition, macrophages positive for iNOS were
rarely found in human ovarian cancer and were only localized in the periphery \textsuperscript{105}. Induction of angiogenesis by the production of angiogenic factors such as vascular epithelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PD-ECGF) has often been linked with the TAM. In breast cancer patients, TAM density positively correlated with VEGF expression and microvessel density, while all of them negatively correlated with disease-free survival \textsuperscript{106}. TAM have been shown to express high levels of both the scavenger receptor A \textsuperscript{107} and the mannose receptor \textsuperscript{95}, which is compatible with the M2 macrophage phenotype.

On the other hand, TAM have the potential to directly control tumor cell proliferation when appropriately stimulated, and thus exert tumor suppressing functions. In two murine models of melanoma, engineered to produce granulocyte/macrophage-CSF (GM-CSF), macrophage density inversely correlated with tumorigenicity; these macrophages produced macrophage metalloelastase (MME or MMP-12) and angiostatin, which suppressed the growth of metastases \textsuperscript{108}. MHC class II-negative myeloma cells injected into mice were readily infiltrated by macrophages and were crucial in inhibiting tumor growth after activation by CD4\textsuperscript{+} T cell-derived IFN-γ \textsuperscript{109}. Finally, CpG-oligodeoxynucleotide treatment allowed the control of weakly immunogenic tumors, such as the B16 melanoma and the NXS2 neuroblastoma by macrophages \textsuperscript{110}.

3.1.2.2.2 Myeloid-Derived Suppressor Cells

In cancer patients and tumor-bearing mice, a subpopulation of myeloid cells, characterized as myeloid-derived suppressor cells (MDSC), myeloid suppressor cells (MSC) or immature myeloid cells (iMC), has been described to accumulate and exert immunosuppressive function \textsuperscript{111,112}. MDSC represent a heterogeneous population of myeloid cells comprising of immature macrophages, granulocytes and DC; the addition of cytokines is able to differentiate these cells into mature macrophages, granulocytes or DC \textit{in vitro} \textsuperscript{113}. In mice, MDSC are identified by the expression of the markers CD11b and Gr-1, while other markers have also been described for this population, such as CD31 \textsuperscript{113}, CD115 \textsuperscript{114} and CD124 \textsuperscript{114}. Interestingly, this population can be detected in low numbers also in the BM, blood and spleen of healthy mice, lacking however immunosuppressive function. Human equivalent MDSC were originally described in patients with head and neck cancer as CD34\textsuperscript{+} cells \textsuperscript{115}. Afterwards, they were also characterized in the peripheral blood of patients with squamous cell carcinoma, non-small cell lung cancer and breast
cancer. Human MDSC also exhibit an immature phenotype, as shown by the expression of the markers CD34, CD33, CD13 and the absence of CD15.

Accumulating findings suggest that tumor-derived factors are responsible for the generation of MDSC. Conditioned media from tumor cell lines could inhibit the differentiation of DC from their precursors in vitro. In addition, immunosuppressive cells arose from bone marrow cells after culture with Lewis lung carcinoma S/N. MDSC expansion in tumor-bearing hosts has been correlated with T cell dysfunction. Indeed, it was shown that MDSC induce decrease or loss of the expression of the TCR ζ chain, inhibit the CD3/CD28-induced T cell proliferation by the production of nitrogen and oxygen intermediates as well as the CD8+ T cell IFN-γ production. Recently, it was shown that MDSC suppress IL-2-mediated NK cell cytotoxicity via interfering with Stat5 activation. For this purpose, depletion of MDSC in therapeutic settings is under investigation. In mice, an anti-Gr-1 mAb has been used to deplete MDSC and enhance anti-tumor responses. However, a similar treatment is difficult to be applied in humans, since total granulocyte depletion removes also neutrophils which could render the individual susceptible to infections.

### 3.1.3 The adaptive immune system

The two main components of the adaptive immune system are B and T lymphocytes. B cells constitute part of the humoral-mediated response which specializes in the recognition and elimination of the extracellular pathogens, while T cells generate cell-mediated immune responses which target the intracellular pathogens. B lymphocyte maturation takes place within the bone marrow and is accompanied by the acquisition of a unique antigen-binding receptor on their surface, called the B cell receptor (BCR); the BCR constitutes a membrane-bound antibody. Upon antigen encounter, B cells differentiate into memory and effector cells, called plasma cells; the latter produce antibodies in a secreted form.

T lymphocytes also arise in the BM but, subsequently, migrate to the thymus for their maturation. During their maturation, T cells express a unique antigen binding molecule, the T cell receptor (TCR). Both the BCR and TCR are products of somatic gene rearrangement during maturation. The genes encoding these receptors are assembled from variable and constant fragments through recombination activation gene (RAG) protein-mediated somatic recombination. However, the TCR in contrast to the BCR can only recognize antigen bound to
MHC molecules. T cells are distinguished by the presence of either CD4 or CD8 membrane glycoproteins on their surface. The CD4+ T cells recognize antigens in the context of MHC class II molecules on the surface of antigen-presenting cells, while the CD8+ T cells recognize antigens presented by MHC class I molecules found on all cells of a healthy individual. After a CD4+ T cell recognizes and interacts with a MHC class II-peptide complex, it is activated and secretes various cytokines, which play an important role in activating other immune cells, as B cells, CD8+ T cells, macrophages, etc. Thus, CD4+ T cells play a central role in both humoral and cell-mediated immunity. CD8+ T cells exhibit high cytotoxic activity upon activation and act on eliminating potentially dangerous cells, like virus-infected cells, tumor cells or cells of a foreign tissue graft.

Besides the conventional B and T cells, adaptive immunity encompasses the so-called innate-like lymphocytes, i.e. the B1 cells, the marginal-zone B cells, natural killer T (NKT) cells and subsets of \( \gamma\delta \) T cells; the diversity of antigen receptors expressed by these cells is restricted and not entirely random and their specificities are skewed towards a predefined set of ligands.

In certain individuals, the discrimination of self from non-self malfunctions, leading to an immune attack upon the host, a condition termed as autoimmunity. During an autoimmune response, self-reactive clones of B or T cells are activated, generating humoral or cell-mediated responses against self-antigens. Normal healthy individuals are believed to possess self-reactive lymphocytes. However, since the presence of these cells does not necessarily lead to autoimmunity, mechanisms which regulate their activity exist.

### 3.1.3.1 Mechanisms of T cell tolerance

Random rearrangement of the TCR genes generates an enormous TCR repertoire, leading to specificities which in theory could also recognize soluble antigens, self antigens or antigens presented in non-self MHC molecules. Positive selection of T cells in the thymus ensures that TCR expressed on mature T cells will bind only to self MHC molecules; the T cells that fail to do so are eliminated by apoptosis. In a second step termed negative selection, T cells bearing a high-affinity receptor for self antigens presented in self MHC are also eliminated. This selection procedure leads to the apoptosis of ~98% of all T cells in the thymus and ensures the generation of T cells, which are self-MHC restricted and self-tolerant. B cell progenitors undergo a similar process in the BM. This control of self-responsiveness that occurs during lymphocyte
development in the central lymphoid organs is known as central tolerance. One constraint of central tolerance is the requirement for the autoantigens to be present in the thymus. Although many tissue-specific antigens are represented in the thymus, at least at the mRNA level, whether or not they are expressed as proteins at levels sufficient to induce T cell deletion is not clear. In fact, healthy individuals have been shown to harbor self-reactive T cells in the periphery and these T cells are more likely to bear a low-affinity TCR for self-antigens. Mechanisms of peripheral tolerance protect us from these escaping self-reactive clones.

In the periphery, recognition of a peptide–MHC complex on an APC can result either in the activation and clonal expansion of the T cell or in some cases in a state of non-responsiveness called anergy. In contrast to central tolerance, anergy does not lead to the apoptosis of the T cell but allows its survival in the periphery for an extensive period of time in a hyporesponsive state. Whether a T cell will be activated or subjected to anergy in the presence of peptide-MHC complex is determined by the presence or absence of sufficient co-stimulatory signals, delivered through the CD28-B7-1/2 interaction, or the ligation of ligands like the cytotoxic T lymphocyte associated antigen 4 (CTLA-4), a homologue of CD28 with inhibitory function. Dendritic cells (DC) have emerged as key APC in this process, regulating immunity versus tolerance. In fact, immature DC have been described to inhibit T cell responses, and are thus characterized as tolerogenic DC. This second level of control which regulates the responsiveness of mature lymphocytes against unwanted responses to self in the periphery is known as peripheral tolerance.

In addition to the mechanisms of clonal deletion and anergy that physically eliminate or functionally inactivate potentially hazardous self-reactive lymphocytes, other mechanisms of tolerance include T cell ignorance of self antigens and the phenotype skewing. T cell ignorance occurs either because the antigen is expressed in sites not easily accessible to T cells or because the amount of antigen does not reach the threshold required to trigger a T cell response. In the phenotype skewing, effective tolerance might be maintained, even in the presence of an active immune response, depending on the nature of the response. Th2 cytokines, in particular, have been linked with downregulation of autoimmunity in experimental autoimmune encephalomyelitis and diabetes.

Finally, there is accumulating evidence that a subset of CD4+ T cells, named regulatory T cells, actively suppresses the activation and expansion of self-reactive T cells, thereby preventing autoimmune diseases.
3.1.3.2 Regulatory T cells

Naturally occurring regulatory T cells (Treg) constitute 5-10% of all CD4+ T cells and possess potent ability to suppress immune cell effector functions in in vitro functional assays. They are present in the normal thymus, as a functionally mature and distinct T cell subpopulation. Thus, Treg are already specialized for suppressive function before antigen encounter. This differentiates them from other types of regulatory T cells, such as Tr1 and Th3 cells, which under certain conditions arise in the periphery from naive T cells following antigen exposure; these are termed induced Treg (iTreg), in contrast to the naturally occurring Treg (nTreg), which arise from the thymus. The TCR repertoire of Treg is broad and more diverse than the one of the naive T cells, however, more self-reactive than other T cells. They appear to either escape thymic deletion or may indeed be positively selected as part of an ‘anti-self’ repertoire. This may explain why some Treg are in a more proliferative state than other T cells in the periphery of normal animals, presumably due to the recognition of self-antigens. Treg do not produce pro-inflammatory cytokines upon antigenic stimulation and therefore do not harm the host, despite their high self-reactivity. Naturally occurring Treg are widely believed to exert their suppressive effect in a contact-dependent manner, in contrast to induced Treg which exert their suppressive function via cytokines, despite the fact that controversy has emerged from in vivo data, where in various models nTreg were shown to act through cytokine-dependent manner.

3.1.3.2.1 Phenotypic characterization of Treg

Treg are characterized by the expression of cell surface markers, which are usually also found on activated T cells, like the α chain of the IL-2R (CD25), the glucocorticoid-induced TNF-receptor family-related gene/protein (GITR) and the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4, CD152). Currently, the most specific and reliable molecular marker for the identification of Treg in rodents and humans is the transcription factor Foxp3, which is expressed in a highly Treg-specific manner and controls the development and function of Treg. Although Foxp3 expression is more restricted to CD4+ Treg identified in mice, human Foxp3 expression is not as restricted as the mouse counterpart; several reports document its expression in effector T cells upon activation, although at a relatively lower level when compared to Treg. Retroviral transduction of mouse Foxp3 to mouse CD4+CD25+ T cells can convert them to
Treg-like cells, both phenotypically and functionally\textsuperscript{153,154}. Surprisingly, when human Foxp3 and/or Foxp3δ\textsubscript{2}, an isoform of Foxp3 present on human Treg, are ectopically overexpressed in human CD4\textsuperscript{+} T cells, it does not lead to the acquisition of significant suppressor activity \textit{in vitro}\textsuperscript{163}, suggesting that the mouse and human Foxp3 may have some differences.

The gene \textit{foxp3} was identified as the defective gene in the \textit{Scurfy} mouse strain, which has a X-linked recessive mutation leading to lethality in hemizygous males or homozygous females within a month after birth\textsuperscript{164}. Mutations of the human gene FOXP3, the ortholog of the murine \textit{foxp3} gene, were subsequently found to be the cause of the Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked immunodeficiency (IPEX) syndrome, which leads to the development of organ-specific autoimmune diseases, inflammatory bowel disease (IBD), allergic dermatitis, food allergy, hematological disorders, hyperimmunoglobulinemia E, and serious infections\textsuperscript{165,166}. Although IPEX is a rare disease, its clinical picture and its causative factor as a deficiency or malfunction of Treg underlies the role of Treg in maintaining immunologic self-tolerance.

Recent analysis of Foxp3-reporter mice and intracellular staining of the Foxp3 protein revealed a correlation between the ontogeny of CD25-expressing Treg and Foxp3-expressing T cells\textsuperscript{167}. This analysis confirmed the finding that natural Treg become detectable in the periphery of normal mice a few days after birth and thymectomy around day 3 after birth abrogates the thymic production of natural Treg from the beginning of their ontogeny\textsuperscript{168}. There is substantial evidence that CD25 is not a mere indicator of the chronically activated state of Treg, but is a functionally essential molecule for their survival and function. For example, IL-2, IL-2R\alpha (CD25) and IL-2R\beta (CD122) deficiencies produce similar fatal lymphoproliferative inflammatory disease, as the Foxp3 deficiency, with autoimmune components, generally characterized as ‘IL-2 deficiency syndrome’\textsuperscript{169-173}; while deficiency of the common γ chain completely abrogates the development of the other T cells as well. In addition, neutralization of circulating IL-2 resulted in selective reduction of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} T cells in the thymus and periphery. These findings collectively indicate that the IL-2 and transcription factor Foxp3 are key control molecules for the development and function of natural CD4\textsuperscript{+}CD25\textsuperscript{+} Treg. Since Foxp3 is a nuclear factor, the combination of CD4 and CD25 markers have been more commonly used for the isolation or depletion of Treg.
3.1.3.2.2 Treg and autoimmunity

Treg play an indispensable role in the mechanism of self-tolerance, since depletion of these cells from normal animals by the use of a mAb directed against CD25 leads to the spontaneous development of various autoimmune diseases, such as autoimmune gastritis, thyroiditis, type I diabetes (TID) and IBD; reconstitution of the animals with normal CD4⁺CD25⁺ T cells prevents these disorders 174,175. In addition, the incidence of autoimmune disease in IPEX patients, which have a mutated Foxp3 gene, is approximately 90%, with approximately 80% incidence of TID within a year after birth 176. Some patients, in fact, already manifest the disease at the time of birth 176. The high incidence of autoimmune disease in IPEX patients suggests that most of normal individuals may harbor potentially pathogenic self-reactive T cell clones, capable of mediating common autoimmune diseases. This is an indication that dominant self-tolerance is physiologically operating in humans as well as rodents with the purpose of preventing autoimmune disease.

Treg not only inhibit autoimmune responses but also suppress a variety of immune responses to non-self antigens. Depletion of CD4⁺CD25⁺ T cells from animals enhances immune responses to microbes, triggers allergic responses and breaks feto-maternal tolerance during pregnancy. In addition, depletion of Treg provokes effective tumor immunity to autologous tumor cells in otherwise nonresponding animals 138. Conversely, natural Treg can be exploited to treat autoimmune diseases and to establish immunologic tolerance to non-self antigens as in organ transplantation.

3.1.3.2.3 Treg and tumor immunity

Although Treg play a crucial role in preventing autoimmunity by inducing immune tolerance, they might inhibit anti-tumor immunity and promote tumor growth by suppressing host immune responses against non-self antigens. Sakaguchi and colleagues first demonstrated that the removal of CD4⁺CD25⁺ T cells can induce antitumor responses, establishing a ‘common basis’ between tumor immunity and autoimmunity 177. In later studies, many groups reported elevated percentages of CD4⁺CD25⁺ Treg in the total T cell population isolated from tumor tissues or peripheral blood in a variety of cancers, including lung cancer 178, breast cancer 179, ovarian cancer 180, melanoma 181, liver cancer 182, gastric cancer 183 and lymphoma 184. From these studies, two are of a particular interest. Curiel et al. reported that Treg preferentially accumulate...
in ovarian tumors and in malignant ascites attracted by CCL22 and that the high percentage of Treg is associated with the poor prognosis of cancer patients. This study suggests that Treg may mostly interfere with the function of effector T cells and not so much with their priming phase. Viguier at al., on the other hand, found higher accumulation of Treg in the draining lymph nodes infiltrated by melanoma cells, suggesting that Treg contribute to the local immunosuppressive milieu. Increasing number of evidence indicates that Treg in the tumor microenvironment inhibit anti-tumor immunity, which represents a major obstacle for developing effective therapeutic cancer vaccines.

### 3.1.3.2.4 Treg and innate immunity

The suppressive effect of Treg on the adaptive responses mediated by T cells is well documented. However, the effect of Treg on cells of the innate immune system is less well studied. A potential role for Treg in dampening NK cell functions was first suggested by Sakaguchi in a murine leukemia model. More recently, it was reported that Treg depletion in vivo led to the rejection of NK cell sensitive cell lines expressing RAE-1, the ligand for the activating receptor NKG2D in vivo. In vitro, NK cell cytotoxicity was largely inhibited by Treg through a TGF-β-dependent and IL-10-independent mechanism. These studies suggest that Treg directly inhibit NK cell activation towards NKG2D ligand-expressing targets in vitro and in vivo. In another recent study, Treg could induce NK cell death in a granzyme B- and perforin-dependent manner. In a model of hybrid resistance during allogeneic BM transplantation, Treg removal significantly enhanced NK cell-mediated BM graft rejection.

It has been proposed that Treg can directly suppress monocyte/macrophage activation and their effector functions in vitro. Pre-incubation of human blood monocytes with Treg or the presence of Treg in the monocyte culture impaired cytokine production and the upregulation of HLA II, CD40 and CD80 activation markers by monocytes upon LPS stimulation. Kryczek et al. reported upregulation of the inhibitory molecule B7-H4 on isolated human monocytes mediated by Treg. In addition, B7-H4 expression on macrophages infiltrating ovarian tumors inversely correlated with the patients’ survival. The ability of Treg to steer monocyte differentiation towards an alternative activated phenotype, marked by an upregulation of CD206 and CD163 markers, was also demonstrated in vitro. In addition to macrophages, one study provided data that Treg could inhibit reactive oxygen intermediates and cytokine
production by neutrophils, although this phenomenon was only observed after in vitro treatment of both Treg and neutrophils with LPS. Finally, in a model of acute in vivo ablation of Treg, it was observed that various types of innate immune cells, including NK1.1+ cells and macrophages (F4/80+CD11c− cells) increased in numbers in the secondary lymphoid organs after Treg ablation. Nevertheless, it is possible that Treg do not exert their suppressive effect on innate immune cells via direct interaction but in an indirect manner as visualized for T cells. Treg inhibited the activation of autoreactive T cells in the LN by impeding the formation of stable contacts between T cells and DC.

3.1.3.2.5 Manipulation of the suppressive function of Treg

Given the observations that Treg exercise a negative role in tumor immunity, a key question in cancer immunotherapy is how to eliminate or to reverse the suppressive function of Treg. Cyclophosphamide is a chemotherapeutic agent with anti-mitotic action, used to treat various types of cancer. High doses of this drug lead to immunosuppression, the basis of other clinical uses, as preventing organ rejection in organ transplantation. Low doses of this drug, however, lead to enhanced immune responses against a variety of antigens. The chemotherapeutic activity of cyclophosphamide was readily linked with the elimination of tumor-induced suppressor T cells and later, namely, with its effect on Treg. Controversy still exists, however, on whether cyclophosphamide solely decreases Treg cell numbers, interferes with their suppressive function or influences both numbers and suppressive ability, and on the mechanism of action. Another approach used in clinical studies is the administration of an anti-CTLA-4 mAb; however, the antitumor effects of CTLA-4 blockade were shown to be due to increased T cell activation rather than inhibition or depletion of the CTLA-4-expressing Treg. Presently, several investigators are targeting the specific elimination of Treg with a fusion protein of diphtheria toxin and the IL-2 cytokine (ONTAK, Denileukin diftitox, DAB389IL-2). Administration of ONTAK could efficiently eliminate CD25-expressing Treg and yielded effector functions in patients with melanoma or renal cancer. Another recombinant immunotoxin, LMB-2, has been used for the same purpose. LMB-2 is a fusion of a single-chain Fv fragment of the CD25-specific, anti-Tac mAb to a truncated form of the bacterial Pseudomonas exotoxin A, which has been shown to selectively eliminate human Treg in vitro without impairing the function of the remaining lymphocytes. In vivo, the administration of
this toxin in metastatic melanoma patients before vaccination led to a transient reduction in circulating Treg numbers without augmenting, however, the efficiency of the vaccination.

An alternative approach to Treg depletion in vivo is to reverse the suppressive function of Treg. It has been demonstrated that TLR signaling activation on DC can render naïve T cells refractory to suppression mediated by Treg in mice. In humans, Poly-G oligonucleotides, which are recognized by TLR8, can directly reverse the suppressive effect of Treg in the absence of DC. Poly-G oligonucleotides could not reverse the suppressive activity of murine Treg, because TLR8 is not functional in mice. In addition, activation of TLR2 with its ligand Pam3Cys directly increased the proliferation of murine Treg and temporally reversed their suppressive function. Other TLR ligands, however, fail to do so. Human TLR5, for example, enhanced rather than reversed the suppressive function of Treg. Further studies are needed to define the role of other TLR on the function of mouse and human Treg and effector cells.

3.2 Type I IFN

Type I IFN are a family of glycoproteins which includes several members, namely IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ, IFN-ω and IFN-ζ, also known as limitin. All the members bind to the same receptor complex known as the IFN-α receptor (IFNAR), which constitutes of two subunits, the IFNAR1 and the IFNAR2. 13 isoforms have been described for IFN-α, and one for IFN-β, -ε, -κ, -τ and -ζ; although IFN-ω has only one functional form, several pseudogenes have been identified. Type I IFN and IFN-induced proteins have a crucial role in the defense against viruses and are unique in vertebrates. They are produced in response to viral infections by many cell types, including lymphocytes, macrophages, fibroblasts, endothelial cells, osteoblasts etc. These proteins trigger the expression of more than 100 genes, the products of which have diverse antiviral activities. Type I IFN can be induced early during infection, and can thus activate promptly the cells of the innate immune system such as NK cells and macrophages.

In principle, all virally infected cells can produce type I IFN, resulting in autocrine and paracrine IFN-mediated signaling, which confers an antiviral state on the infected and neighboring cells. Type I IFN production has been originally studied in virus-infected fibroblasts, where the role of IRF-3 and IRF-7 has been demonstrated. Both of these factors reside in
the cytoplasm and require phosphorylation in their C-terminal region for their activation and translocation to the nucleus, where they activate their target genes. *In vivo* observations further supported the importance of these factors. *Irf3*−/− mice are vulnerable to encephalomyocarditis virus (EMCV) infection. Subsequent generation of the *Irf7*−/− mice showed that these mice are more susceptible to infections of both DNA and RNA viruses, such as Herpes simplex virus (HSV)-1 and EMCV, than wt or *Irf3*−/− mice, establishing IRF-7 as a master regulator of type I IFN-mediated immune responses. The IRF-7-dependent pathway is often referred to as the classical pathway of IFN gene induction.

Alternatively, type I IFN production can be elicited in response to viral or bacterial nucleic acids by the engagement of TLR, namely TLR3, TLR7/TLR8 and TLR9. These receptors detect viral double-stranded RNA, single stranded RNA or unmethylated CpG motifs present in microbial DNA, respectively. An exception to this rule seems to be the IFN-β production in response to TLR4 ligands, which are not nucleic acids. TLR4 is activated by LPS or the lipid A component of Gram-negative bacteria, as well as some viral components. Plasmacytoid DC (pDC) can produce systemic levels of IFN-α after engagement of TLR and thus are often referred to as specialized type I IFN producing cells.

### 3.2.1 Regulatory effects of type I IFN

Although first characterized based on their potent antiviral functions, type I IFN can also mediate a variety of immunoregulatory effects, suggesting that they can be important links between innate and adaptive immune responses. First of all, it has been shown that type I IFN can regulate their own expression. Particular IFN genes, namely IFN-β and/or IFN-α4, are first induced in virally infected cells; the products of their target genes can then act on the neighboring cells to induce the expression of other IFN-α subtypes. The most well known effect of type I IFN is the enhancement of antigen processing and antigen presentation via the MHC class I pathway, leading thus to an enhancement of CD8+ T cell-mediated responses. Type I IFN contribute to the maturation of DC and the induction of co-stimulatory molecules. Type I IFN have been proposed to induce the expression of IL-15, which has been linked with the enhanced NK cell and memory CD8+ T cell proliferation early during viral infections. In contrast, type I IFN have been shown to negatively regulate the expression of IL-12 in mice both *in vitro* and *in vivo* during infection with MCMV and in human *in vitro* generated DC co-
cultured with CD4$^+$ T cells. Since IL-12 is a potent stimulator of IFN-γ production, type I IFN can control the subsequent immune response. Interestingly, IFN-γ production was enhanced by type I IFN in a IL-12-independent manner during a LCMV infection.

Type I IFN or chemical type I IFN inducers, i.e. tilerone, or analogues of viral nucleic acids such as polyinosinic-polycytidylic acid [poly(I:C)] were shown to enhance NK cell cytotoxicity and their *in vivo* trafficking. Only recently, however, it was clearly demonstrated – with the use of an *in vivo* mouse model of inducible depletion of DC – that type I IFN do not directly activate NK cells but rather indirectly via DC, which in turn produced and trans-presented IL-15 to NK cells *in vivo*. Finally, in addition to their regulatory effects, type I IFN affect the development and homeostasis of B and T cells, DC and osteoclasts.

### 3.2.2 Type I IFN and tumor

In addition to their antiviral activity, exogenously administered type I IFN have been shown to suppress the growth of transplantable tumors of different origins as well as pulmonary metastases in mice. Type I IFN could have a direct inhibition on tumor cell proliferation *in vitro* by promoting cell growth arrest in the G1 and S phases. In addition, type I IFN treatment resulted in vascular endothelial cell damage that preceded tumor necrosis and restored the levels of other tumor-promoting factors, such as down-regulating matrix metalloproteinase (MMP)-9 and up-regulating E-cadherin. For these reasons, exogenously administered type I IFN have also been used in clinical applications for the treatment of a range of malignancies such as hairy cell leukemia, melanoma, renal cell carcinoma and Kaposi sarcoma. Nevertheless, clinical success has been limited due to the short half-life of type I IFN, which is less than 5h, and the side effects from the high dose administration required for effective therapy.

Besides the well documented role of type I IFN administration for tumor treatment, there are few reports documenting a role for endogenous type I IFN in the control of tumor growth of transplantable tumors. Although much progress has been made in understanding the mechanisms of type I IFN responses to infection and inflammation, it is less clear how type I IFN production is stimulated during an anti-tumor response, which cells produce them, which cells respond to them and what is the importance of type I IFN for anti-tumor immunity.

Recently, a role for type I IFN in tumor immunoediting has been described. MCA-induced sarcomas arising in IFNAR1$^{-/-}$ mice exhibited an unedited phenotype, i.e. they were...
immunogenic and were rejected in a T cell-dependent manner when transplanted into wt recipients. In addition, type I IFN have been shown to control NK cell-mediated anti-tumor responses against MCA-induced sarcomas, MHC class I deficient RMA-S tumors and cytokine immunotherapy of lung metastases. Interestingly, a role for IFN-α in downregulating the recognition of tumor cells by NK cells has also been proposed by the inhibition of the expression of H60 and, to a lesser extent, of MULT1 on MCA-induced sarcoma lines from 129/Sv mice after in vitro culture with IFN-α.
4 AIM OF THE STUDY

In cancer patients, many tumors downregulate expression of MHC class I molecules in order to escape direct recognition by CD8+ T cells, which is MHC class I restricted 261. At the same time, these tumors become more susceptible to NK cell attack due to the lack of self MHC class I 63. However, upon inoculation of higher tumor cell numbers, tumors escape the control of the immune system. Therefore, it is essential to define mechanisms to strengthen the immune response against a high tumor cell load and tumors with poor immunogenicity. Strategies to enhance anti-tumor immune responses include activation of anti-tumor effector functions via the administration, for example, of cytokines 262,263 or, on the other hand, the removal of suppressive signals counteracting activation.

In this regard, the aim of our study is to strengthen the immune response against a MHC class I-deficient tumor, the RMA-S lymphoma. For this purpose, the role of immunosuppressive Treg in inhibiting innate immune cell functions during the anti-tumor response in vivo is addressed. In order to achieve this, we experimentally remove Treg before s.c. inoculation of mice with RMA-S tumor cells and analyze the emerging anti-tumor effector functions.

The second part of this study deals with the role of type I IFN in the anti-tumor immunity against the RMA-S lymphoma. Type I IFN have been well characterized for their contribution in anti-viral responses 264. In parallel, they have been extensively used in clinical practice to treat various types of cancers 265. Few reports exist, however, documenting the production of type I IFN during an anti-tumor response as well as their contribution to the control of tumor growth. In order to pinpoint the role of type I IFN in the anti-tumor response, we are monitoring the growth of RMA-S lymphoma in mice unresponsive to type I IFN (IFNAR1−/− mice), and characterizing the anti-tumor mechanisms which are under their control.
5 MATERIALS AND METHODS

5.1 MATERIALS

5.1.1 Laboratory equipment

(listed in alphabetical order)

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anesthesia machine, Vapor 19.1</td>
<td>Drägerwerk AG</td>
</tr>
<tr>
<td>Beta-counter, MicroBeta TriLux 1450 LSC</td>
<td>PerkinElmer</td>
</tr>
<tr>
<td>Bio-plex array system</td>
<td>Bio-rad</td>
</tr>
<tr>
<td>Cell culture incubator, Heraeus BBD 6220 (CO₂)</td>
<td>Kendro</td>
</tr>
<tr>
<td>Centrifuge, Heraeus Multifuge 4 K-R/3 S-R</td>
<td>Kendro</td>
</tr>
<tr>
<td>Centrifuge 5415 R (table)</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>Centrifuge, Sorvall Evolution RC</td>
<td>Kendro</td>
</tr>
<tr>
<td>ELISA microplate reader, GENios</td>
<td>TECAN</td>
</tr>
<tr>
<td>FACS sorter, FACSDiva</td>
<td>BD</td>
</tr>
<tr>
<td>FACS sorter, FACSVantage SE</td>
<td>BD</td>
</tr>
<tr>
<td>Flow cytometer, FACSCalibur</td>
<td>BD</td>
</tr>
<tr>
<td>Flow hood, Heraeus Hera Safe</td>
<td>Kendro</td>
</tr>
<tr>
<td>Fridge, premium</td>
<td>Liebherr</td>
</tr>
<tr>
<td>Freezer -20°C, comfort/profi line</td>
<td>Liebherr</td>
</tr>
<tr>
<td>Freezer -86°C, VIP series</td>
<td>Sanyo</td>
</tr>
<tr>
<td>Gamma-counter, Cobra auto-gamma</td>
<td>Packard, PerkinElmer</td>
</tr>
<tr>
<td>Gamma cell 1000</td>
<td>Atomic Energy of Canada Ltd</td>
</tr>
<tr>
<td>Harvester 96-well automated, TOMTEC</td>
<td>PerkinElmer</td>
</tr>
<tr>
<td>Heat sealer</td>
<td>PerkinElmer</td>
</tr>
<tr>
<td>Ice machine</td>
<td>Hoshizaki</td>
</tr>
<tr>
<td>Incubator Shaker, Innova 4200</td>
<td>New Brunswick Scientific</td>
</tr>
<tr>
<td>Magnetic stirrer, MR3001 K</td>
<td>Heidolph</td>
</tr>
<tr>
<td>Microscope, WILOWERT30</td>
<td>Neolab</td>
</tr>
<tr>
<td>Microscope (Stereo Microscope)</td>
<td>Hund</td>
</tr>
</tbody>
</table>
Microscope C1Si (confocal) | Nikon  
---|---  
Minifuge, GalaxyMini | VWR  
N₂ tank, CryoSystem 6000 | MVE  
pH meter | WTW  
Photometer, Ultraspec 3100 | Amersham Biosciences  
Pump, Econo Pump | Bio-Rad  
Rotating wheel | Labor-Brand  
Scales, PB602-S | Heidolph  
Scales (micro), AG285 | Heidolph  
Vortex, VortexGenie2 | VWR/Scientific Industries  
Water bath, Heraeus Julabo TW20 | Kendro  

### 5.1.2 Cell culture products

<table>
<thead>
<tr>
<th>Product</th>
<th>Source</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard tissue culture flasks/filter screw caps – 25 cm²</td>
<td>TPP</td>
<td>90026</td>
</tr>
<tr>
<td>Standard tissue culture flasks/filter screw caps – 75 cm²</td>
<td>TPP</td>
<td>90076</td>
</tr>
<tr>
<td>Standard tissue culture flasks/filter screw caps – 150 cm²</td>
<td>TPP</td>
<td>90151</td>
</tr>
<tr>
<td>Tissue culture flasks/filter screw caps – 182 cm²</td>
<td>Greiner</td>
<td>660175</td>
</tr>
<tr>
<td>96-well U-bottom with lid – Standard TC</td>
<td>BD</td>
<td>353077</td>
</tr>
<tr>
<td>96-well flat-bottom with lid – Standard TC</td>
<td>BD</td>
<td>353072</td>
</tr>
<tr>
<td>96-well flat-bottom with transwell insert</td>
<td>Corning</td>
<td>3381</td>
</tr>
<tr>
<td>48-well flat-bottom with lid – Standard TC</td>
<td>BD</td>
<td>353078</td>
</tr>
<tr>
<td>24-well flat-bottom with lid – Standard TC</td>
<td>BD</td>
<td>353047</td>
</tr>
<tr>
<td>12-well flat-bottom with lid – Standard TC</td>
<td>BD</td>
<td>353043</td>
</tr>
<tr>
<td>6-well flat-bottom with lid – Standard TC</td>
<td>BD</td>
<td>353046</td>
</tr>
<tr>
<td>50ml conical tubes Falcon™</td>
<td>BD</td>
<td>352070</td>
</tr>
<tr>
<td>15ml conical tubes</td>
<td>Greiner</td>
<td>188271</td>
</tr>
<tr>
<td>Product</td>
<td>Source</td>
<td>Cat. No.</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>5 ml round-bottom polypropylene test tube</td>
<td>BD</td>
<td>352008</td>
</tr>
<tr>
<td>5 ml round-bottom polystyrene test tube with cell strainer</td>
<td>BD</td>
<td>352235</td>
</tr>
<tr>
<td>0,6 ml round-bottom test tube</td>
<td>Greiner</td>
<td>101101</td>
</tr>
<tr>
<td>Eppendorf tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 µm cell strainer Falcon™</td>
<td>BD</td>
<td>352350</td>
</tr>
<tr>
<td>40 µm cell strainer Falcon™</td>
<td>BD</td>
<td>352340</td>
</tr>
<tr>
<td>Cryovial®, 2ml sterile</td>
<td>Roth</td>
<td>E309.1</td>
</tr>
<tr>
<td>Nalgene™ Cryo 1°C Freezing Container, “Mr. Frosty”</td>
<td>Nunc</td>
<td>5100-0001</td>
</tr>
<tr>
<td>35mm FluoroDish™</td>
<td>WPI</td>
<td>FD35-100</td>
</tr>
<tr>
<td>HiTran Protein G HP, 5ml</td>
<td>GE Healthcare</td>
<td>17-0405-01</td>
</tr>
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</table>

### 5.1.3 Cell culture media

<table>
<thead>
<tr>
<th>Product</th>
<th>Source</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640 (1x) w/o L-Glutamine</td>
<td>GIBCO-Invitrogen</td>
<td>31870</td>
</tr>
<tr>
<td>D-MEM (1x) (High Glucose) with L-Glutamine, 4500 mg/L D-Glucose, w/o sodium pyruvate</td>
<td>GIBCO-Invitrogen</td>
<td>41965</td>
</tr>
<tr>
<td>D-PBS (1x) w/o Ca, Mg, sodium bicarbonate</td>
<td>GIBCO-Invitrogen</td>
<td>14190</td>
</tr>
<tr>
<td>IMDM Iscoves Modified Dulbecco Medium (1x)</td>
<td>GIBCO-Invitrogen</td>
<td>21980032</td>
</tr>
<tr>
<td>Fetal Bovine Serum, Origin: EU Approved</td>
<td>GIBCO-Invitrogen</td>
<td>10270</td>
</tr>
<tr>
<td>Penicillin/Streptomycin-Solution</td>
<td>GIBCO-Invitrogen</td>
<td>15140</td>
</tr>
<tr>
<td>10000 U/ml penicillin, 10000 µg/ml streptomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glutamine 200 mM (100x), 29.2 mg/ml</td>
<td>GIBCO-Invitrogen</td>
<td>25030</td>
</tr>
<tr>
<td>Non-essential amino acids (100X)</td>
<td>GIBCO-Invitrogen</td>
<td>11140035</td>
</tr>
<tr>
<td>Sodium pyruvate MEM 100mM</td>
<td>GIBCO-Invitrogen</td>
<td>11360088</td>
</tr>
<tr>
<td>β-mercaptoethanol 50mM</td>
<td>GIBCO-Invitrogen</td>
<td>31350010</td>
</tr>
<tr>
<td>Trypsin –EDTA (1x) HBSS w/o Ca²⁺/Mg²⁺ w/ EDTA</td>
<td>GIBCO-Invitrogen</td>
<td>25300</td>
</tr>
<tr>
<td>Dimethylsulphoxide Hybri Max® (DMSO)</td>
<td>Sigma-Aldrich</td>
<td>D2650</td>
</tr>
<tr>
<td>Cell Dissociation Solution Non-enzymatic 1x</td>
<td>Sigma-Aldrich</td>
<td>C5914</td>
</tr>
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</table>
### 5.1.4 Solutions

(listed in alphabetical order)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACK lysis buffer</td>
<td>0.829 g NH₄Cl, 0.1 g KHCO₃, 0.38 mg EDTA, 100 ml ddH₂O, pH 7.3</td>
</tr>
<tr>
<td>FACS buffer</td>
<td>1x PBS, 0.02 % (v/v) NaN₃ in PBS, 1% FCS, 0.5 mM EDTA</td>
</tr>
<tr>
<td>Freezing medium</td>
<td>1x FCS, 10% DMSO</td>
</tr>
<tr>
<td>MACS buffer</td>
<td>1x PBS, 1% FCS, 0.5 mM EDTA</td>
</tr>
<tr>
<td>Primary cell culture medium</td>
<td>1x RPMI, 10% FCS, 2 mM L-glutamine, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 1 mM Sodium Pyruvate, 1x Non-essential amino acids, 0.25 mM β-mercaptoethanol</td>
</tr>
<tr>
<td>PBS (10x)</td>
<td>1.37 M NaCl, 27 mM KCl</td>
</tr>
</tbody>
</table>
100 mM $\text{Na}_2\text{HPO}_4$ (anhydrous)
20 mM $\text{KH}_2\text{PO}_4$

**Antibody purification:**

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Concentration</th>
<th>Component</th>
<th>Source</th>
<th>Product Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding buffer</td>
<td>20 mM</td>
<td>Sodium phosphate</td>
<td>ddH$_2$O, pH 7.0</td>
<td></td>
</tr>
<tr>
<td>Elution buffer</td>
<td>0.1 M</td>
<td>Glycin HCl</td>
<td>ddH$_2$O, pH 2.7</td>
<td></td>
</tr>
<tr>
<td>Neutralization buffer</td>
<td>1 M</td>
<td>Tris-HCl</td>
<td>ddH$_2$O, pH 9</td>
<td></td>
</tr>
<tr>
<td>Precipitation buffer</td>
<td>29.1 g</td>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>ddH$_2$O</td>
<td></td>
</tr>
</tbody>
</table>

### 5.1.5 Chemicals

(listed in alphabetical order)

<table>
<thead>
<tr>
<th>Product</th>
<th>Source</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-methyl- L -tryptophan (1MT)</td>
<td>Sigma-Aldrich</td>
<td>860646</td>
</tr>
<tr>
<td>Brefeldin A</td>
<td>Sigma-Fluka</td>
<td>B7651</td>
</tr>
<tr>
<td>Carboxyfluorescein succinimidyl ester (CFSE)</td>
<td>Sigma-Fluka</td>
<td>21888</td>
</tr>
<tr>
<td>Chromium-51</td>
<td>PerkinElmer</td>
<td>NEZ030005MC</td>
</tr>
<tr>
<td>Collagenase, type IV from <em>Clostridium histolyticum</em></td>
<td>Cell systems</td>
<td>LS004188</td>
</tr>
<tr>
<td>DNase I</td>
<td>Sigma</td>
<td>DN25-1G</td>
</tr>
<tr>
<td>FACS lysing solution (10x)</td>
<td>BD</td>
<td>349202</td>
</tr>
<tr>
<td>Heparin-Sodium, 25 000 Units</td>
<td>B Braun</td>
<td>1708.00.00</td>
</tr>
<tr>
<td>IFN-γ, recombinant mouse</td>
<td>BD</td>
<td>51-9000889</td>
</tr>
</tbody>
</table>
Indomethacin  
Isoflurane  
Lipopolysaccharide (LPS)  
*Lycopersicum esculentum* lectin  
[methyl-3H] Thymidine  
N°-Monomethyl-D-arginine monoacetate  
N°-Monomethyl-L-arginine monoacetate  
PMSF  
Paraformaldenhyde (PFA)  
Propidium Iodide (PI)  
TNFa, recombinant human  
TRAIL, recombinant mouse  
Triton X-100  
Saponin from quillaja bark

5.1.6 Antibodies

5.1.6.1 Antibodies for FACS analysis

The following antibodies against mouse antigens were used (listed in alphabetical order):

<table>
<thead>
<tr>
<th>Ab specificity</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Source</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7-H4</td>
<td>PE</td>
<td>188</td>
<td>rat IgG2a, κ</td>
<td>1:100</td>
<td>eBioscience</td>
<td>12-5972</td>
</tr>
<tr>
<td>CD3</td>
<td>FITC</td>
<td>145-2C11</td>
<td>arménian hamster</td>
<td>1:100</td>
<td>BD</td>
<td>553062</td>
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<tr>
<td>CD3</td>
<td>APC</td>
<td>145-2C11</td>
<td>arménian hamster IgG, κ</td>
<td>1:100</td>
<td>BD</td>
<td>553066</td>
</tr>
<tr>
<td>CD4</td>
<td>FITC</td>
<td>GK1.5</td>
<td>rat IgG2a</td>
<td>1:100</td>
<td>BD</td>
<td>553046</td>
</tr>
<tr>
<td>CD4</td>
<td>PECy5</td>
<td>H129.19</td>
<td>rat IgG2a</td>
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<td>BD</td>
<td>553654</td>
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<tr>
<td>CD8</td>
<td>PE</td>
<td>53-6.7</td>
<td>rat IgG2a, κ</td>
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<td>553033</td>
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<tr>
<td>Antibody</td>
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<td>Isotype</td>
<td>Species</td>
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<td>Catalog Number</td>
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<td>---------</td>
<td>----------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>CD11b FITC</td>
<td>M1/70</td>
<td>rat IgG2b</td>
<td>1:100</td>
<td>BD</td>
<td>557396</td>
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</tr>
<tr>
<td>CD11b APC</td>
<td>M1/70</td>
<td>rat IgG2b</td>
<td>1:100</td>
<td>BD</td>
<td>553312</td>
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<tr>
<td>CD11c FITC</td>
<td>HL3</td>
<td>hamster IgG1</td>
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<tr>
<td>CD25 FITC</td>
<td>7D4</td>
<td>rat IgM</td>
<td>1:100</td>
<td>BD</td>
<td>553071</td>
<td></td>
</tr>
<tr>
<td>CD25 PE</td>
<td>PC61</td>
<td>rat IgG1</td>
<td>1:100</td>
<td>BD</td>
<td>553866</td>
<td></td>
</tr>
<tr>
<td>CD45.1 FITC</td>
<td>A20</td>
<td>mouse IgG2a</td>
<td>1:1000</td>
<td>BD</td>
<td>553775</td>
<td></td>
</tr>
<tr>
<td>CD45.1 PE</td>
<td>A20</td>
<td>mouse IgG2a</td>
<td>1:100</td>
<td>BD</td>
<td>553776</td>
<td></td>
</tr>
<tr>
<td>CD80 (B7-1) PE</td>
<td>16-10A1</td>
<td>rat IgG2a, κ</td>
<td>1:100</td>
<td>BD</td>
<td>553692</td>
<td></td>
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<tr>
<td>CD86 (B7-2) PE</td>
<td>GL1</td>
<td>rat IgG2a, κ</td>
<td>1:100</td>
<td>BD</td>
<td>553692</td>
<td></td>
</tr>
<tr>
<td>CD95 FITC</td>
<td>Jo2</td>
<td>armenian hamster IgG2, λ</td>
<td>1:20</td>
<td>BD</td>
<td>554257</td>
<td></td>
</tr>
<tr>
<td>CD119 (IFN-γR, a chain)</td>
<td>biotin</td>
<td>GR20</td>
<td>rat IgG1</td>
<td>1:100</td>
<td>BD</td>
<td>558771</td>
</tr>
<tr>
<td>CD273 (B7-DC) PE</td>
<td>TY25</td>
<td>rat IgG2a, κ</td>
<td>1:100</td>
<td>BD</td>
<td>557796</td>
<td></td>
</tr>
<tr>
<td>CD274 (B7-H1) PE</td>
<td>MIH5</td>
<td>rat IgG2, λ</td>
<td>1:100</td>
<td>BD</td>
<td>558091</td>
<td></td>
</tr>
<tr>
<td>CD275 (B7-H2) PE</td>
<td>HK5.3</td>
<td>rat IgG2a, κ</td>
<td>1:100</td>
<td>eBioscience</td>
<td>12-5985</td>
<td></td>
</tr>
<tr>
<td>CD276 (B7-H3) PE</td>
<td>M3.2D7</td>
<td>rat IgG2a</td>
<td>1:100</td>
<td>eBioscience</td>
<td>12-5973</td>
<td></td>
</tr>
<tr>
<td>F4/80 Alexa488</td>
<td>BM8</td>
<td>rat IgG2a</td>
<td>1:100</td>
<td>Caltag</td>
<td>MF48020</td>
<td></td>
</tr>
<tr>
<td>Foxp3 PE</td>
<td>FJK-16s</td>
<td>rat IgG2a</td>
<td>1:100</td>
<td>eBioscience</td>
<td>12-5773</td>
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<tr>
<td>Gr-1 APC</td>
<td>RB6-8C5</td>
<td>rat IgG2a</td>
<td>1:100</td>
<td>BD</td>
<td>553129</td>
<td></td>
</tr>
<tr>
<td>H-2Kb PE</td>
<td>AF6-88.5</td>
<td>mouse IgG2a, κ</td>
<td>1:100</td>
<td>BD</td>
<td>553570</td>
<td></td>
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<tr>
<td>I-A^B PE</td>
<td>AF6-120.1</td>
<td>mouse IgG2a</td>
<td>1:200</td>
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<td>553552</td>
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<tr>
<td>IFN-γ FITC</td>
<td>XMG1.2</td>
<td>rat IgG1</td>
<td>1:50</td>
<td>BD</td>
<td>554411</td>
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<tr>
<td>Ly-6C/G (Gr-1) APC</td>
<td>RB6-8C5</td>
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<td>BD</td>
<td>553129</td>
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NK1.1 PE PK136 mouse IgG2a 1:1500 BD 557391

<table>
<thead>
<tr>
<th>Isotypes</th>
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<tr>
<td>Isotype</td>
<td>FITC</td>
<td>R3-34</td>
<td>rat IgG1</td>
<td>BD 554684</td>
</tr>
<tr>
<td>Isotype</td>
<td>PE</td>
<td>MOPC-21</td>
<td>mouse IgG1</td>
<td>BD 559320</td>
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<tr>
<td>Isotype</td>
<td>PE</td>
<td>R35-95</td>
<td>rat IgG2a</td>
<td>BD 553930</td>
</tr>
<tr>
<td>Isotype</td>
<td>APC</td>
<td>A95-1</td>
<td>rat IgG2b</td>
<td>BD 553991</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>APC</td>
<td></td>
<td>1:1000</td>
<td>BD 554067</td>
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5.1.6.2 Antibodies for in vitro activation

The following antibody against mouse CD3 was used in vitro for the activation of T cells:

<table>
<thead>
<tr>
<th>Ab specificity</th>
<th>Clone</th>
<th>Isotype</th>
<th>Source</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>145-2C11</td>
<td>armenian hamster IgG1, κ</td>
<td>BD</td>
<td>553057</td>
</tr>
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</table>

5.1.6.3 In vivo administered antibodies

The following antibodies against mouse antigens were injected i.p. in mice for the depletion of cell populations or the neutralization of cytokines (listed in alphabetical order):

<table>
<thead>
<tr>
<th>Ab specificity</th>
<th>Clone</th>
<th>Isotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>GK1.5</td>
<td>rat IgG2a</td>
<td>Purification from hybridoma</td>
</tr>
<tr>
<td>CD8</td>
<td>2.43</td>
<td>rat IgG2b</td>
<td>Bioexpress</td>
</tr>
<tr>
<td>CD25</td>
<td>PC61</td>
<td>rat IgG1</td>
<td>Purification from hybridoma</td>
</tr>
<tr>
<td>CD95L</td>
<td>MFL 4</td>
<td>armenian hamster IgG</td>
<td>Kindly provided by Prof. Yagita, Juntendo University School of Medicine, Kyoto, Japan</td>
</tr>
<tr>
<td>IFN-β</td>
<td>7FD3</td>
<td>rat IgG1</td>
<td>Purification from hybridoma</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>XMG1.2</td>
<td>rat IgG1</td>
<td>Bioexpress</td>
</tr>
<tr>
<td>IL-10R</td>
<td>YL03.1B1.3a</td>
<td>rat IgG1</td>
<td>DNAX</td>
</tr>
<tr>
<td>NK1.1</td>
<td>PK136</td>
<td>mouse IgG2a</td>
<td>Bioexpress</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1D11</td>
<td>mouse IgG1</td>
<td>Bioexpress</td>
</tr>
</tbody>
</table>
5.1.7 Cell lines

The cell lines that were used in this study are listed below (in alphabetical order):

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Medium*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4G2</td>
<td>anti-CD16/CD32 hybridoma</td>
<td>RPMI 1640</td>
<td>166</td>
</tr>
<tr>
<td>7FD3</td>
<td>anti-IFN-β hybridoma</td>
<td>RPMI 1640</td>
<td>Kindly provided by Dr. Rainer Zawatzky, DKFZ</td>
</tr>
<tr>
<td>EC7.1</td>
<td>MHC class I-deficient variant of RMA-S</td>
<td>IMDM</td>
<td>267</td>
</tr>
<tr>
<td>Jurkat</td>
<td>human T cell leukemia</td>
<td>RPMI 1640</td>
<td>268</td>
</tr>
<tr>
<td>L929</td>
<td>mouse fibroblast</td>
<td>DMEM</td>
<td>269</td>
</tr>
<tr>
<td>PC61</td>
<td>anti-CD25 hybridoma</td>
<td>RPMI 1640</td>
<td>270</td>
</tr>
<tr>
<td>RMA</td>
<td>mouse T cell lymphoma</td>
<td>RPMI 1640</td>
<td>271</td>
</tr>
<tr>
<td>RMA-S</td>
<td>TAP2-deficient variant of RMA</td>
<td>RPMI 1640</td>
<td>271</td>
</tr>
<tr>
<td>RMA-RAE-1γ</td>
<td>RAE-1γ transfectant of RMA</td>
<td>RPMI 1640</td>
<td>69</td>
</tr>
<tr>
<td>YAC-1</td>
<td>mouse lymphoma</td>
<td>RPMI 1640</td>
<td>9</td>
</tr>
</tbody>
</table>

*All media were supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin with the exception of PC61 which was supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, 1 mM sodium pyruvate and 0.25 mM β-mercaptoethanol.

Cell lines were regularly checked for mycoplasma contamination using PCR based assay.

5.1.8 Magnetic Cell Sorting (MACS) beads and columns

<table>
<thead>
<tr>
<th>Product</th>
<th>Source</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-CD4 beads</td>
<td>Miltenyi Biotec</td>
<td>130-049-201</td>
</tr>
<tr>
<td>anti-CD8 beads</td>
<td>Miltenyi Biotec</td>
<td>130-049-401</td>
</tr>
<tr>
<td>anti-CD11b beads</td>
<td>Miltenyi Biotec</td>
<td>130-049-601</td>
</tr>
<tr>
<td>anti-CD90 beads</td>
<td>Miltenyi Biotec</td>
<td>130-049-101</td>
</tr>
</tbody>
</table>
 anti-DX5 beads | Miltenyi Biotec | 130-052-501
---|---|---
 Regulatory T cell Isolation kit | Miltenyi Biotec | 130-091-041
---|---|---
 anti-APC beads | Miltenyi Biotec | 130-090-855
---|---|---
 MS Columns | Miltenyi Biotec | 130-042-201
---|---|---
 LS Columns | Miltenyi Biotec | 130-042-401
---|---|---
 LD Columns | Miltenyi Biotec | 130-042-901

### 5.1.9 Kits

<table>
<thead>
<tr>
<th>Product</th>
<th>Source</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE anti-mouse/rat Foxp3 Staining Set</td>
<td>eBioscience</td>
<td>72-5775</td>
</tr>
<tr>
<td>Mouse IFN-γ OptEIA™ Set</td>
<td>BD</td>
<td>555138</td>
</tr>
<tr>
<td>OptEIA™ Reagent Set B</td>
<td>BD</td>
<td>550534</td>
</tr>
<tr>
<td>Mouse IFN-γ in vivo capture assay</td>
<td>BD</td>
<td>558491</td>
</tr>
<tr>
<td>Bio-plex cytokine reagent kit</td>
<td>Biorad</td>
<td>171-304000</td>
</tr>
<tr>
<td>Bio-plex cell lysis kit</td>
<td>Biorad</td>
<td>171-304011</td>
</tr>
<tr>
<td>Bio-Plex mouse serum diluent kit</td>
<td>Biorad</td>
<td>171-305005</td>
</tr>
<tr>
<td>Bio-plex mouse IFN-γ assay</td>
<td>Biorad</td>
<td>171-G11934</td>
</tr>
<tr>
<td>Bio-plex mouse IL-1β assay</td>
<td>Biorad</td>
<td>171-G12819</td>
</tr>
<tr>
<td>Bio-plex mouse IL-6 assay</td>
<td>Biorad</td>
<td>171-G10738</td>
</tr>
<tr>
<td>Bio-plex mouse IL-10 assay</td>
<td>Biorad</td>
<td>171-G11356</td>
</tr>
<tr>
<td>Bio-plex mouse IL-13 assay</td>
<td>Biorad</td>
<td>X60-000ZFYB</td>
</tr>
<tr>
<td>Bio-plex mouse IL-12 (p70) assay</td>
<td>Biorad</td>
<td>171-G13758</td>
</tr>
<tr>
<td>Bio-plex mouse MIP-1α assay</td>
<td>Biorad</td>
<td>171-G16477</td>
</tr>
<tr>
<td>Bio-plex mouse MIP-1β assay</td>
<td>Biorad</td>
<td>X60-005KMTI</td>
</tr>
<tr>
<td>Bio-plex mouse MIP-2 assay</td>
<td>Biorad</td>
<td>XD0-000001T</td>
</tr>
<tr>
<td>Bio-plex mouse VEGF assay</td>
<td>Biorad</td>
<td>XD0-000007B</td>
</tr>
<tr>
<td>BCA™ Protein Assay kit</td>
<td>Pierce</td>
<td>23227</td>
</tr>
</tbody>
</table>
5.2 MICE

C57BL/6 wild-type (wt) and C57BL/6-Ly5.1 congenic mice were purchased from Charles River Laboratories (Sulzfeld, Germany and Erembodegem, Belgium). C57BL/6-Ly5.1, IFNAR1-/- and RAG2-/-IFNAR1-/- mice were bred in our animal facility. IFN-γ receptor-/- (IFN-γR-/-), perforin-/- (Prf-/-) and inducible Nitric Oxide Synthase-/- (iNOS-/-) C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Mice were housed in specific pathogen-free conditions and used in experiments at 6-8 wk of age. All experiments were performed according to local animal experimental ethics committee guidelines.

5.3 METHODS

5.3.1 Cell culture methods

5.3.1.1 Determination of cell number

An aliquot of cell suspension was diluted 1:1 with trypan blue solution (0.05 % w/v) to distinguish dead cells and cells were counted with a Neubauer counting chamber (0.1 mm depth). The number of live cells per ml is calculated as following:

\[
\text{average cell number} / \text{chamber square (0.1 mm}^3) \times \text{dilution factor} \times 10^4
\]

5.3.1.2 Freezing and thawing of cells

For freezing of cells, cell suspensions were centrifuged (1200 rpm, 10 min, RT) and the pellet was resuspended in freezing medium at a concentration of 5 x 10⁶ cells/ml. 2 ml aliquots in freezing tubes were placed in ice-cooled freezing containers and placed for 24 h at -80°C. Frozen cells were subsequently stored in liquid nitrogen.

Cell thawing was performed quickly in a 37°C water bath until ~ 10% of suspension remained frozen. The suspension was immediately diluted into 9 ml of appropriate cold medium and
centrifuged (1200rpm, 10 min, RT). Cells were resuspended in medium and cultured at 37°C, 5% CO₂.

5.3.1.3 Splitting of adherent cells
For the splitting of adherent cells, the culture medium was removed from the flasks, the cells were washed once with pre-warmed PBS, and Trypsin-EDTA or Cell Dissociation Solution was added in sufficient amount to cover the cell layer. Cells were then incubated at 37°C for ~5 min, checking in parallel the progress of detachment under the microscope. After complete detachment, culture medium was added to the flask, cells were collected in Falcon tubes and centrifuged (1200 rpm, 10 min, RT). Cells were subsequently split at the appropriate ratio into new flask or used in experiments.

5.3.1.4 Splitting of suspension cells
Cells in suspension reaching an optimal density were split in the appropriate ratio, ranging between 1:5 up to 1:20 by adding the appropriate volume of medium.

5.3.2 Mouse tumor model
For the analysis of tumor growth in vivo, tumor cell lines were harvested in the exponential growth phase. Tumor cells were washed 3 times with PBS and resuspended in PBS at a concentration of 1 x 10⁷ cells/ml for RMA or RMA-S cells or 1 x 10⁶ cells/ml, primary to injection. Groups of 5-10 mice were injected s.c. in the shaved left flank with 100 µl of tumor cell suspension. Tumor growth was assessed 3 times weekly with a caliper measuring along the perpendicular axes of the tumors and expressed as the product of the two diameters. For rechallenge experiments, mice were rested for at least 3 months after primary tumor cell inoculation before secondary challenge.
5.3.2.1 Isolation of organs and preparation of single cell suspensions

5.3.2.1.1 Blood

For blood isolation, animals were sacrificed using CO₂; mouse blood was obtained by puncture of the orbital vein of mice. For FACS staining, 30 µl of heparin were added to 1 ml of blood. For the detection of IFN-γ in the serum or adoptive transfer memory experiments, blood was coagulated in 1.5 ml tubes for 1 h at 4°C. The serum was separated by centrifugation (1500 rpm, 10 min, 4°C).

5.3.2.1.2 Spleen and LN

Animals were sacrificed by dislocation of the neck; spleen and peripheral LN were excised using sterile forceps and scissors and kept in ice-cold PBS + 5% FCS medium. Single cell suspensions were obtained by mincing the spleen or LN through a 40 μm cell strainer. For the lysis of erythrocytes, splenocytes were treated with buffered ammonium chloride potassium phosphate solution (ACK-buffer) for 1 min at RT and then washed with PBS + 5% FCS (1400 rpm, 10 min, 4°C). Cells were resuspended in PBS + 5% FCS.

5.3.2.1.3 Tumor infiltrating leukocytes

For the preparation of single cell suspensions from tumors, tumors were excised using sterile forceps, cut into small pieces with a scalpel and digested with 5 mg/ml collagenase type IV and 0.5 mg/ml DNase I for 15 min at 37°C. At the end of the incubation, the digested tumor was smashed through a 70 μm-pore cell strainer, cells were collected in a 50 ml Falcon tube and washed once with PBS + 10% FCS (1400 rpm, 10 min, 4°C).

5.3.2.1.4 Bone marrow (BM) cells

Animals were sacrificed by dislocation of the neck; hind legs were dissected and bones were freed from all sinews and muscle tissue using sterile forceps and scissors. The femur and tibiae were separated by breaking the knee and the heel, washed briefly in 80 % ethanol and placed in ice-cold PBS. To rinse out the BM cells, the ends of femur and tibiae were cut and 5-10 ml of
ice-cold PBS were forced through the bone cavity with a 27G needle. The isolated BM cells were washed three times with PBS before *i.v.* injection into the mice.

### 5.3.2.2 *In vivo* depletion and neutralization experiments

For the *in vivo* depletion of Treg, 300 µg of anti-CD25 mAb were injected *i.p.* 2 days before tumor cell inoculation. The depletion efficiency was >95% in blood after 2 days. In some experiments, mice were additionally depleted of NK1.1<sup>+</sup> cells or CD4<sup>+</sup> T cells by *i.p.* injection of 200 µg anti-NK1.1 or anti-CD4 mAbs, respectively, on days -2, +2, +9, and +16. At day 10 after tumor cell inoculation and at the endpoint, the efficiency of these depletions was >90% in blood, spleen and tumor tissue, as assessed by flow cytometric analysis. For the *in vivo* neutralization of IFN-γ, 300 µg of anti-mouse IFN-γ mAb were injected *i.p.* three times weekly beginning at the day of tumor cell inoculation. For the *in vivo* neutralization of CD95L, 250 µg of anti-mouse CD95L mAb were injected *i.p.* every 3 days beginning from day -1 before tumor inoculation. The anti-apoptotic activity of the anti-CD95L mAb was confirmed *in vitro* by the inhibition of T cell activation-induced cell death, which is known to be mediated via the CD95-CD95L pathway (data not shown). For the *in vivo* neutralization of TGF-β, 2 mg of anti-TGF-β mAb were administered *i.p.* 2 times weekly, starting from day -2 before tumor inoculation. For the *in vivo* blocking of IL-10R, 1 mg of anti-IL-10R mAb was injected *i.p.* once weekly beginning from day -2 before tumor inoculation.

### 5.3.2.3 Bone marrow transplantation

Wt, IFNAR1<sup>-/-</sup> or RAG2<sup>-/-</sup>/IFNAR1<sup>-/-</sup> mice were irradiated with two doses of 450 rad with a 3h interval in between. After 4 hours of rest, irradiated mice were injected with 4 x 10<sup>6</sup> bone marrow cells in 200 µl PBS collected from donor wt or IFNAR1<sup>-/-</sup> mice. Mice were maintained on amoxycilin antibiotic diluted in drinking water for 6 weeks after reconstitution. At 6 weeks after injection, chimeras were analyzed for the reconstitution of immune cell populations by flow cytometry of peripheral blood cells after staining with antibodies to CD45.1 and CD45.2.
5.3.2.4 Memory experiments

5.3.2.4.1 In vivo memory experiments

Mice which rejected RMA-S tumors after anti-CD25 treatment were rested for at least 3 months after primary tumor cell inoculation. The reconstitution of the Treg compartment at this time was confirmed in the blood of mice by flow cytometric analysis for CD4 and CD25 markers. For rechallenge experiments, mice were injected s.c. in the left flank with $1 \times 10^6$ RMA-S or RMA cells and tumor growth was monitored. In some experiments, mice were depleted of CD4$^+$ or CD8$^+$ T cells or NK1.1$^+$ cells in combination either with CD4$^+$ or CD8$^+$ T cell depletion. Naïve mice injected for the first time with tumor cells were used as a control.

5.3.2.4.2 Adoptive transfer memory experiments

In adoptive transfer experiments, CD4$^+$ and CD8$^+$ T cells were isolated by magnetic separation from the spleens and LN of naïve mice or mice which had previously rejected RMA-S tumors and washed 3 times with PBS. The blood serum was also prepared from the same mice. $1 \times 10^7$ purified CD4$^+$ T cells or $6 \times 10^7$ CD8$^+$ T cells or 250 µl of blood serum were injected i.v. to naïve C57BL/6 hosts, one day before s.c. RMA-S tumor cell inoculation.

5.3.3 Antibody purification

5.3.3.1 Purification of the anti-CD25 mAb

For the purification of the anti-CD25 mAb, PC61 hybridoma cell line was expanded until confluence. Cell cultures were centrifuged (3500 rpm, 7 min, 4°C) and S/N was collected. Total protein was precipitated with ammonium sulfate added at slow rate to the S/N until saturation. The S/N was shaken on a magnetic stirrer for ~1h at RT. After centrifugation (7500 rpm, 30 min, 4°C), pellets were resuspended in 100 ml ddH$_2$O and dialyzed O/N at 4°C in 1x PBS. The dialyzed mAb was sterile filtered and purified by binding on a protein G column with the means of a peristaltic pump, pre-washed once with ethanol, and once with the binding buffer. The mAb solution was passed through the column twice at a flow rate 4 ml/min. The bound mAb was eluted from the column with 30-50 ml elution buffer and fractions were collected in 15 ml falcon tubes containing 200 µl neutralization buffer. Additional neutralization buffer was added to
adjust the pH to 7. Optical density (O.D.) was measured at 260 nm; the fractions which had an O.D. over 0.6 were pooled. The pooled mAb was dialyzed O/N in 1x PBS and sterile filtered. Protein concentration was analyzed with the BCA kit and purity with a SDS-gel. Aliquots of the mAb were kept at -20°C. The protein G column was washed with ethanol and stored at 4°C.

5.3.3.2 Purification of the anti-IFN-β mAb
For the purification of the anti-IFN-β mAb, the 7FD3 hybridoma cell line was expanded until confluence. FCS was excluded from the cell culture medium during the last step of culture. Cell cultures were centrifuged (3500 rpm, 7 min, 4°C) and S/N was collected. Total protein was precipitated with ammonium sulfate added at slow rate to the S/N until 50% saturation. The S/N was shaken on a magnetic stirrer for ~1h at RT. After centrifugation (7500rpm, 30min, 4°C), pellets were resuspended in ddH₂O in a volume corresponding to 0.5% of the starting volume and dialyzed O/N at 4°C in 1x PBS. The dialyzed mAb was sterile filtered. Protein concentration was analyzed with the BCA kit and purity with a SDS-gel. Aliquots of the mAb were kept at 4°C.

5.3.4 Fluorescence-activated cell sorting (FACS)

5.3.4.1 FACS staining and analysis
For staining of cells, 0.5 – 1 x 10⁶ cells were washed once with FACS buffer (1200 rpm, 10 min, 4°C for cell lines or 1400 rpm, 10 min, 4°C for primary mouse cells) and incubated with 10% Fc block (2.4G2 supernatant) for 10 min on ice to block Fc receptors. Subsequently, the primary antibodies were added and cells were further incubated for 30 min at 4°C. For biotinylated antibodies, cells were washed once with FACS buffer and stained with conjugated streptavidin for additional 30 min at 4°C. After staining, cells were washed once, fluorescence was assessed by a FACSCalibur and data were analyzed with the CellQuest software. Peripheral blood leucocytes were stained by adding the primary antibodies directly into the blood samples. At the end of the incubation, erythrocytes were lysed with FACS Lysis Buffer for 1 min at RT, followed by washing with FACS buffer. Streptavidin was added at a second step when necessary. Working concentrations of antibodies are denoted under chapter 5.1.6.1.
5.3.4.2  FACS sorting

For FACS sorting, up to $1 \times 10^8$ total tumor cells were washed once with PBS + 0.1% FCS (1400 rpm, 10 min, 4°C,) and incubated with 10% Fc block (2.4G2 supernatant) for 10min on ice to block Fc receptors. Subsequently, the primary antibodies were added and cells were further incubated for 30 min at 4°C. After staining, cells were washed once with PBS + 0.1% FCS, resuspended in PBS + 1% FCS and filtered through a cell strainer to remove cell clusters. Cell sorting was performed with FACSDiva and FACSVantage.

5.3.5  MACS sorting

In some cases, primary cell subpopulations were sorted with magnetic beads, according to the manufacturer’s protocol.

5.3.6  Determination of cytokines

5.3.6.1  Intracellular FACS staining

For the detection of IFN-γ producing cells, single cell suspensions were prepared from tumor tissue of PBS and anti-CD25 treated mice. Cells were stained for extracellular markers and subsequently fixed with 50 µl of 4% PFA for 10 min at RT. Cells were washed once (1400 rpm, 10 min, 4°C,) with cold FACS buffer and permeabilized with 100 µl of 0.5% saponin for 10 min at RT. 10% Fc block and 10 µg/ml rat IgG were added for additional 10 min to block unspecific binding. Cells were subsequently washed once (1400 rpm, 10 min, 4°C) with cold FACS buffer containing 0.5% saponin. Anti-IFN-γ or the appropriate isotype control was added and cells were incubated for 30 min at 4°C. Cells were washed once (1400 rpm, 10 min, 4°C) with cold FACS buffer containing 0.5% saponin and once (1400 rpm, 10 min, 4°C) with cold FACS buffer, before analyzed with the CellQuest software on a FACSCalibur.

5.3.6.2  Enzyme-Linked ImmunoSorbent Assay (ELISA)

CD8$^+$ T cells were purified from spleens and LN by magnetic cell sorting or from tumor tissue of tumor bearing mice by FACS sorting. $1 \times 10^5$ CD8$^+$ T cells were cultured either alone or in the presence of RMA-S or EC7.1 cells at a ratio 1:1 for 18h. At the end of the incubation, S/N were
collected and stored at -20°C until used. IFN-γ levels were determined by ELISA for murine IFN-γ, according to the manufacturer’s instructions.

### 5.3.6.3 In vivo IFN-γ capture assay

For the determination of IFN-γ in the serum of naïve or tumor bearing mice treated with PBS or anti-CD25 mAb, biotinylated anti-IFN-γ mAb was injected i.v. into the mice on days 0, 3, 5 and 9 after RMA-S tumor cell inoculation and blood samples were collected after 24 or 48 h. Blood serum was prepared and the levels of IFN-γ were determined according to the manufacturer’s instructions.

### 5.3.6.4 Bio-plex protein array assay for cytokine measurement

For the determination of cytokine and chemokine production by tumor infiltrating macrophages, CD11b^+F4/80^+ cells were isolated from tumors of control and Treg depleted mice on day 10 after RMA-S tumor cell inoculation by flow cytometric sorting (purity > 95%). 1 x 10^5 macrophages were cultured in 200µl primary cell culture medium in a U-bottom 96-well plate and after 12 or 24h supernatants were collected. For the determination of cytokine and chemokine production in tumor tissue, lysates were prepared from total tumor tissue in the presence of PMSF as protease inhibitor. Total protein concentration was calculated with the BCA kit and the concentration of all lysate samples was adjusted optimally to 300 µg/ml with the Bio-plex lysis buffer. All supernatants and lysates were further diluted 1:1 with mouse serum before the assay. Cytokine and chemokine release was determined with the Bio-plex Protein Array system, according to the manufacturer’s instructions. The sensitivity of the assay for IFN-γ detection was 2 pg/ml, for MIP-1β 1.5 pg/ml, for MIP-2 3 pg/ml, for IL-6 1 pg/ml, for IL-13 3 pg/ml and for IL-1β 1.6 pg/ml.

### 5.3.7 Proliferation and suppression assays

#### 5.3.7.1 Treg suppression assay

CD4^+ T cells were isolated from mouse splenocytes based on a negative magnetic selection procedure; Treg (CD4^+CD25^+ T cells) and Tcon (CD4^+CD25^- T cells) were subsequently selected
from the pure, untouched CD4$^+$ T cells, based on CD25 magnetic separation. Both populations had a purity of ~90%. To document the suppressive activity of Treg, 1 x 10$^5$ Tcon were incubated in U bottom 96-well plates in 200 µl of primary cell culture medium alone or in co-culture with titrating cell numbers of Treg in the following Treg : Tcon ratios: 1:1, 1:2, 1:4, 1:8, 1:16. For the stimulation of proliferation of T cells, 0.5 µg/ml mouse anti-CD3 mAb was added to the culture medium and 1 x 10$^5$ irradiated (30 Gray) CD90-depleted splenocytes served as APC. After 4 days of culture at 37°C in 5% CO$_2$, 1 µCi $^3$H-thymidine, diluted 1:10 in RPMI medium, per well was added for additional 16 h. To control non-specific inhibition due to crowding in the well, 2x Tcon were used as a control. Proliferation was measured using a scintillation counter.

5.3.7.2 Tumor cell growth inhibition assay

CD11b$^+$ cells were isolated with magnetic separation (purity ~50%) or macrophages were purified with FACS sorting (purity > 95% CD11b$^+$F4/80$^+$) from tumors on d10 after tumor cell inoculation. The purified cell populations were cultured in 96-well plates with 1 x 10$^4$ RMA-S cells at the following ratios: CD11b$^+$ cells : RMA-S cells = 5:1, 10:1, 20:1 and 40:1 – depending on the assay – for 24 or 48 h in a U-bottom 96-well plate in 200 µl of primary cell culture medium. RMA-S cells were in parallel cultured in the absence of CD11b$^+$ cells. In some experiments, CD11b$^+$ cells were separated from RMA-S cells by transwell inserts. In some experiments, inhibitors were used to block the activity of iNOS, PGE2 and IDO: 0.5 mM L-NMMA and its stereoisomer D-NMMA as a control, 10µM indomethacin and EtOH diluent control, and 100µM 1MT and HCl diluent control, respectively. Proliferation was assessed by adding 1µCi of $^3$H-thymidine, diluted 1:10 in RPMI medium, to each well, 6h before harvesting. At the end of the incubation, the cells were harvested on filter papers, signal was enhanced by the addition of a scintillator and the radioactivity was quantified by a beta counter. In cases where the plates could not be harvested immediately after the end of the incubation, the plates were placed in -20°C until analyzed for $^3$H-thymidine incorporation. Before harvesting, plates were thawed at 37°C.
5.3.7.3 Documentation of proliferation by CFSE dilution

A total of $1 \times 10^7$ RMA-S cells diluted in 1 ml PBS + 5% FCS were labeled with 1 µM CFSE, which was added to the cells while vortexing. After 10 min incubation at RT on shaker, the cells were washed with PBS + 5% FCS three times (1200 rpm, 10 min, 4°C). During the procedure, the cells were protected from the light. $1 \times 10^4$ CFSE-labeled RMA-S cells were cultured alone or in the presence of CD11b+ cells at a ratio of CD11b+ cells : RMA-S cells = 40 :1 for 48 h in a U-bottom 96-well plate in 200 µl of primary cell culture medium. At the end of the incubation, cells were harvested from the plate, washed once with FACS buffer (1400 rpm, 10 min, 4°C) and fluorescence was analyzed on a FACSCalibur. Dilution of the CFSE dye corresponded to proliferation of the cells.

5.3.8 Killing assays

5.3.8.1 $^{51}$Cr release cytotoxicity assay

RMA-S or YAC-1 tumor cells were labeled with 100µCi $^{51}$Cr for 90min and washed three times with RPMI medium. A total of $1 \times 10^3$ $^{51}$Cr-labeled target cells were seeded in each well of a 96-well U-bottom plate. NK cells, purified with DX5+ magnetic cell sorting, were added at different effector to target (E:T) ratios (100:1, 50:1, 25:1, 12.5:1, 6.25:1, 3.125:1, 1.56:1) in a final volume of 200 µl of primary cell culture medium. The plates were centrifuged at 1200 rpm for 3 min and incubated for 6 h at 37°C in 5% CO2. At the end of the incubation, 100 µl of S/N was collected from each well and the radioactivity was counted in a beta counter. The percentage of cytotoxicity in each well was calculated as:

$$\frac{\text{mean cpm} - \text{minimum (spontaneous) mean release}}{\text{maximum release (total) – minimum (spontaneous) mean release}} \times 100$$

Minimum (spontaneous release) corresponds to the amount of radioactivity released by tumor cells cultured in the absence of NK cells. Maximum release relates to the amount of radioactivity released by tumor cells cultured in the presence of 10% Triton X-100.

5.3.8.2 In vitro induction of apoptosis by death ligands

RMA-S cells were cultured at a concentration of $1 \times 10^5$ cells/ml in the absence or presence of 50 U/ml recombinant mouse IFN-γ for 24h. At the end of the incubation, cells were washed (1200
rpm, 10 min, 4°C) twice and re-plated at a concentration of 1 \times 10^6 \text{ cells/ml} in a 6-well plate for 6h in the absence or presence of either 0.1 \mu M \text{ TNF-\alpha} or 1 \mu g/ml \text{ TRAIL}. In parallel, L929 cells were cultured in the absence or presence of TNF-\alpha, and Jurkat cells in the absence or presence of TRAIL, as positive controls to TNF-\alpha- and TRAIL-induced apoptosis \textit{in vitro}. At the end of the incubation, cells were harvested, washed once (1200 rpm, 10 min, 4°C) with FACS buffer and stained with 1\mu l of PI. Dead cells, defined as PI+ cells, were determined by flow cytometric analysis as percentage of total cells.

5.3.9 Visualization of angiogenesis by confocal microscopy

Tumor vasculature was visualized by fluorescent angiography using a FITC-labeled \textit{Lycopersicon esculentum} lectin injected \textit{i.v.} (100 \mu g diluted in 100 \mu l of PBS) in the tail vein of mice. Two min after lectin injection, mice were sacrificed by cervical dislocation. Tumors were harvested, placed on 0.17 mm thick coverslips and examined unfixed under fluorescent confocal microscopy (Nikon C1Si confocal microscope with a 488-nm argon ion laser). Detection of the \textit{Lycopersicon esculentum} lectin was achieved at 503–537 nm. Datasets typically represented 1 \times 1 \times 0.1 \text{ mm} (512 \times 512 \text{ pixels} \times 61 \text{ planes}) with a 1.65 \mu m step interval. The analysis used the entire z-series of all three channels.

5.3.10 Statistical analysis

Differences between groups were calculated using standard Student’s \textit{t} test. Differences in tumor growth between groups of mice were calculated using Koziol test. Values of \textit{p} < 0.05 were considered to be statistically significant.
6 RESULTS

6.1 REGULATORY T CELLS SUPPRESS ANTI-TUMOR IMMUNITY AGAINST A MHC CLASS I DEFICIENT LYMPHOMA

6.1.1 Regulatory T cells suppress IFN-γ dependent leukocyte accumulation in lymphoma

6.1.1.1 Accumulation of Treg in RMA-S tumor-bearing mice

RMA-S lymphoma cell line is a MHC class I-deficient variant of the RBL-5 tumor. According to the original characterization by Kärre and colleagues, RMA-S tumor cells are efficiently controlled by NK cells, when injected into mice at relatively low cell numbers. For our study, we used a higher tumor cell number (1 x 10^6 cells) leading to progressive tumor growth after s.c. injection. In order to investigate the role of Treg in the anti-tumor immunity against a MHC class I-deficient tumor, we examined spleens and tumor tissues from RMA-S tumor-bearing mice for the presence of Treg. Flow cytometric analysis revealed that the percentages of CD4⁺CD25⁺ T cells among CD4⁺ T cells were significantly increased in spleens of tumor-bearing mice (Figure 6.1A). High percentages of CD4⁺CD25⁺ T cells among CD4⁺ T cells were also observed within the tumor infiltrating leukocytes. Since CD25 expression is also upregulated on CD4⁺CD25⁻ effector T cells after activation, we determined the percentages of CD4⁺ T cells expressing the Treg specific transcription factor Foxp3. Our results confirmed the significantly increased percentages of Treg in spleens of tumor-bearing mice and high percentages of Treg in the tumor tissue (Figure 6.1B). Interestingly, CD25 expression was not observed in any cell population other than Treg. CD25 expression correlated with suppressive ability, since CD4⁺CD25⁺ T cells were able to suppress the proliferation of CD4⁺CD25⁻ T cells in an in vitro co-culture in a dose-dependent manner (Figure 6.1C). Our results demonstrate that in the RMA-S tumor model Treg accumulate in the spleen and are present in high percentages within the tumor tissue.
Figure 6.1. Increased numbers of Treg in mice bearing RMA-S tumors. (A-B) C57BL/6 mice were injected s.c. with RMA-S cells. After 21 days spleens and tumors were removed and analyzed for the presence of CD4+CD25+ (A) or CD4+Foxp3+ (B) T cells by flow cytometry. Representative dot plots show CD4 and CD25 staining (A) or CD4 and Foxp3 staining (B) of total splenocytes (left panels). Right panels depict quantification of CD4+CD25+ (A) or CD4+Foxp3+ (B) T cells in spleens of control and tumor-bearing mice as well as in tumor tissue. The percentages of CD25+ cells (A, gate R2) or Foxp3+ cells (B, gate R2) among total CD4+ T cells (gate R1) were calculated. Data show the mean ± SD of eight animals per group and are representative of three experiments. *, p<0.01 using Student’s t test. (C) Tcon (CD4+CD25– T cells) from C57BL/6 naïve mice were stimulated with anti-CD3 mAb and irradiated syngeneic T cell-depleted splenocytes in the presence of varying numbers of Treg (CD4+CD25+ T cells). Cells were cultured for 96 h and proliferation was assessed by ³H-thymidine-incorporation, added for additional 16 h to the culture. To control for nonspecific inhibition due to crowding in the well, 2x Tcon were used as a control. Data show the mean ± SD of triplicate cultures and are representative of three experiments. *, p<0.05, **, p<0.01, ***, p<0.005, using Student’s t test.

6.1.1.2 Depletion of Treg abrogates RMA-S tumor growth

To examine the impact of Treg on the immune response against RMA-S tumor, Treg were depleted by i.p. injection of anti-CD25 mAb 2 days prior to RMA-S tumor cell inoculation. Of note, CD25 expression was not detectable on RMA-S cells cultured in vitro or isolated ex vivo.
from tumor-bearing mice (Figure 6.2). When mice were depleted of Treg, tumor growth initially progressed similar to the control group (Figure 6.3A). However, 10 days after tumor cell injection, tumors started to decrease in size and were eventually rejected in the majority of Treg depleted mice, whereas tumors continued to progressively grow in the PBS-treated control group. Similar results were obtained upon s.c. inoculation of RMA cells transduced with RAE-1, a ligand for the NK cell activating receptor NKG2D \(^{69,70}\) (Figure 6.3B). These results demonstrate that Treg suppress the rejection of NK cell sensitive tumors, like the MHC class I-deficient RMA-S and the RAE-1 expressing RMA tumor cells.

![Figure 6.2. RMA-S cells do not express CD25.](image)

**Figure 6.2. RMA-S cells do not express CD25.** (A-C) Representative histogram plots documenting CD25 expression, as assessed by flow cytometry, on RMA-S cells cultured *in vitro* (A), RMA-S cells isolated from the tumors of RMA-S tumor bearing mice 21 days after *s.c.* tumor cell inoculation (B) and total splenic CD4\(^+\) T cells (C). Staining with the specific antibody is indicated by the black line and the corresponding isotype control by the grey filled area. The data are representative of two experiments.

### 6.1.1.3 NK1.1\(^+\) cells and CD4\(^+\)CD25\(^-\) T cells mediate the rejection of RMA-S tumors in the absence of Treg

In order to investigate whether NK1.1\(^+\) cells were required for the rejection of RMA-S tumors in the absence of Treg, we depleted NK1.1\(^+\) cells alone or in combination with Treg depletion. Depletion of NK1.1\(^+\) cells alone led to accelerated tumor growth in comparison to the PBS-treated control group, emphasizing the importance of this cell population for the control of RMA-S tumor growth (Figure 6.3C, left panel). In the absence of Treg, NK1.1\(^+\) cell depletion initially resulted in an accelerated tumor growth, followed by a decrease in tumor size after day 10 of tumor inoculation (Figure 6.3C, right panel). This remarkable regression in the absence of NK1.1\(^+\) cells, however, did not lead to complete tumor eradication, possibly due to the higher initial tumor load caused by the absence of NK1.1\(^+\) cells. In summary, NK1.1 depletion led to enhanced tumor growth at early time points as depicted for day 7, irrespective of the presence or
Figure 6.3. NK1.1+ cells and CD4+CD25− T cells mediate rejection of RMA-S tumors in the absence of Treg. (A) C57BL/6 mice were treated with PBS (left panel, n=8) or anti-CD25 mAb (right panel, n=6), before s.c. inoculation with RMA-S cells and tumor growth was monitored. (B) C57BL/6 mice were treated with PBS (left panel, n=10) or anti-CD25 mAb (right panel, n=10), before s.c. inoculation with RMA-RAE-1γ cells and tumor growth was monitored. (C) C57BL/6 mice were treated with anti-NK1.1 (left panel, n=8) or anti-CD25 and anti-NK1.1 mAb (right panel, n=7), inoculated s.c. with RMA-S cells and tumor growth was monitored. (D) Bar graphs showing two representative time points of tumor growth as described in panels A and C. (E) Groups of 9 C57BL/6 mice were treated with PBS or anti-CD4 mAb, inoculated s.c. with RMA-S cells and tumor growth was monitored. Two representative time points of tumor growth are depicted in bar graphs. (F) C57BL/6 RAG2− mice were injected i.v. with PBS (left panel, n=7) or 2x10⁶ CD4+ T cells (middle panel, n=7) or 2x10⁶ CD4+CD25− T cells (right panel, n=5), 8 days before s.c. inoculation with RMA-S cells, and tumor growth was monitored. (D-E) Error bars depict SD of individual mice in the experiment. (A-C and F) Each line represents one single mouse. The results are representative of four (A), three (C) and two (B, E and F) experiments. *, p<0.005, **, p<0.0005 using Student’s t test.
absence of Treg (Figure 6.3D, left panel), whereas Treg depletion had a strong impact on day 19 representing a later time point of tumor growth (Figure 6.3D, right panel). These data suggest that the initial phase of RMA-S tumor growth is controlled by NK1.1+ cells regardless of the presence of Treg.

In several tumor models, depletion of the whole CD4+ T cell compartment, comprising Treg, conventional CD4+ T cells and a subset of NKT cells, had a similar impact on tumor growth as compared to Treg depletion alone. Interestingly, in our model, depletion of the whole CD4+ T cell compartment led to progressive tumor growth, which was even enhanced compared to the control group at a later time point, as depicted for day 14 (Figure 6.3E). Therefore, in the absence of Treg, conventional CD4+CD25+ T cells are important for the rejection of RMA-S tumors. The importance of CD4+CD25+ T cells in the anti-tumor response against RMA-S cells was analyzed in RAG2-/- mice. Total CD4+ T cells including Treg, or CD4+CD25+ conventional T cells, isolated from naïve wt mice, were adoptively transferred into RAG2-/- recipients before s.c. inoculation of RMA-S tumor cells. Tumor growth in these mice was compared with tumor growth in RAG2-/- mice which did not receive any cells (PBS group). Adoptive transfer of the whole CD4+ T cell compartment hardly affected RMA-S tumor growth when compared to mice which did not receive any cells (Figure 6.3F, left and middle panel). Most importantly, mice which received CD4+CD25+ T cells exhibited reduced tumor growth compared to mice which received the whole CD4+ T cell compartment (Figure 6.3F, middle and right panel). This observation implies that Treg suppress CD4+CD25+ T cells during the anti-tumor response against RMA-S cells.

In conclusion, in the absence of Treg, NK1.1+ cells and CD4+ T cells are required for efficient rejection of high numbers of RMA-S tumor cells.

6.1.1.4 Depletion of Treg leads to the generation of anti-tumor memory responses against RMA-S tumor cells

In a further step, we determined whether RMA-S tumor rejection in the absence of Treg also leads to the generation of immunological memory. Mice, which had rejected RMA-S tumors in the absence of Treg, were rechallenged with RMA-S tumor cells after being rested for 3 months. Mice, inoculated with tumor cells for the first time, were used as controls. In mice depleted of CD25+ T cells during the primary response, the Treg compartment had completely recovered on
Figure 6.4. **Protective memory is generated in the absence of Treg.** (A-F) C57BL/6 mice were treated with anti-CD25 mAb before s.c. inoculation with RMA-S cells, rejected RMA-S tumors and rested for at least 3 months. These mice are designated as memory mice. (A) Three months after RMA-S tumor cell inoculation, the presence of CD4^+CD25^+ T cells was assessed by flow cytometry in the blood of memory (upper panels) versus naïve (lower panels) mice. Representative dot plots show CD4 and CD25 staining of PBL (left panels) or gated CD4^+ T cells (right panels). The percentages of CD25^+ cells (gate R3) among total CD4^+ T cells (gate R2) were calculated. (B) Naïve C57BL/6 mice (n=24, closed circles) or memory mice (n=17, open circles) were inoculated s.c. with RMA-S cells and tumor growth was monitored. (C) Naïve C57BL/6 mice (n=6) or memory mice (n=4) were inoculated s.c.
with RMA cells and tumor growth was monitored. (D) Memory mice were treated with PBS (n=4), anti-CD4 (n=4) or anti-CD8 (n=4) mAb, as designated, and inoculated s.c. with 1 x 10^6 RMA-S cells. Naïve C57BL/6 mice (n=5) were also inoculated with RMA-S cells and tumor growth was monitored. (E) Memory mice were treated with PBS (n=5) or a combination of anti-CD4/NK1.1 (n=5) or anti-CD8/NK1.1 (n=5) mAb, as designated, and inoculated s.c. with RMA-S cells. Naïve C57BL/6 mice (n=5) were also inoculated with RMA-S cells and tumor growth was monitored. (F) CD4^+ or CD8^+ T cells or blood serum were isolated from memory or naïve mice, as designated, and transferred into naïve recipients before s.c. inoculation with RMA-S cells. Mice which did not receive any cells or serum were used as a control. A representative time point (day 18) of tumor growth is depicted in bar graphs. *, p<0.01 using Student’s t test. The results are representative of four (B), two (A, D and F) and one (C and E) experiments.

the time of rechallenge, as determined by flow cytometric analysis 3 months after the primary challenge (Figure 6.4A). Thus, Treg were present at normal cell numbers at rechallenge. Upon rechallenge, protective immunity against the RMA-S tumor cells was observed in 100% of mice, whereas progressive tumor growth occurred in almost all of the mice that were injected with RMA-S cells for the first time (Figure 6.4B). Mice that had rejected RMA-S tumors after Treg depletion were also protected against the MHC class I-positive RMA tumor cells (Figure 6.4C).

In order to assess the nature of the memory induced in the absence of Treg, mice were depleted of various cell compartments before rechallenge with RMA-S cells. CD4^+ or CD8^+ T cell depletion alone was not sufficient to abrogate the memory response observed upon a secondary challenge with RMA-S cells (Figure 6.4D). Interestingly, simultaneous depletion of NK1.1^+ and CD4^+ cells or NK1.1^+ and CD8^+ cells only partially abrogated the memory response to a secondary challenge of RMA-S cells (Figure 6.4E). To gain further insight into the cell population(s) which mediate(s) memory responses against RMA-S tumor cells, CD4^+ T cells, CD8^+ T cells and blood serum were isolated from mice that had previously rejected RMA-S tumors after Treg depletion and were rested for at least 3 months after tumor cell inoculation; these mice are designated as memory mice from hereon. The cells or serum were subsequently transferred into naïve hosts prior to challenge with RMA-S cells. As shown in Figure 6.4F, transfer of CD4^+ T cells and, more prominently, of CD8^+ T cells isolated from memory mice had a protective effect for the hosts, since it led to reduced tumor growth in comparison to the mice which did not receive any cells or which received CD4^+ T cells or CD8^+ T cells isolated from naïve mice. The role of CD8^+ T cells in RMA-S anti-tumor immunity is discussed in paragraph [4.1.3]. Transfer of serum isolated either from memory or naïve mice did not have any influence in tumor growth, excluding a role for humoral immunity in the RMA-S model. In summary our
results demonstrate that immune responses induced during Treg depletion led to the generation of protective cell-mediated immunological memory.

6.1.1.5 Neutralization of IFN-\(\gamma\) abrogates RMA-S tumor rejection mediated by Treg depletion *in vivo*

IFN-\(\gamma\) was shown to mediate anti-tumor immune responses by several mechanisms \(^2,274\). Therefore, we sought of determining the levels of IFN-\(\gamma\) in the RMA-S model in the presence and absence of Treg. First, we assessed whether depletion of Treg would induce systemically increased levels of IFN-\(\gamma\). Measurement of *in vivo* cytokine production is problematic owing to the rapid utilization, catabolization, and excretion of the cytokines quickly after secretion. We, therefore, used an *in vivo* IFN-\(\gamma\) capture assay, an adaptation of the standard cytokine ELISA \(^275\). This assay facilitates the measurement of cytokines in serum by increasing their *in vivo* half lives up to 1000-fold after *in vivo* administration of a biotinylated neutralizing mAb. Thus, we determined the levels of IFN-\(\gamma\) in the blood serum prepared from control or Treg depleted mice, 3, 5 and 9 days after RMA-S tumor inoculation and compared them with those found in the blood serum of naïve mice. No statistically significant difference in the levels of IFN-\(\gamma\) between control and Treg depleted mice or between naïve and tumor bearing mice was found (Figure 6.5A). These data suggest that during the anti-tumor response against RMA-S cells, no systemic IFN-\(\gamma\) production occurs.

In a second step, we determined IFN-\(\gamma\) levels, locally, in the tumor tissue. Significantly increased levels of this cytokine were detected in RMA-S tumors of Treg depleted mice relative to the PBS group (Figure 6.5B). By intracellular staining, we observed that IFN-\(\gamma\) was predominantly produced by CD8\(^+\) T cells, NK cells and to a lower extent by CD4\(^+\) T cells in the tumor tissue (Figure 6.5C). Most importantly, the percentages of IFN-\(\gamma\)-producing cells were increased in the absence of Treg, suggesting a suppressive effect by Treg. Thus, we studied the contribution of IFN-\(\gamma\) to RMA-S tumor rejection in the absence of Treg. Figure 6.5D shows that, although CD25\(^+\) cell depletion alone led to tumor rejection, combined treatment of mice with anti-CD25 and neutralizing anti-IFN-\(\gamma\) mAb abrogated this effect resulting in tumor growth with similar kinetics as in the control mice. The presence of IFN-\(\gamma\) was important during the first week after tumor inoculation, since neutralization of IFN-\(\gamma\) solely after day 7 of tumor inoculation only
minimally affected tumor growth in the absence of Treg (data not shown). These results demonstrate that Treg suppress IFN-γ production and that IFN-γ is crucial for the rejection of RMA-S tumors in the absence of Treg.

Figure 6.5. IFN-γ is required for RMA-S tumor rejection in the absence of Treg. (A) C57BL/6 mice were treated with PBS or anti-CD25 mAb, as indicated, and were inoculated s.c. with RMA-S cells. Three, 5 and 9 days after tumor cell inoculation, mice were injected i.v. with anti-IFN-γ biotin-conjugated mAb and blood serum was collected 24 or 48h after injections. IFN-γ levels in the serum were subsequently determined with an in vivo capture assay. Naïve C57BL/6 mice which were not inoculated with RMA-S cells were used as a control. The data show the mean ± S.D. of 2-3 mice per group and time point. (B) C57BL/6 mice were treated with PBS or anti-CD25 mAb, as
indicated. 10 days later tumors were removed and IFN-γ levels were determined in total tumor lysates by Bioplex analysis. Data show the mean ± SD of lysates from seven animals per group. *, p<0.05 using Student’s t test. (C) C57BL/6 mice were treated with PBS (n=3) or anti-CD25 mAb (n=3), as indicated. 10 days later tumors were removed, pooled single cell suspensions were prepared, and IFN-γ positive CD4+ T cells, CD8+ T cells or NK cells (NK1.1+CD3-) were determined by intracellular staining. (D) C57BL/6 mice were treated with PBS (left panel, n=8) or anti-CD25 mAb (middle panel, n=8) or anti-CD25 and anti-IFN-γ mAb (right panel, n=5), inoculated s.c. with RMA-S cells and tumor growth was monitored. (E) Representative histogram plot documenting CD119 (IFN-gR, a chain) expression on RMA-S cells, as assessed by flow cytometry. Staining with the specific antibody is indicated by the black line and the corresponding isotype control by the grey filled area. (F) C57BL/6 IFN-γR-/- mice were treated with PBS (n=8) or anti-CD25 mAb (n=8), as indicated, before s.c. inoculation with RMA-S cells, and tumor growth was monitored. (D and F) Each line represents one single mouse. The results are representative of two (C, D and F) and one (A, B and E) experiments.

Next, we tested whether IFN-γ acted directly on RMA-S cells or on cells of the host to mediate tumor rejection in the absence of Treg. To address this question, we injected RMA-S cells, which express IFN-γR (Figure 6.5E), into IFN-γR-/- mice. In this experimental setup, only the tumor cells responded to IFN-γ. Subsequent monitoring of tumor growth revealed that IFN-γR-/- mice exhibited enhanced tumor growth in comparison to the wt controls, indicating the contribution of IFN-γ signaling on host cells for tumor control in the presence of Treg (Figure 6.5F). More importantly, depletion of Treg had no impact on tumor growth in IFN-γR-/- mice in comparison to IFN-γR-/- mice injected with PBS (Figure 6.5F). This finding suggests that IFN-γ signaling in host cells is essential for tumor rejection in the absence of Treg.

6.1.1.6 Perforin, FasL and inhibition of angiogenesis are not involved in the rejection of RMA-S tumor in the absence of Treg

In order to determine the mechanism leading to RMA-S tumor rejection in the absence of Treg, we investigated the role of perforin and the CD95-CD95L pathway. Perforin-mediated cytotoxicity has been shown to be important for the control of RMA-S tumor growth by NK cells. While perforin-/- mice showed enhanced RMA-S tumor growth in comparison to the wt group in the presence of Treg, reduced tumor growth was observed in these mice after Treg depletion (Figure 6.6A). The CD95-CD95L pathway is known to be important for CD4+ T cell-mediated cytotoxicity and FACS staining revealed that RMA-S cells express CD95 (Figure 6.6B). Nevertheless, neutralization of CD95L in vivo by injection of MFL-4 mAb did not
Figure 6.6. Perforin, CD95L and inhibition of angiogenesis are not involved in the rejection of RMA-S tumors in the absence of Treg. (A) Groups of 8 perforin$^{-/-}$ ($Prf^{-/-}$) mice were treated with PBS or anti-CD25 mAb, as indicated, before s.c. inoculation with RMA-S cells, and tumor growth was monitored. The results are representative of two experiments. (B) Representative histogram plot documenting CD95 expression on RMA-S cells, as assessed by flow cytometry. Staining with the specific antibody is indicated by the black line and the corresponding isotype control by the grey filled area. (C) Groups of 5 C57BL/6 wt mice were treated with anti-CD25 and/or anti-CD95L mAb, as indicated, before s.c. inoculation with RMA-S cells and tumor growth was monitored. (A and C) Each line represents one single mouse. (D) RMA-S cells cultured in the absence or presence of 50U/ml IFN-$\gamma$ for 24h were additionally cultured for 6h in the absence or presence of TNF-$\alpha$ or TRAIL and dead cells, defined as PI$^+$ cells, were determined at the end of incubation by flow cytometric analysis. L929 and Jurkat cells were used as positive control for TNF-$\alpha$- or TRAIL-induced in vitro apoptosis. (E) Groups of 3 C57BL/6 mice were treated with PBS, anti-CD25 mAb, and/or anti-IFN-$\gamma$ mAb, as indicated, before s.c. inoculation with RMA-S cells. Nine days later, mice were dissected and vessels leading to tumors were enumerated under a dissecting microscope. (F) Groups of 3 C57BL/6 mice were treated with PBS or anti-CD25 mAb, as indicated, before s.c. inoculation with RMA-S cells. Ten days
later, FITC-labeled *Lycopersicon esculentum* lectin was injected *i.v.* into mice and the tumor vasculature was observed in unfixed tumors by confocal microscopy.

influence neither tumor growth in the presence of Treg nor tumor rejection observed in the absence of Treg (Figure 6.6C). These data suggest that the anti-tumor effector mechanisms induced in the absence of Treg could still function in the absence of perforin and when CD95L was neutralized. In addition, cell viability of RMA-S cells, which were untreated or sensitized with IFN-γ, was not affected by incubation with TNF-α or TRAIL *in vitro* (Figure 6.6D).

Since IFN-γ was shown to inhibit tumor-induced angiogenesis 278, we determined vessel number and integrity in the presence or absence of Treg on day 10 of tumor growth, because at this time point the mean tumor sizes of control and Treg depleted mice were similar (Figure 6.3A). To address this question, PBS treated and Treg depleted mice, which were in addition neutralized or not for IFN-γ, were dissected on day 9 after tumor cell injection and vessels leading to tumors were enumerated under a dissecting microscope. No difference in the number of vessels connected to the tumor was documented among mice of the different groups (Figure 6.6E). For a more elaborate analysis of the tumor vasculature, FITC-labeled *Lycopersicon esculentum* lectin, an endothelial cell selective reagent, was injected into mice on day 10 of tumor growth and the tumor vasculature was observed by confocal microscopy. No differences regarding vessel number and vessel localization were observed between PBS and anti-CD25 treated mice (Figure 6.6F). In addition, no extravasion of the FITC-lectin was detected in any of the mice of control or Treg depleted groups, indicating that the vessels infiltrating the tumor tissue were functional in both groups. These data render it unlikely that angiogenesis is affected in the absence of Treg.

6.1.1.7 In the absence of Treg, increased numbers of leukocytes accumulate in the RMA-S tumor tissue in an IFN-γ dependent manner

Tumor-infiltrating lymphocytes have been found in many tumors and their numbers have been correlated with control or progression of tumor growth. In a recent study, a high density of immune cells, including T cells, correlated with longer survival in patients with colorectal cancer 279. Thus, we investigated the accumulation of leukocytes in the RMA-S tumors in the presence or absence of Treg. We determined the quantity of leukocytes as percentages of total live cells in
the tumor in mice treated with PBS or anti-CD25 mAb on days 7-10. To clearly distinguish between tumor cells and infiltrating leukocytes within the tumor tissue, we injected Ly5.2+ RMA-S tumor cells into congenic C57BL/6-Ly5.1+ mice. Flow cytometric analysis revealed that infiltrating Ly5.1+ cells represented higher percentages of total cells in the tumors of Treg depleted mice (Figure 6.7A). There was a significant increase in both the percentages and absolute cell numbers of leukocytes accumulating within the tumor mass, when Treg were depleted (7.46 ± 2.46 x 10^5 cells in the PBS group versus 14.6 ± 4.3 x 10^5 cells in the anti-CD25 group, p<0.0005). Absolute tumor cell numbers and tumor volume were virtually identical between the two groups at the investigated time points (data not shown).

Since we found a requirement of IFN-γ for tumor rejection in the absence of Treg (Figure 6.5D), we asked whether the presence of IFN-γ is required for the accumulation of leukocytes. We neutralized IFN-γ in the presence or absence of Treg and determined percentages of Ly5.1+ leukocytes infiltrating the Ly5.2+ tumors. Interestingly, the enhanced leukocyte accumulation in the absence of Treg was abrogated when IFN-γ was neutralized. IFN-γ neutralization alone did not lead to a reduction in tumor-infiltrating leukocytes in comparison to the control group (Figure 6.7A). In summary, these results indicate that Treg hamper leukocyte accumulation in the tumors, and this accumulation is IFN-γ dependent.

### 6.1.1.8 Increased numbers of NK cells, conventional CD4+ T cells and macrophages are detected in the RMA-S tumors in the absence of Treg

By analyzing individual leukocyte subpopulations, we observed that CD11b+ cells, NK cells (NK1.1+CD3-) and conventional CD4+ T cells (CD4+CD25-) were all increased in the absence of Treg (Figure 6.7B). The overall cellular composition of the infiltrating leukocyte compartment was comparable in both groups, with CD11b+ cells comprising up to 80% of all leukocytes. As observed with the whole Ly5.1+ compartment, the increased CD11b+ and CD4+ T cell accumulation in the absence of Treg was abrogated upon IFN-γ neutralization. Strikingly, almost no NK cells were detected in the tumor after IFN-γ neutralization (Figure 6.7B, insert). Figure 6.7B demonstrates that CD11b+ cells were the most prominent leukocyte subpopulation infiltrating the RMA-S tumor tissue after depletion of Treg. We further characterized the various CD11b+ cell subtypes and determined their accumulation in the absence of Treg. Macrophages, defined as CD11b+F4/80+ cells, dendritic cells (DC), defined as CD11b+CD11c+ cells and...
Figure 6.7. Increased numbers of leukocytes accumulate in the RMA-S tumors in an IFN-γ dependent manner in the absence of Treg. (A-C) C57BL/6-Ly5.1+ mice were treated with PBS (white bars) or anti-CD25 mAb (black bars) or anti-IFN-γ mAb (grey bars) or anti-CD25 and anti-IFN-γ mAb (checkered bars) and inoculated s.c. with Ly5.2+ RMA-S cells. Seven to 10 days later, tumors were removed and infiltrating Ly5.1+ (A), CD11b+, NK (NK1.1+CD3+) and CD4+CD25+ cells (B), or CD11b+F4/80+ cells (C) were determined by flow cytometric analysis and quantified as percentages of total live cells in the tumor. (D) C57BL/6-Ly5.1+ mice were treated with PBS or anti-CD25 mAb or anti-CD4 mAb or anti-CD25 and anti-NK1.1 mAb and inoculated s.c. with Ly5.2+ RMA-S cells. Ten days later, tumors were removed and infiltrating CD11b+F4/80+ cells were determined by flow cytometric analysis and quantified as percentages of total live cells in the tumor. (A-B) Data are pooled from 4 independent experiments using 5 animals per group. (C-D) Data show the mean ± SD of 3-5 animals per group and are representative of three (C) and one (D) experiments. *, p<0.05, **, p<0.005 in comparison to the PBS group, using Student’s t test. n.a. = not analyzed.

granulocytes, defined as CD11b+Gr-1+ cells, were all detected in significantly higher numbers within the tumor tissue on day 10 of tumor growth (Figure 6.7C). Similar to the whole CD11b+ compartment, the increased accumulation of all these populations in the absence of Treg was dependent on IFN-γ (Figure 6.7C). CD11b is also expressed on NK cells, characterizing their final maturation stage. Approximately 85% of the NK cells infiltrating the tumor tissue on day
10 of tumor growth were positive for the CD11b marker (Figure 6.18). However, the overall NK cell population is only a minor fraction of the whole CD11b+ population (Figure 6.7B). Cell depletion experiments revealed that predominantly CD4+ T cells and NK cells were important for the observed enhanced macrophage accumulation (Figure 6.7D). In summary, our data demonstrate that Treg suppress the accumulation of NK cells, CD4+CD25− T cells and CD11b+ cells, including macrophages, in the RMA-S tumors and this accumulation is IFN-γ dependent. In addition, NK cell accumulation in the tumors is absolutely dependent on IFN-γ, irrespective of the presence of Treg.

![Figure 6.8](image)

**Figure 6.8. Treg suppress the anti-tumor response against RMA-S independently of TGF-β or IL-10.** (A) Groups of 8 C57BL/6 mice were treated with PBS (left panel) or anti-TGF-β mAb (middle panel) or the appropriate isotype control (mouse IgG1, right panel), were s.c. inoculated with RMA-S cells and tumor growth was monitored. Data are representative of two experiments. (B) Groups of 8 C57BL/6 mice were treated with PBS (left panel), anti-IL-10R mAb (middle panel) or the appropriate isotype control (rat IgG1, right panel), were s.c. inoculated with RMA-S cells and tumor growth was monitored. (A and B) Each line represents one single mouse.

6.1.1.9 Treg suppress RMA-S anti-tumor immunity independently of TGF-β or IL-10

Finally, we wanted to address the question via which mechanism do Treg exert their suppressive effect on the anti-tumor immunity against RMA-S tumor. In certain *in vivo* models, Treg were shown to mediate their suppressive activity via the production of TGF-β. Thus, we examined whether neutralization of TGF-β in mice injected with RMA-S tumor cells would abrogate Treg mediated suppression and thus lead to a similar outcome on tumor growth as Treg depletion.
Figure 6.8A shows that after neutralization of TGF-β, RMA-S cells grew progressively similar to control mice. These data suggest that, in our model, Treg-mediated suppression is independent of TGF-β. Another important Treg-derived factor reported to mediate suppression is IL-10 \(^{281}\). \textit{In vivo} administration of a blocking antibody against the IL-10R was not able to affect RMA-S tumor growth, when compared to the administration of an isotype-matched control (Figure 6.8B). In conclusion our data show that in the RMA-S tumor model Treg do not use neither TGF-β nor IL-10 to exert their suppressive function.

### 6.1.2 Regulatory T cells suppress macrophage activation in lymphoma

#### 6.1.2.1 Increased amounts of MHC class II on macrophages in the absence of Treg

The observation that CD11b\(^+\) cells were the most prominent cell population infiltrating the RMA-S tumors in the absence of Treg, along with the importance of CD4\(^+\) T cells for tumor rejection, prompted us to study MHC class II expression on tumor-infiltrating leukocytes on day 10 of tumor growth. Interestingly, as shown in Figure 6.9A and 6.9B, left panel, higher percentages of MHC class II-expressing macrophages (CD11b\(^+\)F4/80\(^+\)) were found in the tumors of Treg depleted mice (49.6 ± 7.9% versus 28.4 ± 6.4% in the PBS group, p<0.05). Furthermore, the geometric mean fluorescence intensity (MFI) of MHC class II was also significantly increased on macrophages infiltrating the RMA-S tumors of the Treg depleted mice (Figure 6.9B, right panel). Notably, the MHC class II upregulation in the absence of Treg was abrogated by neutralization of IFN-γ, whereas IFN-γ neutralization alone did not have any significant effect on macrophage MHC class II expression (Figure 6.9B). Expression levels of the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) on tumor-infiltrating macrophages were similar between PBS treated and Treg depleted groups, although – concerning CD80 – the MFI was significantly enhanced when compared to blood monocytes of tumor bearing mice (Figure 6.9C).

B7-H4, an inhibitory member of the B7 family, has been shown to be induced on human monocytes after \textit{in vitro} co-culture with Treg \(^{191}\). In addition, B7-H4 expression on macrophages infiltrating ovarian tumors correlated with the patients’ survival \(^{192}\). Expression levels of B7-H4 were determined on tumor-infiltrating macrophages in the RMA-S model. Increased expression
Figure 6.9. Increased MHC class II expression of tumor-infiltrating macrophages in the absence of Treg. (A-B) C57BL/6 mice were treated with PBS, anti-CD25 mAb, and/or anti-IFN-γ mAb, as indicated, before s.c. inoculation with RMA-S cells and 10 days later tumors were removed. (A) Tumor-infiltrating CD11b+F4/80+ cells were gated and analyzed for MHC class II expression, as shown in the histograms (one representative analysis out of six mice per group is shown). Staining with the specific antibody is indicated by the black line and the corresponding isotype control by the grey filled area. (B) Percentages of MHC class II-expressing CD11b+F4/80+ cells (left panel) and MFI of MHC class II of CD11b+F4/80+ cells (right panel) in the tumor tissue of PBS (white bars) or anti-CD25 mAb (black bars) or anti-IFN-γ mAb (grey bars) or anti-CD25 and anti-IFN-γ mAb (checkered bars) treated mice. Data show the mean ± SD of three animals per group and are representative of two experiments. *, p< 0.05, using Student’s t test. (C-E) C57BL/6 mice were treated with PBS or anti-CD25 mAb, as indicated, before s.c. inoculation with RMA-S cells, and 10 days later, tumors (C-E) and blood (C, right panel) were removed. CD11b+F4/80+ cells were gated and analyzed for CD86 and CD80 (C), B7-H4 (D) B7-H1, B7-H2, B7-H3 and B7-DC (E) expression. (C-E) Data show the mean ± SD of three animals per group.
of cell surface B7-H4 was detected on tumor-infiltrating macrophages as compared to blood monocytes of tumor bearing hosts; however, no differences in B7-H4 expression were observed on tumor-infiltrating macrophages between PBS and anti-CD25 treated mice (Figure 6.9D). When B7-H4 expression was monitored by intracellular staining, high levels of this molecule were detected on both blood and tumor-infiltrating macrophages; however, again, no differences were detected between PBS and anti-CD25 treated mice (data not shown). Finally, we analyzed the tumor-infiltrating macrophages for the expression of other B7 family members, namely B7-H1, B7-H2, B7-H3 and B7-DC. B7-DC, and to a lesser extent B7-H3, expression was induced on tumor-infiltrating macrophages as compared to blood monocytes of tumor bearing hosts (data not shown). However, no difference was observed between PBS and anti-CD25 treated mice (Figure 6.9E). B7-H1 and B7-H2 were highly expressed by both blood and tumor-infiltrating macrophages, but no significant difference could be documented between PBS and anti-CD25 treated groups (Figure 6.9E). In conclusion, our data suggest that in the RMA-S tumor model expression of B7-H1, B7-H2, B7-H3, B7-H4 and B7-DC on tumor-infiltrating macrophages is not controlled by Treg.

### 6.1.2.2 Enhanced macrophage activity in the absence of Treg

In order to further characterize the macrophages infiltrating the RMA-S tumors in the presence or absence of Treg, cytokine production of freshly isolated tumor-infiltrating macrophages was assessed after 12 h of culture in medium alone (Figure 6.10A, upper panel) or in the presence of RMA-S cells in combination with LPS to induce maximum release of cytokines (Figure 6.10A, lower panel). The results demonstrate consistently higher levels of MIP-1β, MIP-2, MIP-1α, IL-6, IL-13 and IL-1β produced by macrophages isolated from tumors of Treg depleted mice compared to control mice (Figure 6.10A). Similar amounts of cytokines and chemokines were observed in cultures in the absence or presence of RMA-S cells without the addition of LPS, as shown representatively for MIP-2 and IL-6, suggesting that the presence of tumor cells did not influence the amounts of cytokines produced upon LPS stimulation (Figure 6.10B). IL-12 (p70), IFN-γ and VEGF were not detected. Analysis of the macrophage cytokine profile after 24 h of culture revealed a similar pattern (data not shown). In vivo IFN-γ neutralization did not affect the production of certain cytokines, such as IL-1β, while it pronouncedly increased the production of IL-6 (Figure 6.10C).
Figure 6.10. Increased chemokine and cytokine production by tumor-infiltrating macrophages in the absence of Treg. (A-B) C57BL/6 mice were treated with PBS (white bars) or anti-CD25 mAb (black bars), before s.c. inoculation with RMA-S cells, and 10 days later, tumors were removed. Tumor-infiltrating CD11b⁺F4/80⁺ cells were purified and cultured in vitro either with medium (A, upper panel) or RMA-S cells in a 5:1 ratio in combination with LPS (A, lower panel) or RMA-S cells in a 5:1 ratio alone (B) for 12 h and analyzed for chemokine and cytokine production by Bioplex. (C) C57BL/6 mice were treated with anti-CD25 mAb (white bars) or anti-CD25 and anti-IFN-γ mAb (black bars), before s.c. inoculation with RMA-S cells and 10 days later tumors were removed. Tumor-infiltrating CD11b⁺ cells were purified and cultured in vitro either with medium for 24 h and analyzed for cytokine production by Bioplex. (A-C) Data are representative of three (A and B) and one (C) experiments. n.d. = not detectable.
Next, we examined whether macrophages, found in tumors of Treg depleted mice, could directly inhibit RMA-S tumor growth. For this purpose, CD11b+ cells were enriched from tumors of control or Treg depleted mice; alternatively macrophages (CD11b+ F4/80+) were isolated by flow cytometric sorting (purity > 95%). These cells were cultured for 48 h with RMA-S cells and proliferation was monitored. Enriched CD11b+ cells (Figure 6.11A, left panel) as well as macrophages purified by flow cytometric sorting (Figure 6.11A, right panel) suppressed RMA-S tumor cell growth at similar levels at a 10:1 ratio. In addition, they were significantly more potent when isolated from tumors of Treg depleted mice compared to cells from the control group (Figure 6.11A). Similar results were obtained after 24 h of co-culture, as shown representatively for enriched CD11b+ cells (Figure 6.11B). No proliferation was detected when macrophages were cultured in the absence of tumor cells. CD11b+F4/80- cells isolated from Treg depleted mice also suppressed RMA-S proliferation, in contrast to CD11b+F4/80- cells isolated from PBS treated mice (Figure 6.11C). Treg were not present during the tumor cell growth inhibition assay indicating that the difference in the suppression between the two groups was not due to Treg contaminating the culture of macrophages derived from PBS treated mice (data not shown). Interestingly, RMA-S cell growth suppression by CD11b+ cells was completely abrogated when IFN-γ was neutralized both in the presence and to a lesser extent – which was not statistically significant – in the absence of Treg, pinpointing a role for IFN-γ in enhancing the tumoristatic function of macrophages (Figure 6.11D).

6.1.2.3 Macrophages kill RMA-S cells independently of the enzymatic activity of iNOS, PGE2 and IDO

In order to examine the mechanism of suppression of RMA-S proliferation by macrophages, we used a trans-well system to separate the macrophages from the RMA-S tumor cells during a 48h co-culture. Interestingly, the suppression of RMA-S proliferation was still observed when the cells were not in contact (Figure 6.12A), suggesting that a soluble factor was responsible for the effect. The suppression of RMA-S proliferation was not due to cell cycle arrest, since the presence of macrophages did not have any influence on the CFSE dilution of labeled RMA-S cells (Figure 6.12B). Interestingly, dead RMA-S cells, as documented by PI staining, could not be detected in the co-culture of macrophages and RMA-S cells, unless the two populations were separated with a trans-well insert (Figure 6.12C). At the same time, macrophages became CFSE+.
Figure 6.11. Enhanced macrophage activity in the absence of Treg. (A-D) C57BL/6 mice were treated with PBS or anti-CD25 and/or anti-IFN-γ mAb, as indicated, before s.c. inoculation with RMA-S cells and 10 days later, tumors were removed. (A) Enriched CD11b⁺ cells (left panel) or purified macrophages (CD11b⁺F4/80⁺, >95% pure) (right panel) were isolated from tumors and co-cultured in vitro with RMA-S cells at the indicated ratios. After 48h of co-culture, tumor cell proliferation was monitored by ³H-thymidine incorporation assay. (B) Enriched CD11b⁺ cells were isolated from tumors and co-cultured in vitro with RMA-S cells at the indicated ratios. After 24h of co-culture, tumor cell proliferation was monitored by ³H-thymidine incorporation assay. (C) Purified CD11b⁺F4/80⁺ or CD11b⁺F4/80⁻ cells were isolated from tumors (>95% pure) and co-cultured in vitro with RMA-S cells at a 10:1 ratio. After 48h of co-culture, tumor cell proliferation was monitored by ³H-thymidine incorporation assay. (D) Enriched CD11b⁺ cells were isolated from tumors and co-cultured in vitro with RMA-S cells at the indicated ratios. After 48h of co-culture, tumor cell proliferation was monitored by ³H-thymidine incorporation assay. (A-D) Data represent mean ± SD of triplicate cultures and are representative of three (A and B) and one (C and D) experiments. *, p<0.05, **, p<0.005, ***, p<0.0001 using Student’s t test.
Figure 6.12. Macrophages kill and phagocytose RMA-S cells in vitro. (A-D) C57BL/6 mice were treated with PBS or anti-CD25 mAb, as indicated, before s.c. inoculation with RMA-S cells and 10 days later tumors were removed. (A) Enriched CD11b⁺ cells were isolated from tumors and co-cultured in vitro with RMA-S cells in the absence or presence of a transwell insert at a 10:1 ratio. After 48h of co-culture, tumor cell proliferation was monitored by [³H]-thymidine incorporation assay. The data represent mean ± SD of triplicate cultures. (B) RMA-S cells (unstained, upper left panel) were labeled with CFSE (t = 0, lower left panel) and cultured in the absence (upper right panel) or presence (lower right panel) of CD11b⁺ cells originating from PBS treated mice. After 48h of co-culture, CFSE dilution was detected by flow cytometric analysis. The data originate from pooled triplicate cultures. (C) Enriched CD11b⁺ cells were isolated from tumors of PBS treated mice and co-cultured in vitro with RMA-S cells, in the absence (left panel) or presence (right panel) of a transwell insert, at the indicated ratios. After 48h of co-culture, dead RMA-S cells were determined as PI⁺ cells by flow cytometric analysis. (D) Enriched CD11b⁺ cells were isolated from tumors of PBS treated mice and co-cultured in vitro with CFSE-labeled RMA-S cells at a 40:1 ratio. After 48h of co-culture, CFSE staining on CD11b⁺ cells was documented by flow cytometric analysis.
when they were in the same chamber with CFSE-labeled RMA-S cells (Figure 6.12D). The same results were observed when macrophages were isolated either from PBS or anti-CD25 treated mice. In summary, our results suggest that the macrophages induce RMA-S cell death \textit{in vitro} via a soluble factor and then uptake RMA-S cells by phagocytosis.

![Figure 6.13](image)

**Figure 6.13.** iNOS, PGE2 and IDO are not involved in the suppression of RMA-S tumor growth by macrophages. (A) Groups of 8 wt or inducible Nitric Oxide Synthase\(^{-/-}\) (\(iNOS^{-/-}\)) mice were treated with PBS or anti-CD25 mAb, as indicated, before \(s.c.\) inoculation with RMA-S cells, and tumor growth was monitored. Each line represents one single mouse. (B-C) C57BL/6 mice were treated with PBS or anti-CD25 mAb, as indicated, before \(s.c.\) inoculation with RMA-S cells, and 10 days later, tumors were removed. Enriched CD11b\(^+\) cells were isolated from tumors and co-cultured \textit{in vitro} with RMA-S cells at the indicated ratios in the presence of L-NMMA (B), indomethacin (C, left panel), 1MT (C, right panel) or the appropriate controls, as indicated. After 48h of co-culture, tumor cell proliferation was monitored by \(^3\)H-thymidine incorporation assay. Data represent mean ± SD of triplicate cultures.

One effector molecule of activated macrophages is nitric oxide (NO) produced by the enzyme inducible nitric oxide synthase (iNOS), which has been shown to have tumor-suppressing or tumor-promoting functions \(^{282}\). In the presence and absence of Treg, tumor growth in \(iNOS^{-/-}\) mice progressed similarly to the wt controls (Figure 6.13A). In addition, the presence of a NO inhibitor, NG-monomethyl-L-arginine (L-NMMA), did not affect macrophage-mediated inhibition of tumor cell proliferation \textit{in vitro} (Figure 6.13B), arguing against a role of NO in the
RMA-S model. Prostaglandin E synthase 2 (PGE2) and indoleamine-pyrrole 2,3-dioxygenase (IDO) had been initially described as tumor-derived factors involved in immune suppression; however, many publications have identified these enzymes in various APC populations, including macrophages. We addressed the role of these two enzymes by the addition of the specific inhibitors indomethacin, to block the activity of PGE2, and 1MT, which blocks IDO activity, during the 48h co-culture of RMA-S cells with macrophages. The addition of inhibitors for either of these molecules was not able to reverse the suppressive effect of macrophages (Figure 6.13C). In summary, macrophages induce RMA-S cell death during their in vitro co-culture via a soluble factor, which is independent of the enzymatic activity of iNOS, PGE2 and IDO.

6.1.3 Regulatory T cells suppress CD8$^+$ T cell recognition of TAP-deficient tumors

6.1.3.1 CD8$^+$ T cells contribute to RMA-S tumor rejection in the absence of Treg by direct recognition of RMA-S cells

Our depletion experiments implied a role of CD8$^+$ T cells in the memory response against RMA-S tumors (Figure 6.4D-F). In addition, CD8$^+$ T cells were the most prominent IFN-γ producing cell population in the tumor tissue on day 10 after tumor cell inoculation (Figure 6.5C). These findings prompted us to further investigate the role of CD8$^+$ T cells in the primary anti-tumor response against RMA-S. Interestingly, depletion of the CD8$^+$ T cell compartment abrogated RMA-S tumor rejection in the absence of Treg, suggesting that, together with NK cells and CD4$^+$ T cells (Figure 6.3C-F), CD8$^+$ T cells also contribute to RMA-S tumor eradication during the primary anti-tumor response (Figure 6.14A). In addition, when we analyzed RMA-S tumor infiltrates on day 10 after tumor cell inoculation, in the presence or absence of Treg, we found increased accumulation of CD8$^+$ T cells in the absence of Treg (Figure 6.14B), as shown for other leukocyte populations in our model (Figure 6.7B-C). These data suggest that Treg suppress CD8$^+$ T cell accumulation in the tumor, as well as CD8$^+$ T cell mediated anti-tumor activity against RMA-S tumor.
The recognition of tumor cells by CD8$^+$ T cells in vivo can be either a direct process, by the presentation of tumor peptides on the surface of tumor cells in the context of MHC class I molecules or an indirect process, by the presentation of tumor antigens on the surface of APC, via a mechanism known as cross-presentation.\(^{284}\) In the first case, a mechanism of direct recognition of TAP$^{-}$ tumors by CD8$^+$ T cells has been recently described\(^ {267,285}\). This recognition is possible due to the fact that cells with an impairment in the antigen processing pathway – as is the case for RMA-S cells – are not completely negative for MHC class I expression (Figure 6.15, left panel), but can present a variety of peptides originating within the endoplasmic reticulum.\(^ {267}\) Thus, we addressed the question whether CD8$^+$ T cells in our model can directly recognize RMA-S tumor cells. For this purpose, we isolated highly purified CD8$^+$ T cells from the LN, DLN and tumor tissue of PBS and anti-CD25 treated mice 10 days after RMA-S tumor cell inoculation and co-cultured them in vitro with fresh RMA-S cells. Supernatants were collected after 18h of culture and levels of IFN-$\gamma$ were determined by ELISA, as a readout for CD8$^+$ T cell activation. Figure 6.16A, left panel shows that CD8$^+$ T cells isolated from the DLN of Treg depleted mice produced IFN-$\gamma$ in vitro in the presence of RMA-S cells. This was not the case for CD8$^+$ T cells isolated from the DLN of PBS treated mice or CD8$^+$ T cells isolated from the non-DLN (LN) either of tumor bearing or naïve mice. Most interestingly, CD8$^+$ T cells infiltrating the RMA-S tumors of both PBS and anti-CD25 treated mice potently produced IFN-$\gamma$ in vitro in the presence of RMA-S cells. 

Figure 6.14. CD8$^+$ T cells contribute to the rejection of RMA-S tumors in the absence of Treg. (A) C57BL/6 mice were treated with PBS (left panel, n=8) or anti-CD25 mAb (middle panel, n=6), or anti-CD25 and anti-CD8 mAb (right panel, n=7) before s.c. inoculation with RMA-S cells and tumor growth was monitored. Each line represents one single mouse. (B) C57BL/6-Ly5.1$^+$ mice were treated with PBS (white bars) or anti-CD25 mAb (black bars) and inoculated s.c. with Ly5.2$^+$ RMA-S cells. Ten days later, tumors were removed and infiltrating CD8$^+$ T cells, determined by flow cytometric analysis, were quantified as percentages of total live cells in the tumor. *, p<0.05, using Student’s t test.
cells and this production was significantly higher when CD8\(^+\) T cells were isolated from the tumors of Treg depleted animals (Figure 6.16A, right panel). IFN-\(\gamma\) production by CD8\(^+\) T cells in this \textit{in vitro} set-up was absolutely dependent on MHC class I, since co-culture of CD8\(^+\) T cells with EC7.1 cells, the MHC class I-negative variant of RMA-S (Figure 6.15, right panel), abrogated IFN-\(\gamma\) production (Figure 6.16A). Finally, no IFN-\(\gamma\) production was detected when CD8\(^+\) T cells were cultured alone (Figure 6.16A). In conclusion, our data show that CD8\(^+\) T cells isolated from RMA-S tumor bearing mice can directly recognize RMA-S cells, via a MHC class I dependent manner and this recognition is enhanced in the absence of Treg.

![Figure 6.15. RMA-S cells express low levels of MHC class I. Representative histogram plots documenting MHC class I expression, as assessed by flow cytometry, on RMA-S (left panel) and EC7.1 cells (right panel). Staining with the specific antibody is indicated by the black line and the corresponding isotype control by the grey filled area.](image)

In a second set of experiments, we isolated CD8\(^+\) T cells from spleens and LN of mice which had rejected RMA-S tumors after Treg depletion and were then rested for at least three months to allow the recovery of the Treg compartment. These mice are designated as memory mice. In parallel, CD8\(^+\) T cells from spleens and LN of naïve mice were also isolated. In both cases, CD8\(^+\) T cells were co-cultured with RMA-S cells \textit{in vitro}, and IFN-\(\gamma\) production was determined in the supernatants after 18h of co-culture. As shown in Figure 6.16B, CD8\(^+\) T cells isolated from spleens or LN of naïve mice did not produce any detectable amounts of IFN-\(\gamma\) upon co-culture with RMA-S cells. In contrast, CD8\(^+\) T cells isolated from spleens or, most prominently, from LN of memory mice produced significant amounts of IFN-\(\gamma\). This production was dependent on MHC class I, because when CD8\(^+\) T cells were co-cultured with EC7.1 cells, IFN-\(\gamma\) production was not observed (Figure 6.16B). These data indicate that CD8\(^+\) T cells isolated from secondary lymphoid organs of mice, which had rejected RMA-S tumors after Treg depletion, can directly recognize RMA-S cells.
Figure 6.16. Enhanced direct recognition of RMA-S tumor cells by CD8+ T cells in the absence of Treg. (A) C57BL/6 mice were treated with PBS or anti-CD25 mAb, as indicated, and inoculated s.c. with RMA-S cells. Mice which were not inoculated with RMA-S cells are designated as naive. Ten days later, draining LN (DLN), non-draining LN (LN) and tumors were removed. CD8+ T cells were purified from LN and DLN by MACS sorting (purity > 80%) or from tumors by FACS sorting (purity > 95%) and cultured alone or in the presence of RMA-S or EC7.1 tumor cells for 18h. IFN-γ production was measured in the S/N by ELISA. (B) C57BL/6 mice were treated with anti-CD25 mAb before s.c. inoculation with RMA-S cells, rejected RMA-S tumors and rested for at least 3 months. These mice are designated as memory mice. Mice which were not inoculated with RMA-S cells are designated as naive. CD8+ T cells were isolated from the spleens or LN of naïve or memory mice, and co-cultured for 18h alone or in the presence of RMA-S or EC7.1 tumor cells. (A-B) Data represent the mean of duplicates in ELISA ± SD and are representative of one (A) and three (B) experiments. *. p < 0.001 in comparison to the PBS group, using Student’s t test. n.d. = not detectable.
6.1.4 Immune intervention in established RMA-S tumors

6.1.4.1 Depletion of Treg in established RMA-S tumors

We showed that Treg depletion before RMA-S tumor cell inoculation led to tumor rejection (Figure 6.3A). Furthermore, we addressed the question whether Treg depletion could enhance anti-tumor immunity to mediate the rejection of already established RMA-S tumors. For this purpose, we injected mice s.c. with RMA-S cells and allowed the tumors to grow. 10 days after tumor cell inoculation, 200µg of anti-CD25 depleting mAb or PBS as a control were injected intra- and peritumorally, and tumor growth was observed. Figure 6.17A shows that RMA-S tumor growth could be efficiently controlled when the anti-CD25 mAb was administered 2 days prior to tumor cell inoculation, while late Treg depletion was not able to influence RMA-S tumor growth, when compared to the control group that was treated with PBS. These data suggest that depletion of Treg is not by itself sufficient to influence tumor growth of established RMA-S tumors.

6.1.4.2 Depletion of NK cells in established RMA-S tumors

NK cells are absolutely required for RMA-S tumor eradication (Figure 6.3C). Their absence led to enhanced tumor growth already in early time points after RMA-S tumor inoculation (Figure 6.3D, left panel) and this enhanced tumor growth was still present in later time points (Figure 6.3D, right panel). In later time points of tumor growth, however, it is not obvious whether this increased tumor growth is due to the absence of NK cells or to the initial tumor load, which is higher when NK cells are not present. In order to investigate the contribution of NK cells in later time points of RMA-S tumor growth in the presence or absence of Treg, mice were treated with PBS or anti-CD25 mAb before s.c. injection with RMA-S cells; 10 days after tumor cell inoculation, anti-NK1.1 depleting mAb or PBS as a control was administered i.p. and tumor growth was monitored. Depletion of NK cells 2 days prior to tumor cell inoculation led to enhanced tumor growth either in the presence or absence of Treg (Figure 6.17B). Interestingly, late depletion of NK cells had only a minor effect on RMA-S tumor growth, in the presence (Figure 6.17B, upper panel) or in the absence of Treg (Figure 6.17B, lower panel). These data
suggest that NK cells have only a minor contribution to the anti-RMA-S tumor immune response in later time points of tumor growth.

**Figure 6.17. Depletion of Treg or NK cells in established RMA-S tumors does not affect tumor growth.** (A) Groups of 8 C57BL/6 mice were treated with PBS (left panel) or anti-CD25 mAb administered either on day -2 (middle panel) or on day +10 (right panel), s.c. inoculated with RMA-S cells and tumor growth was monitored. (B) Groups of 7 C57BL/6 mice were treated with PBS or anti-CD25 mAb on day -2 and/or NK1.1 mAb administered starting either from day -2 or day +10, as indicated, s.c. inoculated with RMA-S cells and tumor growth was monitored. (A-B) Each line represents one single mouse.

Acquisition of NK cell function during development correlates with high expression of the CD11b marker by NK cells. In addition, it was reported that various lineages of tumors
interfere with NK cell functions, by interrupting their maturation in the bone-marrow. To address the question whether RMA-S tumor impedes the functional maturation of NK cells, we monitored CD11b expression on NK cells in various organs during RMA-S tumor progression. Figure 6.18 shows that CD11b expression on NK cells was reduced in the BM, blood, spleen and tumor tissue, starting from day 10 after RMA-S tumor cell inoculation, while this reduction became more prominent at later time points of tumor growth, as representatively shown for days 17 and 21 after tumor cell inoculation. Similar results were found when CD43 was used as a maturation marker for NK cells (data not shown). These data correlate late NK cell dysfunction with reduced CD11b expression in RMA-S tumor bearing mice, without excluding, however, that alternative mechanisms may be involved.

Figure 6.18. Decreased CD11b expression on NK cells during RMA-S tumor progression. C57BL/6 mice were injected s.c. with RMA-S cells. After 7, 10, 17 and 21 days, bone marrow, spleens, blood and tumors were removed and analyzed for CD11b expression on NK cells by flow cytometry. Data show the mean ± SD of four tumor bearing and two naïve animals per time point and are representative of two experiments. *, p<0.05, **, p<0.01 using Student’s t test.
6.2 Control of anti-tumor immunity by type I IFN

6.2.1 Enhanced RMA-S tumor growth in the absence of type I IFN signaling

In order to assess the role of type I IFN in the anti-tumor immunity against RMA-S, we s.c. injected $1 \times 10^6$ RMA-S tumor cells in wt or IFNAR1$^{-/-}$ mice, which lack the IFNAR1 subunit of the IFN-α/β receptor complex. Interestingly, we found that mice which could not respond to type I IFN exhibited enhanced RMA-S tumor growth, which was statistically significant (Figure 6.19A). This phenotype could also be observed in wt mice when injected with a neutralizing anti-IFN-β mAb (Figure 6.19B). These data show that RMA-S tumor growth is under the control of endogenously produced type I IFN.

Figure 6.19. IFNAR1$^{-/-}$ mice exhibit enhanced RMA-S tumor growth. (A) C57BL/6 wt (left panel, n=5) or IFNAR1$^{-/-}$ (right panel, n=7) mice were s.c. inoculated with RMA-S cells and tumor growth was monitored. wt versus IFNAR1$^{-/-}$, p > 0.01, using Koziol test. (B) C57BL/6 wt mice were treated with PBS (left panel, n=5) or anti-IFN-β mAb (middle panel, n=5) or rat IgG1 mAb (right panel, n=5), inoculated s.c. with RMA-S cells and tumor growth was monitored. (A-B) Each line represents one single mouse. The results are representative of two independent experiments.
6.2.2 Requirement of hematopoietic and non-hematopoietic compartment for responsiveness to type I IFN

We next addressed the question whether the cell targets of type I IFN in our model were derived from the hematopoietic or non-hematopoietic compartment of the host. For this purpose, we reconstituted lethally irradiated wt and RAG2−/−IFNAR1−/− mice with BM cells isolated from wt or IFNAR1−/− mice. Thus, we created the following BM chimeras: wt → wt, in which reconstitution leads to type I IFN responsiveness in both the hematopoietic and non-hematopoietic compartments; wt → RAG2−/−IFNAR1−/− mice, in which type I IFN responsiveness is reconstituted only in the hematopoietic cells; and IFNAR1−/− → wt, in which type I IFN responsiveness is present only in the non-hematopoietic compartment. The use of congenic markers (Ly5.1/Ly5.2) was employed for the discrimination of host versus donor cells (Table 6.1). Five weeks after BM reconstitution, the efficiency of the reconstitution was assessed in the blood of mice by flow cytometric analysis and varied between 60 and 90% in individual mice (data not shown). Six weeks after BM reconstitution, RMA-S tumor growth was assessed in the chimeric mice, in comparison to non-reconstituted IFNAR1−/− mice. Figure 6.20 shows that reconstitution of the type I IFN responsiveness in either the hematopoietic or non-hematopoietic compartment of the host only partially restored the enhanced RMA-S tumor growth observed in the IFNAR1−/− mice. These results show that type I IFN sensitivity within both hematopoietic and non-hematopoietic cells is required for RMA-S tumor growth control.

Figure 6.20. IFNAR1 signaling on both hematopoietic and non-hematopoietic cells is important in RMA-S anti-tumor response. Groups of 9 C57BL/6 wt or RAG2−/−IFNAR1−/− mice were lethally irradiated and reconstituted with BM derived either from wt or IFNAR1−/− mice, as indicated. Six weeks after BM reconstitution, chimeric mice, as well as IFNAR1−/− mice (n=5) were s.c. injected with RMA-S cells and tumor growth was monitored. Each line represents one single mouse. The results are representative of two experiments.
Table 6.1. Generation of BM chimeras with differential defect in IFNAR1 signaling either on hematopoietic or non-hematopoietic cells.

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<td>Ly5.2+ IFNAR1&lt;sup&gt;-/-&lt;/sup&gt;</td>
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6.2.3 Contribution of the hematopoietic compartment for responsiveness to type I IFN

6.2.3.1 NK cell cytotoxicity is impaired in IFNAR1<sup>-/-</sup> mice

NK cells are absolutely required for the control of RMA-S tumor growth (Figure 6.3C-D). Therefore, we addressed the question whether NK cell function is impaired in IFNAR1<sup>-/-</sup> mice. For this purpose, NK cells were isolated from the spleens of wt or IFNAR1<sup>-/-</sup> mice by magnetic separation and their cytotoxic ability was tested against the NK cell sensitive tumor cell line YAC-1 or against RMA-S cells in a standard <i>in vitro</i> 6h <sup>51</sup>Cr release assay. Interestingly, NK cells isolated from the spleens of IFNAR1<sup>-/-</sup> mice had significantly reduced ability to kill YAC-1 or RMA-S targets when compared to NK cells from wt mice (Figure 6.21A). Thus, NK cell cytotoxicity is impaired in IFNAR1<sup>-/-</sup> mice.

6.2.3.2 Macrophage tumoristatic activity is not impaired in IFNAR1<sup>-/-</sup> mice

Another important effector cell population identified in the RMA-S tumor model is macrophages, which can suppress RMA-S proliferation <i>in vitro</i> (Figure 6.11). Macrophage tumoristatic activity against RMA-S tumor cells was assessed in the IFNAR1<sup>-/-</sup> mice. Tumor infiltrating CD11b<sup>+</sup> cells isolated from RMA-S tumors grown in wt or IFNAR1<sup>-/-</sup> mice were enriched with magnetic cell sorting 10 days after tumor cell inoculation and co-cultured <i>in vitro</i> with RMA-S tumor cells. RMA-S tumor cell proliferation was assessed after 48h using <sup>3</sup>H-thymidine incorporation assay. As shown in Figure 6.21B, CD11b<sup>+</sup> cells from IFNAR1<sup>-/-</sup> mice were equally efficient to suppress
RMA-S tumor cell growth in vitro as CD11b+ cells isolated from wt mice. These findings suggest that macrophage tumoristatic function is not impaired in IFNAR1−/− mice.

**Figure 6.21. Impaired NK cell but normal macrophage function in the IFNAR1−/− mice.** (A) NK cells were isolated from spleens of wt (white points) or IFNAR1−/− (black points) mice and co-cultured in vitro with 51Cr-labelled YAC-1 (left panel) or RMA-S (right panel) target cells at the indicated ratios. After 6h of co-culture, 51Cr release was measured in the S/N. Data represent mean ± SD of triplicate cultures. *, p<0.05, **, p<0.001, ***, p<0.0001 using Student’s t test. (B) C57BL/6 wt (white bars) or IFNAR1−/− (black bars) mice were s.c. inoculated with RMA-S cells and 10 days later tumors were removed. CD11b+ cells were isolated from the tumors and co-cultured in vitro with RMA-S cells at the indicated ratios. After 48h of co-culture, tumor cell proliferation was monitored by 3H-thymidine incorporation assay. Data represent mean ± SD of triplicate cultures. (C) C57BL/6 wt (white bars) or IFNAR1−/− (black bars) mice were s.c. inoculated with RMA-S cells and 10 days later tumors were removed and infiltrating CD11b+, Gr-1+, CD4+, CD8+ and NK (NK1.1+CD3−) cells were determined by flow cytometric analysis and quantified as percentages of total live cells in the tumor. Data show the mean ± SD of 3 animals per group.

### 6.2.3.3 Intratumoral leukocyte accumulation is not impaired in IFNAR1−/− mice

Increased numbers of myeloid cells \textsuperscript{287} and reduced numbers of NK cells \textsuperscript{259} have been reported in the blood of IFNAR1−/− mice. We addressed the question whether leukocyte accumulation in
the RMA-S tumors is impaired in IFNAR1<sup>−/−</sup> mice. For this purpose, we determined the quantity of various tumor infiltrating leukocyte subpopulations as percentages of total live cells in the tumor in wt and IFNAR1<sup>−/−</sup> mice on day 10 after RMA-S tumor cell inoculation. The mean tumor size of the two groups was comparable at this time point. Flow cytometric analysis revealed that CD11b<sup>+</sup> cells, Gr-1<sup>+</sup> cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and NK cells were infiltrating the tumors of wt and IFNAR1<sup>−/−</sup> mice in similar amounts (Figure 6.21C). These data indicate that, although the numbers of some cell populations are misbalanced in the blood of IFNAR1<sup>−/−</sup> mice, the leukocyte accumulation in the RMA-S tumors is not impaired and is comparable to the wt situation.

6.2.4 Contribution of the non-hematopoietic compartment for responsiveness to type I IFN

6.2.4.1 Angiogenesis is impaired in IFNAR1<sup>−/−</sup> mice

Figure 6.20 shows that type I IFN responsiveness is required in the non-hematopoietic compartment. To address the importance of this finding, angiogenesis pattern and vessel functionality were observed in RMA-S tumors of wt and IFNAR1<sup>−/−</sup> mice. For this purpose, wt or IFNAR1<sup>−/−</sup> mice were injected with FITC-labeled <i>Lycopersicon esculentum</i> lectin on day 10 of tumor growth and the tumor vasculature was observed by confocal microscopy. Long vessels with normal branching were commonly present within the tumors of wt mice, and no extravasated FITC-lectin could be detected (Figure 6.22A, left panel). On the other hand, structural irregularity, heterogeneity and leakiness were common features of the vessels in the RMA-S tumors of IFNAR1<sup>−/−</sup> mice (Figure 6.22A, right panel).

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<td>—</td>
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Table 6.2. Generation of BM chimeras with differential defect in IFNAR1 signaling either on hematopoietic or non-hematopoietic cells.
In a further step, we wanted to address the question whether the observed defect in angiogenesis depends solely on IFNAR1 signaling on non-hematopoietic cells or factors produced by infiltrating leukocytes are also involved. For this purpose, we created BM chimeras, where either the hematopoietic or non-hematopoietic system was impaired in IFNAR1 signaling (Table 2). As expected, normal vessel pattern was observed in the wt tumors, while disoriented vessel formation and destroyed vessels, as documented by the presence of extravasated FITC-lectin, were observed in the tumors of IFNAR1−/− mice (Figure 6.22B). Interestingly, most vessels infiltrating the tumors of either wt → IFNAR1+/− or IFNAR1−/− → wt chimeric mice exhibited an abnormal branching pattern, quite similar to the one observed in the tumors of IFNAR1−/− mice (Figure 6.22B). In summary, these data support that angiogenesis is impaired in the RMA-S tumors of IFNAR1−/− mice and both hematopoietic and non-hematopoietic cells are responsible for this impairment.

Figure 6.22. Impaired angiogenesis in the IFNAR1−/− mice. Groups of 3 C57BL/6 wt or IFNAR1−/− mice (A) or groups of 3 C57BL/6 wt mice or wt > IFNAR1+/− or IFNAR1−/− > wt or IFNAR1+/− > IFNAR1−/− BM chimeras (B) were s.c. inoculated with RMA-S cells. (A-B) Ten days later, FITC-labeled Lycopersicon esculentum lectin was injected into the mice and tumor vasculature was observed in unfixed tumors by confocal microscopy.
7 DISCUSSION

7.1 Suppression of innate immune cells by Treg

In the first part of this study, we investigated the role of Treg on the innate immune response against the lymphoma RMA-S. Our study revealed that Treg suppress RMA-S tumor rejection by inhibiting the IFN-γ-mediated accumulation of leukocytes, including macrophages at the tumor site. Most importantly, we demonstrated that highly activated, tumoristatic macrophages accumulated in the tumor tissue in the absence of Treg, unraveling a novel aspect of Treg-mediated suppression of anti-tumor immune responses in vivo.

During the last years, the role of Treg as professional suppressive cells has been confirmed by several studies. Treg were shown to exert an immunosuppressive role in multiple disease settings, including anti-tumor responses. Indeed, Treg were shown to accumulate in high numbers in both murine and human tumors, as well as in secondary lymphoid organs. This increased accumulation can be due either to proliferation of preexisting Treg or to the conversion of CD4+CD25+ T cells into Treg, which has been described to take place in the periphery under certain conditions. In the s.c. RMA-S mouse lymphoma model, we detected high percentages of CD4+Foxp3+ Treg among CD4+ T cells in secondary lymphoid organs, as well as infiltrating the tumor tissue. Foxp3 expression coincided with CD25 expression on CD4+ T cells, which in turn correlated with suppressive activity in an in vitro assay. Most importantly, in vivo depletion of CD25+ cells led to RMA-S tumor rejection, pinpointing the suppressive role of the CD4+CD25+Foxp3+ Treg in the control of the MHC class I-deficient RMA-S tumor.

There have been many reports addressing the suppressive effect of Treg on T cells in the context of autoimmune diseases, infectious models and tumor immunity. It has been suggested that human Treg have overlapping TCR specificities as Tcon and would be able to suppress in an antigen-specific manner. Thus, the concept that Treg would suppress innate immune cell functions, which are not antigen specific, is intriguing. Nevertheless, there have been some reports which documented the suppressive effect of Treg on cells of the innate immune system. Previous studies showed that Treg inhibited NK cell effector functions in both mouse and human systems. In the absence of Treg, 3LL Lewis Lung carcinoma cells, RAE-1-
transfected B16 melanoma, and RAE-1-transfected RMA-S lymphoma cells, all of which express high levels of the NKG2D ligand RAE-1, were more efficiently controlled in vivo. These data suggest that Treg can directly inhibit NK cell activation towards NKG2D ligand-expressing tumor cells in vivo. Our depletion experiments indicated that NK1.1+ cells are necessary but not sufficient for tumor rejection in the absence of Treg. Notably, in our experiments, the early growth of RMA-S tumors, which do not express NKG2D-ligands, was retarded by NK cells, irrespective of whether Treg were present or absent. Our data indicate that the early NK cell-mediated tumor control, most likely due to direct tumor cell killing, was not hampered by Treg. This suggests that distinct NK cell activation pathways might be differentially controlled by Treg.

We discovered that IFN-γ, produced by NK cells, CD8+ T cells and to a lesser extent by CD4+ T cells, was absolutely required for the eradication of RMA-S cells in Treg depleted mice. In addition, our data revealed that high amounts of IFN-γ were detected in the tumor and that these levels were significantly elevated in the absence of Treg. IFN-γ can exert anti-tumor immunity by various mechanisms. First, IFN-γ can directly suppress tumor growth. Nevertheless, IFN-γ did not influence RMA-S cell proliferation in vitro (data not shown). IFN-γ has also been shown to inhibit angiogenesis in some tumors. In our tumor model, CD31 or MECA-1 staining of tumor sections obtained at day 10 did not show any significant difference in vessel number or localization in the presence or absence of Treg (data not shown). In addition, vessel integrity remained unchanged in the absence or presence of Treg, suggesting that angiogenesis was not altered in Treg depleted mice.

IFN-γ has been shown to facilitate leukocyte migration into tumors, directly or indirectly, by the modulation of certain chemokines and chemokine receptors. In order to gain insight into the immune response within the tumor, we analyzed tumor-infiltrating cells at day 10, before RMA-S tumors were rejected in Treg depleted mice. At this time point, the tumor size between control and Treg depleted mice was similar. We detected that mostly CD11b+ cells -- consisting of macrophages, dendritic cells and granulocytes -- and comparably lower amounts of NK cells, CD4+ and CD8+ T cells accumulated in the tumor tissue. Importantly, all these cell populations were significantly increased in mice depleted of Treg. In this context, it has also been shown that Treg inhibited tissue infiltration of conventional CD4+ T cells in a diabetes model. In addition, injection of Treg into nude mice inhibited accumulation of NK cells to RMA-S cells injected into the peritoneum. In these studies, the Treg mediated mechanisms responsible for the inhibition of cell accumulation were not defined. In a recent study, it was reported that Treg could induce...
NK and CD8\(^+\) T cell death in a granzyme B- and perforin-dependent fashion \(^{187}\), suggesting that this may be the mechanism via which Treg control NK cell numbers also in our tumor model. In a study involving large cohorts of human colorectal cancers, a high density of immune cells, including T cells, correlated with longer survival in patients, suggesting that \textit{in situ} analysis of tumor infiltrating immune cells may be a valuable prognostic tool in the treatment of some types of malignancies \(^{279}\). In the RMA-S \textit{s.c.} tumor model, higher density of infiltrating cells also correlated positively with tumor regression in the absence of Treg. Our experiments further show that the increased CD11b\(^+\) and CD4\(^+\) T and CD8\(^+\) T cell accumulation in the tumors of Treg depleted mice was dependent on IFN-\(\gamma\). Furthermore, the presence of IFN-\(\gamma\) was absolutely required for NK cell accumulation in the tumor tissue.

Finally, IFN-\(\gamma\) is involved in macrophage activation \(^{299}\). Indeed, in the absence of Treg, we did not only find high numbers of macrophages in the tumor tissue but these cells also displayed increased MHC class II expression. The upregulation of MHC class II was dependent on IFN-\(\gamma\). These data suggest that increased IFN-\(\gamma\) levels in the absence of Treg lead to enhanced macrophage activation. We assume that these macrophages, due to enhanced levels of MHC class II, have the potential to induce efficient activation of CD4\(^+\) T cells at the tumor site. In addition, increased numbers of MHC class II \textit{high} DC accumulated in the tumor in the absence of Treg. Taken together, this increase of antigen-presenting cells with high MHC class II expression may be instrumental for efficient presentation of tumor antigens to CD4\(^+\) T cells in the absence of Treg. Indeed, CD4\(^+\) T cells are absolutely required for tumor rejection in these mice. Moreover, the absence of Treg led to the generation of immunological memory.

Tumor-associated macrophages represent the major infiltrating component of many tumors and can have multi-faceted functions depending on the tumor microenvironment \(^{103}\). In several experimental tumor models, activation of an inflammatory response mediated by macrophages promoted tumor progression \(^{300}\). In addition, a subpopulation of myeloid cells, designated as MDSC (myeloid-derived suppressor cells), expressing the markers Gr-1 and CD11b, was shown to suppress T cell responses \(^{301}\). We have evidence that the tumor-infiltrating macrophages in our model did not suppress, but rather activated T cell proliferation (data not shown). Therefore, our data suggest that in our model, tumor-infiltrating macrophages are distinct from myeloid-derived suppressor cells. Studies have shown that macrophages produce inflammatory chemokines and cytokines, which recruit and activate other cell types \(^{103}\). In addition, tumor-infiltrating macrophages have the potential to directly control tumor cell
proliferation when appropriately stimulated, e.g. by potently activated T cells in the presence of IFN-\(\gamma\) or after CpG treatment \(^{109,110}\). Our experiments showed that, in the absence of Treg, macrophages isolated from tumors produced higher amounts of chemokines and inflammatory cytokines, which could lead to efficient cell recruitment and activation. Most importantly, enriched CD11b\(^+\) cells and macrophages isolated from tumors in the absence of Treg inhibited tumor cell growth more potently, as compared to cells isolated from control tumors. The suppressive effect of macrophages on the growth of RMA-S cells \(\textit{in vitro}\) was abrogated when IFN-\(\gamma\) was neutralized \(\textit{in vivo}\) during the anti-tumor immune response. One effector molecule expressed by activated macrophages is nitric oxide (NO) produced by the enzyme inducible nitric oxide synthase (iNOS), which has tumor-suppressing or tumor-promoting functions. In the presence and absence of Treg, tumor growth in iNOS\(^{-/-}\) mice progressed similarly to the wt controls. In addition, the presence of a NO inhibitor did not affect macrophage-mediated inhibition of tumor cell proliferation \(\textit{in vitro}\). Similarly, the addition of inhibitors against the activity of PGE-2 and IDO did not affect RMA-S tumor growth in the presence of macrophages \(\textit{in vitro}\). These data argue against a role of the enzymes NO, PGE-2 and IDO in our model.

Of note, IFN-\(\gamma\) neutralization had a diverse effect on the pro-inflammatory cytokine profile in the absence of Treg; while it did not affect IL-1\(\beta\) production by CD11b\(^+\) cells, it strongly enhanced IL-6 production. This is of importance, since IL-6 in combination with TGF-\(\beta\) has been shown to be necessary for the differentiation of naïve T cells into Th17 cells \(^{302}\). It is also reported that IFN-\(\gamma\) can negatively regulate Th17 cell differentiation and \textit{vice versa} \(^{303}\). It is possible that the presence of IFN-\(\gamma\) in the absence of Treg suppresses Th17 polarization and protects the host from autoimmune reactions, which have often been linked to Th17 cells \(^{304}\). Of note, IL-17 was detected in low amounts in tumor lysates from both control and Treg depleted animals 10 days after RMA-S tumor inoculation (data not shown). It will be of interest to quantify Th17 levels in the RMA-S tumor tissue after IFN-\(\gamma\) neutralization.

It has been reported that Treg suppress macrophage activation and effector function directly \(\textit{in vitro}\) \(^{190,193}\). \textit{In vivo}, Kim \textit{et al.} documented that macrophage expansion was under the control of Treg under steady state conditions in adult mice \(^{195}\). Kryczek \textit{et al.} showed upregulation of the B7 family member B7-H4 (B7S1, B7-S1, B7x) on isolated human monocytes by Treg after direct contact in an \(\textit{in vitro}\) co-culture \(^{191}\). In mice, B7-H4 is expressed upon activation on B cells, T cells and monocytes and was shown to negatively regulate T cell
responses. Its ligand remains to be identified, but evidence support that it is expressed on activated T cells. In addition, B7-H4 expression on macrophages infiltrating ovarian tumors correlated with the patients’ survival. In our tumor model, no difference in the expression of B7-H4 was observed on tumor-infiltrating macrophages between control and Treg depleted mice. Our study reveals a novel role of Treg mediated suppression of macrophage function in a tumor model in vivo. Whether the suppression of macrophages requires a direct Treg/macrophage interaction, as observed for Treg/DC interaction in vivo, or is rather due to indirect effects via inhibition of other immune cells, requires further investigation. Kryczek et al showed that Treg directly suppressed macrophages in an in vitro co-culture. Our experiments demonstrate that in vivo macrophage accumulation at the tumor site was absolutely dependent on CD4+ T cells and to a lesser extent on NK cells. In addition, increased macrophage accumulation in the absence of Treg, MHC class II upregulation and tumoristatic activity of macrophages were all dependent on IFN-γ, which in turn Treg suppressed its production. We assume that, in our model, Treg suppressed IFN-γ production by lymphocytes, which in a second step interfered with macrophage activation and accumulation at the tumor site.

The mechanism that Treg use to exert their suppressive function in the RMA-S model is still under investigation. Neutralization of TGF-β or blocking IL-10R by specific mAbs, two pathways that have been linked to Treg-mediated suppression, did not influence tumor growth and did not lead to tumor rejection in our model, suggesting that neither TGF-β nor IL-10 are required for Treg mediated suppression in vivo. Recently, it was reported that IL-35 is specifically produced by Treg and not effector T cells and can mediate suppression. So far, reagents to block this cytokine in vivo are not available.

Unexpectedly, we discovered an important contribution of CD8+ T cells in the rejection of the MHC class I-deficient RMA-S tumor in the absence of Treg, as well as during the memory response against a secondary challenge to RMA-S cells. In this context, Kelly et al. reported that CD8+ T cells mediated memory responses after rejection of RMA-S cells transfected with CD70 or CD80. The mechanism, however, via which CD8+ T cells would recognize RMA-S cells was not addressed in this studies. In our model, CD8+ T cells isolated from RMA-S tumor tissue during the primary anti-tumor response or from the secondary lymphoid organs of mice which had previously rejected RMA-S tumors after Treg depletion were able to directly recognize RMA-S cells in an in vitro co-culture. It has been reported that the TAP-negative RMA-S cells, which express only very low amounts of MHC class I on their surface, present a range of
antigens – referred to as TEIPP – which are TAP-independent and can be recognized by CD8\(^+\) T cells\(^{267,285}\). It is possible that Treg depletion increases the potential recognition of such peptides by CD8\(^+\) T cells, allowing potent antigen-specific responses to take place even against targets which express very low amounts of MHC class I. This observation might offer new therapeutic approaches against human cancers, which often have low MHC class I expression.

The manipulation of Treg is currently evaluated in clinical trials in tumor patients\(^{209,210}\). It will be informative to monitor numbers and activation status of tumor-infiltrating macrophages in patients undergoing Treg depletion or treated with other immunotherapeutic protocols and to correlate these data with the clinical outcome. The results of our study may have substantial impact on the design of novel strategies in anti-cancer therapy to strengthen both the innate and adaptive immune responses against cancer.

### 7.2 The role of endogenous type I IFN in the anti-tumor response

In the second part of this study, we addressed the role of endogenously produced type I IFN in the anti-tumor response against the MHC class I-deficient lymphoma RMA-S. We showed that RMA-S tumor growth is accelerated in mice, which are non-responsive to type I IFN. Our data demonstrate that this acceleration in tumor growth correlates with defects in NK cell activity and vessel network formation at the tumor site.

Type I IFN have been extensively used in the treatment of various types of cancer, although their mechanism of action is not completely clear\(^{252}\). *In vitro* high doses of type I IFN promote cell cycle arrest in various cell types\(^{248,249}\). It is believed that this may be their mechanism of action after exogenous administration in cancer patients. The study of RMA-S tumor growth in the IFNAR1\(^{-/-}\) mice allows dissecting the effect of type I IFN on the host and on the tumor cells. The accelerated tumor growth observed in these mice after s.c. RMA-S inoculation showed that type I IFN need to signal to the host’s cells for an efficient anti-tumor response. Our findings are in concordance with other reports, which demonstrated a role for type I IFN in tumor immunosurveillance using IFNAR1\(^{-/-}\) 129/Sv mice\(^{258}\). In this study, transplanted tumors originating from MCA-induced fibrosarcomas were under the control of type I IFN-responsive hematopoietic cells. In the RMA-S model, by the generation of BM chimeras, we
showed that cells of both the hematopoietic and non-hematopoietic system responded to type I IFN and were required for an efficient anti-tumor response. The different tumor origin in these models may account for this difference in the immune response. In addition, it was reported that IFN-α did not affect the proliferation of RMA-S cells in vitro. Our results pinpoint a role for endogenously produced type I IFN in the anti-tumor response against the RMA-S tumor, and in addition suggest a mechanism of action, which is dependent on the host’s response to type I IFN.

Type I IFN can exert multi-faceted functions in the hematopoietic system. Type I IFN can promote CD8+ T cell-mediated responses, induce the maturation of DC and indirectly enhance NK cell cytotoxicity. We addressed the question which cell population was impaired during the anti-tumor response in the IFNAR1−/− mice. MHC class I-deficient RMA-S tumor growth is under the control of NK cells. Indeed, we found that NK cells derived from IFNAR1−/− mice exhibited impaired cytotoxicity against two different tumor targets in vitro. This finding is in concordance with a report from another group, which in addition observed reduced NK cell numbers in the spleens of IFNAR1−/− mice. This observation pinpoints to a role for type I IFN in the homeostasis of NK cells, and not only in their function during an immune response. It is known that IFN-β, binding to the IFNAR1, is constitutively produced by fibroblasts and macrophages, so it is possible that these constant levels of IFN-β contribute to NK cell homeostasis. Interestingly, despite the reduced occurrence of NK cells in the spleens of IFNAR1−/− mice, we observed that the accumulation of NK cells – as well as other leukocyte populations – at the tumor site was not impaired. This observation suggests that the factors responsible for the recruitment of leukocytes at the tumor tissue during the anti-tumor response to RMA-S cells are not under the control of endogenous type I IFN. We showed that, in addition to NK cells, in the RMA-S model macrophages are important effector cells, which mediate a tumorstatic effect. It was reported that the response of macrophages to CSF-1 and LPS was impaired in IFNAR1−/− mice. In a co-culture of macrophages and RMA-S cells, the suppressive effect of macrophages on RMA-S tumor growth was indistinguishable between macrophages originating from wt and IFNAR1−/− mice, suggesting that the anti-tumor response of macrophages might not be tightly regulated by type I IFN. Our study correlated the acceleration in tumor growth in IFNAR1−/− mice with an impaired function of NK cells, without excluding, nevertheless, that other cell populations might be affected.

An effect of type I IFN on non-hematopoietic cells has been documented. Treatment with type I IFN caused damage on endothelial cells, which led to tumor necrosis. Anti-angiogenesis
factors which inhibit blood vessel growth are commonly used in cancer immunotherapy based on the notion that angiogenesis is absolutely required for tumor growth. IFN-α has been introduced into clinical applications as an anti-angiogenic factor to treat haemangiomas. In IFNAR1⁻/⁻ mice, we observed increased vascularization, albeit deregulated. The use of in vivo fluorescent angiography revealed a difference in vascular architecture of the tumors of IFNAR1⁻/⁻ versus wt mice, and namely a less organized blood vessel network in the IFNAR1⁻/⁻ mice. In addition, extravasated FITC-lectin was detected. The latter finding pinpoints to the lack of sufficient pericyte recruitment, which form a layer around the endothelial cells. Stainings of tumor sections for pericyte markers should address these questions. It is suggested that angiogenic factors, such as basic fibroblast growth factor (bFGF), downregulate adhesion molecules on endothelial cells, which are involved in leukocyte-vessel wall interactions, such as intercellular adhesion molecule (ICAM)-1. This correlated with decreased leukocyte infiltration of the tumors and thus to reduced immune surveillance. We found that leukocyte accumulation in the tumors of IFNAR1⁻/⁻ mice, as assessed by flow cytometry, was similar to the wt levels, suggesting that this mechanism might not be impaired in IFNAR1⁻/⁻ mice. Nevertheless, it is possible that leukocyte localization within the tumor tissue is different between wt and IFNAR1⁻/⁻ mice. Analysis of tumor tissue sections can address the question whether leukocytes are uniformly distributed throughout the tumor mass in wt and IFNAR1⁻/⁻ mice. In summary, our findings show that endogenous type I IFN are important for the architecture of tumor vasculature and the formation of mature blood vessels; the contribution of an organized vessel network to tumor growth control needs to be elucidated.

The phenotype of impaired anti-tumor response in the IFNAR1⁻/⁻ mice could be mimicked by the administration of an anti-IFN-β neutralizing mAb in vivo. This result implicates IFN-β production during the anti-tumor response, without excluding a role also for the different subtypes of IFN-α. During an anti-viral response, IFN-β and/or IFN-α4 genes are readily induced and subsequently induce the production of the other subtypes of IFN-α. A similar mechanism may as well take place during the anti-tumor response. We were able to detect both IFN-β and IFN-α mRNA in leukocytes infiltrating the RMA-S tumors 10 days after tumor inoculation (data not shown), showing that at least some subtype of IFN-α is produced during the anti-tumor response. Due to the lack of efficiently neutralizing anti-IFN-α mAb, we were not able to address the importance of IFN-α in vivo. The use of an anti-IFN-β neutralizing mAb also
excluded a possible developmental defect in the IFNAR1−/− mice which could be responsible for the observed phenotype. The identification of the type I IFN-producing cell population during the anti-tumor response remains still unclear. In principle, all cells are capable of producing type I IFN during a viral infection, although pDC are specialized IFN-producing cells producing high amounts of type I IFN after TLR stimulation. We could detect IFN-β and IFN-α mRNA on leukocytes of both myeloid and non-myeloid lineage based on CD11b marker discrimination, and in addition IFN-β mRNA on non-hematopoietic cells (data not shown), suggesting that – similar to an anti-viral response – the production of type I IFN during the anti-tumor response can have multiple sources. Nevertheless, the stimuli which can induce the production of type I IFN during the tumor growth remain unknown.

Further investigations on the source and function of endogenous type I IFN during the anti-tumor response will help to identify new therapeutic targets for cancer immunotherapy, and assist the efficacy of the presently applied clinical treatments which are based on the use of type I IFN.
8 References


100. Wiktor-Jedrzejczak, W. & Gordon, S. Cytokine regulation of the macrophage (M phi) system studied using the colony stimulating factor-1-deficient op/op mouse. Physiol Rev 76, 927-47 (1996).


292. Fazilleau, N., Bachelez, H., Gougeon, M.L. & Viguier, M. Cutting edge: size and diversity of CD4+CD25high Foxp3+ regulatory T cell repertoire in humans: evidence for...


## 9 ABBREVIATIONS

(listed in alphabetical order)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>1MT</td>
<td>1-Methyl-L-Tryptophan</td>
</tr>
<tr>
<td>α-</td>
<td>anti-</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium Chloride potassium phosphate buffer</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-Dependent Cell-mediated Cytotoxicity</td>
</tr>
<tr>
<td>APC</td>
<td>AlloPhycoCyanin</td>
</tr>
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<td>APC</td>
<td>Antigen Presenting Cells</td>
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<tr>
<td>BCR</td>
<td>B Cell Receptor</td>
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<tr>
<td>bFGF</td>
<td>basic Fibroblast Growth Factor</td>
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<td>BM</td>
<td>Bone Marrow</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CSF</td>
<td>Colony Stimulating Factor</td>
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<tr>
<td>CFSE</td>
<td>CarboxyFluorescein Succinimidyl Ester</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated Antigen-4</td>
</tr>
<tr>
<td>Cy5</td>
<td>Cyanine 5</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>ddH2O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DLN</td>
<td>Draining LN</td>
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<tr>
<td>DMSO</td>
<td>DiMethylSulfOxide</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
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<tr>
<td>EMCV</td>
<td>EncephaloMyocarditis Virus</td>
</tr>
<tr>
<td>E:T</td>
<td>Effector to Target</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein-IsoThioCyanate</td>
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<td>FSC</td>
<td>Forward SCatter</td>
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<td>g</td>
<td>gram</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte/Macrophage CSF</td>
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<tr>
<td>GITR</td>
<td>Glucocorticoid-induced TNF-receptor family-Related</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<td>HSV</td>
<td>Herpes Simplex Virus</td>
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<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
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<tr>
<td>ICAM</td>
<td>InterCellular Adhesion Molecule</td>
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<tr>
<td>IDO</td>
<td>Indoleamine-pyrrole 2,3-dioxygenase</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
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<td>IFNAR1</td>
<td>Interferon-α Receptor 1</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>InterLeukin</td>
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<tr>
<td>iMC</td>
<td>immature Myeloid Cells</td>
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<tr>
<td>iNOS</td>
<td>inducible Nitric Oxide Synthase</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>IPEX</td>
<td>Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked</td>
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<td>L/D-NMMA</td>
<td>L/D-N^4-MonoMethyl Arginine</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Node</td>
</tr>
<tr>
<td>LPS</td>
<td>LipoPolySaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal Antibody</td>
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<tr>
<td>MACS</td>
<td>Magnetic Cell Sorting</td>
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<tr>
<td>µC</td>
<td>microCurie</td>
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<tr>
<td>MCA</td>
<td>MethylCholAnthrene</td>
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<td>MCMV</td>
<td>Murine CytoMegaloVirus</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma Differentiation-Associated protein 5</td>
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<td>MDSC</td>
<td>Myeloid-DerivedSuppressorCells</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>Mφ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>min</td>
<td>minute</td>
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<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
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<tr>
<td>µl</td>
<td>microlitre</td>
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<td>ml</td>
<td>millilitre</td>
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<td>-------------</td>
</tr>
<tr>
<td>µM</td>
<td>microMolar</td>
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<tr>
<td>mM</td>
<td>miliMolar</td>
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<tr>
<td>MME</td>
<td>Macrophage MetalloElastase</td>
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<tr>
<td>MMP</td>
<td>Matrix MetalloProteinase</td>
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<tr>
<td>MSC</td>
<td>Myeloid Suppressor Cells</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
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<td>NKG2D</td>
<td>NK Group 2 member D</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>NOD</td>
<td>Nucleotide-Oligomerization Domains</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>O/N</td>
<td>Over Night</td>
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<td>PAMP</td>
<td>Pathogen-Associated Molecular Patterns</td>
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<td>PBL</td>
<td>Peripheral Blood Lymphocytes</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>pDC</td>
<td>plasmacytoid DC</td>
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<td>PD-ECGF</td>
<td>Platelet-Derived Endothelial Cell Growth Factor</td>
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<td>pg</td>
<td>picogram</td>
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<td>pH</td>
<td>potential Hydrogeni</td>
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<td>PGE2</td>
<td>Prostaglandin E synthase 2</td>
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<td>Poly(I:C)</td>
<td>PolyInosinic-PolyCytidylic acid</td>
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<td>Prf</td>
<td>Perforin</td>
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<td>PRR</td>
<td>Pattern-Recognition Receptors</td>
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<td>Retinoic Acid Early inducible-1</td>
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<td>RAG</td>
<td>Recombination-Activation Gene</td>
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<tr>
<td>rpm</td>
<td>rounds per minute</td>
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<td>RT</td>
<td>Room Temperature</td>
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<td>s.c.</td>
<td>subcutaneous</td>
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<td>SuperNatant</td>
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<td>SSC</td>
<td>Side SCatter</td>
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<td>Description</td>
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<tr>
<td>TAA</td>
<td>Tumor-Associated Antigens</td>
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<td>Tcon</td>
<td>conventional T cells</td>
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<tr>
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<td>T Cell Receptor</td>
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<td>Type I Diabetes</td>
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<td>Toll-Like Receptors</td>
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<td>TRAIL</td>
<td>TNF–Related Apoptosis-Inducing Ligand</td>
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<td>Regulatory T cells</td>
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<td>TSA</td>
<td>Tumor-Specific Antigens</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>wt</td>
<td>wild type</td>
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</table>
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