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„The role of EGFR in antigen presentation by dendritic cells and macrophages"

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Abstract

It has been previously shown in the lab of Maria Sibilia that EGFR expression in liver macrophages and tumour-associated myeloid cells has a tumour-promoting role in mouse models of hepatocellular carcinoma (HCC) and colorectal cancer (CRC), respectively. Mice lacking EGFR in myeloid cells, but not hepatocytes (in the HCC mouse model) or intestinal epithelial cells (in the CRC mouse model) develop fewer tumours. Additionally, EGFR expression in liver macrophages correlates with poor prognosis in HCC patients and elevated expression of the receptor in myeloid cells of colorectal tumour stroma is associated with tumour progression in patients with metastatic CRC. Furthermore, the EGFR signalling pathway in liver macrophages could be linked to the transcriptional induction and secretion of IL-6, thus promoting tumour progression.

Giving this, we asked the question if this is the only mechanism which leads to tumour progression or if the expression of EGFR in macrophages regulates other basic immunological functions, such as the generation of specific T cell-mediated anti-tumour immune responses. Therefore, my work focused on the investigation of the role of EGFR in antigen presentation and induction of T cell proliferation mediated by macrophages and dendritic cells. Additionally, in a parallel set-up, I also detected and characterised the expression pattern of co-signalling molecules on the surface of these antigen-presenting cells (APCs). These molecules play a tremendously important role in T cell biology, by helping to determine the outcome of T cell responses upon antigen presentation. Moreover, I investigated whether the expression of EGFR in macrophages influences the capacity of these cells to induce different types of effector $CD4^+$ T cells.

There was a trend where EGFR in macrophages played a role in the induction of T_H17 and Treg effector T cells, however I could neither detect EGFR-mediated differences in the expression pattern of co-signalling molecules on macrophages or dendritic cells nor in the efficiency of these APCs to present antigen to T cells.

Nevertheless, additional experiments would need to be carried out, adapting my antigen presentation assay to an *in vivo* model, thus maybe shedding light on how EGFR in macrophages could regulate T cell-mediated immune responses.

Zusammenfassung

Es wurde bereits in der Arbeitsgruppe von Maria Sibilia gezeigt, dass die Expression des EGF Rezeptors in Leber-Makrophagen sowie auch in tumorassoziierten myeloische Zellen eine fördernde Rolle in der Entstehung von Leberzellkarzinom bzw. Dickdarmkrebs in Mäusen hat. Mausmodelle welche den EGF Rezeptor in den myeloischen Zellen, aber nicht in den Hepatoyzten (im Falle des Leberzellkarzinom Mausmodells) oder den Darmepithelzellen (im Falle des Darmkrebs Mausmodells) nicht exprimieren, entwickeln weniger Tumore. Zusätzlich wird die Expression des EGF Rezeptors in Leber-Makrophagen mit einer schlechten Prognoze für Patienten mit Leberzellkarzinom assoziert. Außerdem korreliert eine erhöhte Expression des Rezeptors in den myeloischen Zellen des Stromas mit der Tumorprogression bei Patienten mit einer metastasierenden Form von Dickdarmkrebs. Des Weiteren konnte man den Signaltransduktionsweg des EGF Rezeptors in den Leber-Makrophagen mit der Transkriptionsinduktion und Sekretion von IL-6 verknüpfen, welches die Tumorprogression fördert.

Diesbezüglich haben wir uns die Frage gestellt ob das der einzige Mechanismus ist welcher zur Tumorprogression führt, oder ob der EGF Rezeptor in den Makrophagen nicht vielleicht auch andere grundlegende immunologische Funktionen reguliert, wie z.B. die Erzeugung von spezifischen T-Zell-vermittelten Antitumor-Immunantworten. Daher konzentrierte sich meine Arbeit in der Untersuchung der Rolle des EGF Rezeptors in Antigenpräsentation und Induktion der T-Zellproliferation welche von Makrophagen und dendritischen Zellen vermittelt wird. Zusätzlich habe ich mich gleichzeitig auch auf die Erkennung und Charakterisierung von Co-Signalmolekülen in EGF Rezeptor-defizienten Makrophagen und dendritischen Zellen konzentriert. Diese Moleküle spielen eine enorm wichtige Rolle in der T-Zell-Biologie, indem sie dazu beitragen die T-Zell-Antowrt zu modulieren, nach dem Antigen presäntiert wurde. Zusätzlich, untersuchte ich ob der EGF Rezeptor die Fähigkeit der Makrophagen unterschiedliche Arten von Effektor-$CD4^+$-T-Zellen zu induzieren, beeiflusst.

Es gab eine Trendenz wo der EGF Rezeptor in Makrophagen eine Rolle bei der Induktion von Effektor-T-Zellen wie T_H17 und Tregs gespielt hat. Nichtdestotrotz, konnte ich keine EGF Rezeptor-vermittelten Unterschiede in der Expression von

Co-Signalmolekülen auf Makrophagen oder dendritischen Zellen, noch in deren Effizient Antigen an T-Zellen zu präsentieren, nachwiesen.

Dennoch, müssten zusätzliche Experimente durchgeführt werden, wobei das von mir ausgeführte Antigenpräsetations Assay an einem In-vivo-Modell angepasst werden kann. Dies möge Aufschluss darüber geben, wie der EGF Rezeptor in Makrophagen T-Zell-vermittelnde Immunantworten regulieren könnte.

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1. Introduction

1.1 The Epidermal Growth Factor Receptor

The Epidermal Growth Factor Receptor (EGFR or also known as ErbB-1 or HER1 in humans) is a transmembrane glycoprotein which belongs to the ErbB family of receptor tyrosine kinases (RTKs) that encompasses, additionally to the EGFR, three other closely related subfamilies (ErbB-2/HER2/neu in rodents, ErbB-3/HER3, and ErbB-4/HER4) (Wieduwilt MJ 2008). The receptor is activated by ligand binding to its extracellular domain, inducing homo- or heterodimer formation with other members of the family. Dimerisation is necessary to induce activation of the intracellular tyrosine kinase domain of the receptor and therefore resulting in the autophosphorylation of tyrosine residues in the C-terminus. These residues will then further activate downstream proteins to initiate several signalling cascades, primarily, Ras/MAPK, PI3K/Akt, and JAK/STAT which regulate cellular mechanisms such as cell proliferation, differentiation, and survival (Scaltriti M 2006) (Fig. 1). Activation of EGFR is very important for the embryogenesis and organogenesis in mice and impaired expression of the receptor may result in growth retardation and accumulation of multiple abnormalities in bone, brain, and several epithelial tissues such as skin, hair follicles and eyes (Sibilia M 1995, Sibilia M 1998, Miettinen PJ 1995, Threadgill DW 1995, Wang K 2004, Wong RW 2004).

The EGFR signalling pathway is known to be involved in various types of epithelial cancers in humans. The receptor and its ligands are often abnormally overexpressed in many epithelial tumours and additionally, amplification of the gene and mutations of the tyrosine kinase domain are also detectable in carcinoma patients (Normanno N 2006). Therefore, specific anti-EGFR treatments are one of the key therapeutic strategies used in cancer therapies. There are two major types of therapies targeting this pathway involving treatment of patients with monoclonal antibodies (mABs) or tyrosine kinase inhibitors (TKIs). Cetuximab is an mAb which competes with endogenous ligands for the extracellular binding site and because of its higher affinity, it blocks ligand-binding and therefore inhibits ligand-mediated EGFR activation (Harding J 2005). In contrast, TKIs are small molecules e.g. gefitinib and erlotinib which inhibit the kinase activity of the receptor by engaging to the adenosine

triphosphate (ATP) binding site of the enzyme, thus interfering with its function to induce tyrosine phosphorylation (Wakeling AE 2002).

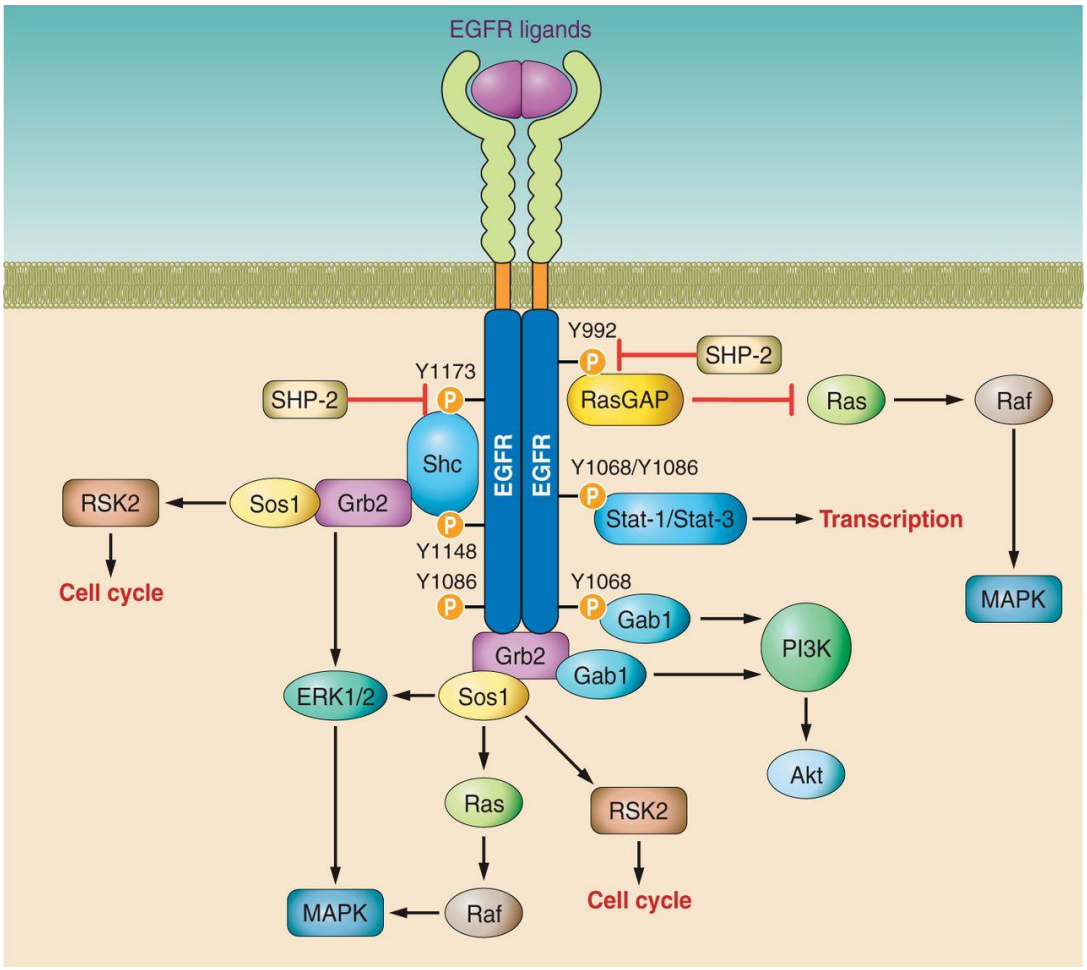


Fig. 1: Several EGFR downstream signalling cascades. *Receptor dimerisation is induced upon extracellular ligand binding, following activation of the intrinsic kinase domain and autophosphorylation of different tyrosine (Y) residues. These will then further activate signalling proteins leading to induction of downstream signalling pathways regulating cell proliferation, differentiation, and survival. The main EGFR downstream signalling pathways involve Ras/MAPK, PI3K/Akt, and JAK/STAT, although other molecules are also known to be regulated by EGFR. Akt – protein kinase B; ERK – extracellular signal-regulated kinase; Gab – Grb2-associated protein; Grb – growth factor receptor-binding protein; MAPK – mitogen activating protein kinase; PI3K – phosphatidylinositol-3-kinase; Raf – rapidly accelerated fibrosarcoma; Ras – rat sarcoma; RSK – ribosomal S6 kinase; Shc – SH2 domain protein C1; SHP – small heterodimer partner; SOS – son of sevenless; STAT – signal transducer and activator of transcription. DOI: 10.1152/physrev.00030.2015*

Although promising in the beginning, most patients who suffer from head and neck, metastatic lung, colorectal or pancreatic cancer will develop resistance to EGFR-targeted therapies (Yewale C 2013, Chen J 2016). Interestingly, a substitution of threonine with methionine at the position 790 (T790M) makes tumours expressing

this mutant form of EGFR, resistant to TKIs therapies, thus mediating a so-called innate resistance (Inukai M 2006). In contrast, mutations such as L858R (substitution of leucine with arginine) or exon 19 deletion, prove to be clinical beneficial, as patients respond very well to TKIs inhibitors. Nonetheless, those patients might acquire resistance to the treatment over time (Lynch TJ 2004). Hence, an increasing effort is invested into studying the mechanism and complexity of resistance to EGFR inhibitors, allowing us to generate and develop new drugs and therapies with increased specificity, thus diminishing therapy-induced resistance in tumours (Chong CR 2013).

Until recently, most of the cancer research had focused on the malignant cells themselves. Nowadays, understanding the nature and complexity of tumour microenvironment might be as important for the development of future therapies as understanding which mechanisms drive malignancies. A tumour consists not only of cancer cells but also immune cells, fibroblast, and endothelial cells, all of which can be hijacked by the malignant cells to sustain and promote tumour growth and even metastasis (Bremnes RM 2011). Therefore, tumour progression is not only dependent on cancer cells but also on the cells present in their microenvironment. According to this, studies have suggested that EGFR in liver macrophages but also in tumour-associated myeloid cells has a tumour-promoting role in the development of hepatocellular carcinoma (HCC) and colorectal cancer (CRC), respectively (Lanaya H 2014, Srivatsa S 2017). The expression of the receptor in Kupffer cells/liver macrophages correlates with poor prognosis in HCC patients and additionally, increased expression in myeloid cells of tumour stroma is associated with tumour progression in patients with metastatic CRC. Furthermore, they unveiled that mice deficient in EGFR expression in myeloid cells develop fewer tumours and that the EGFR signalling pathway in liver macrophages induced transcription and secretion of interleukin 6 (IL-6). Diethylnitrosamine (DEN)-induced liver damage lead to IL-1 production by injured hepatocytes, thus mediating EGFR-dependent IL-6 production by liver macrophages (Lanaya H 2014). Mechanistically, the IL-6 production was mediated by an autocrine feedback where activation of IL-1 receptor/MyD88 signalling in liver macrophages induced expression of the metalloprotease ADAM17 (TACE) which cleaved and released EGFR ligands from the cytoplasm (Chalaris A 2010).

Increased presence of ligands activated EGFR signalling in these cells which induced IL-6 production, thus leading to HCC progression (Lanaya H 2014).

This study concluded that EGFR signalling pathway has a tumour-promoting function in liver macrophages, however we were interested to see if this was the only mechanism leading to tumour progression or if EGFR in macrophages could regulate other immunological functions, such as modulating adaptive immune responses.

1.2 Anti-tumour immune responses

The organism has two lines of defence mechanisms against foreign or even self-material which proves to be harmful. The innate immune response is a non-specific defence mechanism which is generated immediately or within hours after an antigen is sensed in the body. It is based on the ability of the complement system and phagocytic cells to recognize and react to conserved features of harmful molecules such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Tosi MF 2005, Tang D 2012). PAMPs are derived from microorganisms, whereas DAMPs are cell-derived and induce immunity in response to trauma or tissue-damage mediated by e.g. transformed cells (Tang D 2012). If the cells of the innate immune system fail to eliminate the danger, adaptive immunity is initiated through antigen presentation by antigen-presenting cells (APCs). Antigen presentation will give rise to antigen-specific effector $CD4^+$ or $CD8^+$ T cells (Janeway CA Jr 2001). B cells, which are also part of the adaptive immune system, can take up pathogens by themselves and do not need antigen presentation. Nonetheless, they are depended on the help of so-called T helper cells (T_H) to get activated (Parker DC 1993). T_H cells are effector $CD4^+$ T cells which can not only modulate adaptiv immune responses by activating B cells, but also sustain innate immune responses by activating macrophages and other innate immune cells.

Our current understanding of how adaptive immune responses against tumours are modulated involves several steps, all of which have to be met for inducing an effective anti-tumour immune response (Mellman I 2011). At first tumour antigen must be taken up by APCs, mostly dendritic cells (DCs), which will start processing the antigen for its presentation. For their maturation, APCs need a second signal mediated by cytokine

stimulation. Activated APCs will then travel to lymph nodes, where they can present the tumour antigen to naïve T cells generating a protective T cell-mediated immune response (Mellman I 2001). It is known that $CD8^+$ cytotoxic T cells play a very important role in tumour clearance (Titu LV 2002). Moreover, APCs might also trigger natural killer (NK) cell responses which can also lead to tumour immunity (Fregni G 2012). Furthermore, upon antigen presentation different subtypes of effector $CD4^+$ T cells can be induced, which can modulate innate but also adaptive immune responses (Toes REM 1999, Dobrzanski MJ 2013). However, APCs can mediate tolerance through antigen presentation by inducing regulatory T cells (Tregs) and therefore favouring immunosuppression (Steinman RM 2003). Once activated by DCs, effector T cells will travel to the site of tumour, where they can promote their function in eliminating cancer or in some unfortunate cases promote suppression of effector T cell function (Fig. 2).

Interestingly, tumour cells exhibit specific features which allow them to escape immune surveillance. These features are summarised and described as part of the 10 hallmarks of cancer (Hanahan D1 2011). Not only can tumour cells (or infiltrating myeloid cells) inhibit the function of cytotoxic T and NK cells by e.g. expressing co-inhibitory molecules, such as the programmed cell death-ligand 1 (PDL-1) which blocks signal transduction mediated by the T cell receptor (TCR), but they can also downregulate the expression of MHC molecules, therefore reducing antigen presentation to T cells and escaping recognition by the immune system. Additionally, secretion of immunosuppressive mediators and recruitment of Tregs or myeloid-derived suppressor cells sustain immunosuppression and tumour survival (Hanahan D1 2011).

However, a good understanding of how immune cells interact with one another and their needed activation status to generate an anti-tumour response had been shown to be very important for the development of therapies which in the end should boost the immune response and lead to tumour clearance. Vaccination, for once, although initially used as a prophylactic treatment for the prevention of cancer induced by viruses, such as the human papilloma virus (HPV), might also work therapeutically by possibly amplifying the pre-existing response to tumours (Mellman I 2011).

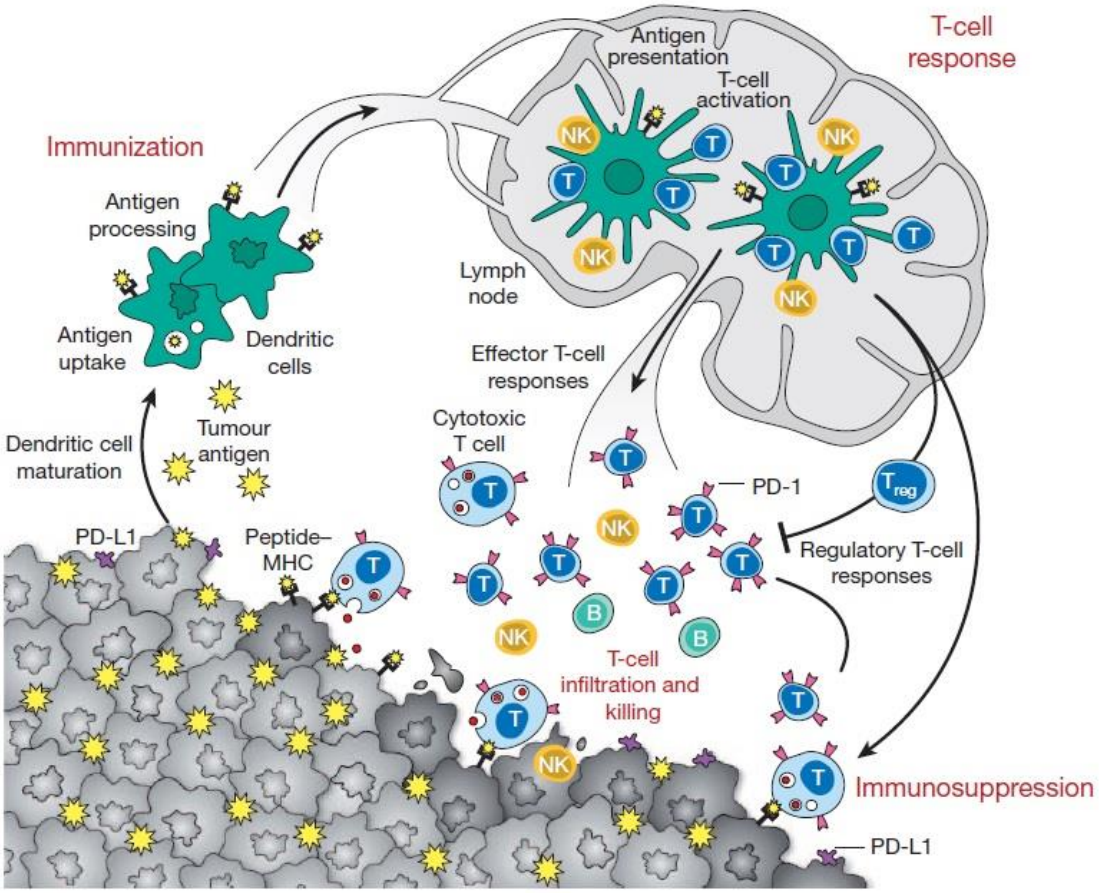


Fig. 2: Generation of T cell-mediated anti-tumour immune responses. *Immunisation is the first step in which a dendritic cell (DC) takes up tumour antigen and starts processing it. DCs will then migrate to draining lymph nodes where the antigen will be presented to naïve T cells. Depending on whether DCs are subjected to an additional maturation stimulus during the capturing of the antigen and its presentation, the cells will induce T cell-mediated anti-tumour immune responses or tolerance through regulatory T cell (Treg)-mediated immunosuppression. Activated T cells will travel back to the site of tumour where they can perform their function by killing tumour cells or modulating innate as well as adaptive immune responses. Immunosuppression can be also generated in the periphery due to the expression of co-inhibitory molecules, such as the programmed cell death-ligand 1 (PD-L1), by tumour or other cells present in the tumour microenvironment. PD-L1 may engage to its receptor programmed cell death protein 1 (PD-1) on the surface of T cells, therefore inhibiting effector T cell function. DOI: 10.1038/nature10673*

1.3 Antigen presentation and T cell response

T cells recognize antigen by their antigen-specific TCR which pairs with the MHC class I or class II molecule expressed on the surface of all nucleated cells or APCs (e.g. DCs, macrophages or B cells), respectively. For the induction of a T cell-mediated immune response, peptide fragments of specific lengths and amino acid signature need to be displayed by the MHC molecules. During the process of antigen presentation mediated by the MHC class I molecule, cytosolic peptides are presented to naïve $CD8^+$ T cells, thus inducing a cytotoxic immune response (Blum JS 2013) (Fig. 3).

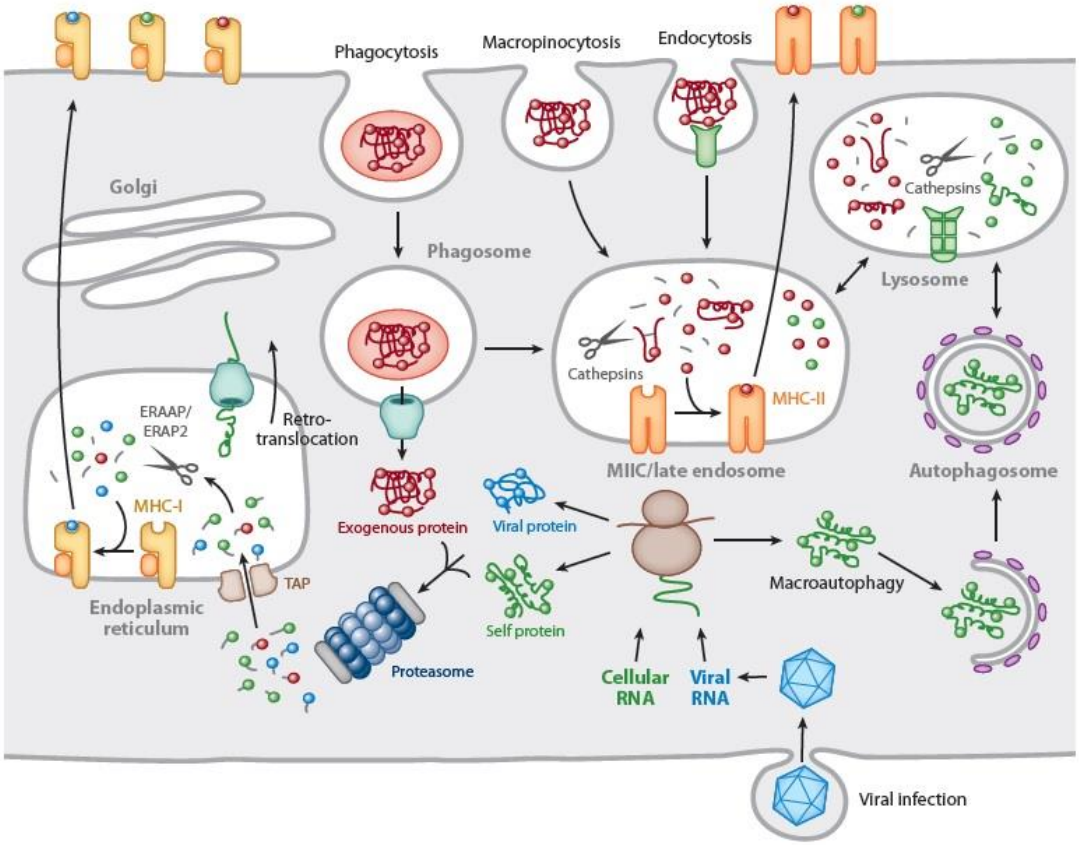


Fig. 3: Processing and loading of antigen onto the MHC class I or class II molecule. *Cytoplasmic proteins are processed by the proteasome, following transportation into the endoplasmic reticulum by the transporter associated with antigen processing (TAP). In the ER, peptides are additionally trimmed by the endoplasmic reticulum aminopeptidase associated with antigen presentation (ERAAP) into the right form before being loaded onto the MHC class I molecule (MHC-I) and translocated to the cell surface. Some APCs, especially DCs, can capture exogenous proteins and after retro-translocation out of the phagosome and in to the cytoplasm those are processed and presented via the MHC-I axis – a process called cross-presentation. However, exogenous antigens are mainly loaded onto the MHC class II molecule (MHC-II). After internalisation of the antigen by endocytosis, macropinocytosis or phagocytosis, antigenic peptides which are generated in the endosome by proteases (e.g. Cathepsins) are finally loaded onto the MCH-II and transported to the cell surface.*
DOI: 10.1146/annurev-immunol-032712-095910

Naïve $CD8^+$ T cells can also be primed by APCs which are not directly infected but take up exogenous bacterial, viral or tumorigenic antigens and present them via the MHC class I axis by the process called cross-presentation (Cruz FM 2017, Grotzke JE 2017). Studies have shown that a fragment of the chicken egg white ovalbumin (OVA) protein, the $OVA_{257-264}$ peptide, is sufficient to be presented as antigen in the context of H-$2K^b$ MHC complex (CD8 co-receptor – MHC class I interaction) to induce T cell proliferation (Rötzschke O 1991). In contrast, the MHC class II molecules are loaded with exogenous peptides which are generated in the endolysosomes (the fusion of an endosome with a lysosome) and are presented to naïve $CD4^+$ T cells, thus leading to the induction of different types of effector T cells (Blum JS 2013) (Fig. 3). The $OVA_{323-339}$ peptide is specific for binding the I-A^b or I-A^d MHC complex (CD4 co-receptor – MHC class II interaction) which is recognized by OT-II or DO11.10 T cells (McFarland BJ 1999, Robertson JM 2000), respectively. In both cases, recognition of this peptide will induce $CD4^+$ T cell-mediated immune responses.

In addition to the MHC molecules, APCs also express co-signalling molecules. Whereas the former ones are just inducing TCR signalling, the latter ones decide upon T cell faith and function. However, to induce T cell activation it is not sufficient to have just one of the mentioned signals (Mueller DL 1989). Co-signalling molecules which are expressed by T cells are divided into two classes. Co-stimulatory molecules promote TCR signalling and induce T cell proliferation, differentiation, cytokine production, and survival, whereas co-inhibitory molecules regulate TCR signalling negatively, therefore inducing tolerance, cell cycle arrest, and even apoptosis (Chen L 2013) (Fig. 4). Interestingly, B7-1 and B7-2, which are expressed by APCs, can engage to two different molecules on the surface of T cell, the CD28 or cytotoxic T cell antigen 4 (CTLA4), therefore promoting co-stimulation or co-inhibition, respectively. Furthermore, interactions mediated by co-signalling molecules between APCs and T cells do not only promote flow of information into T cells but also propagate signals into APCs (e.g. bidirectional effect of B7-1/B7-2 and CTLA4) (Chen L 2013).

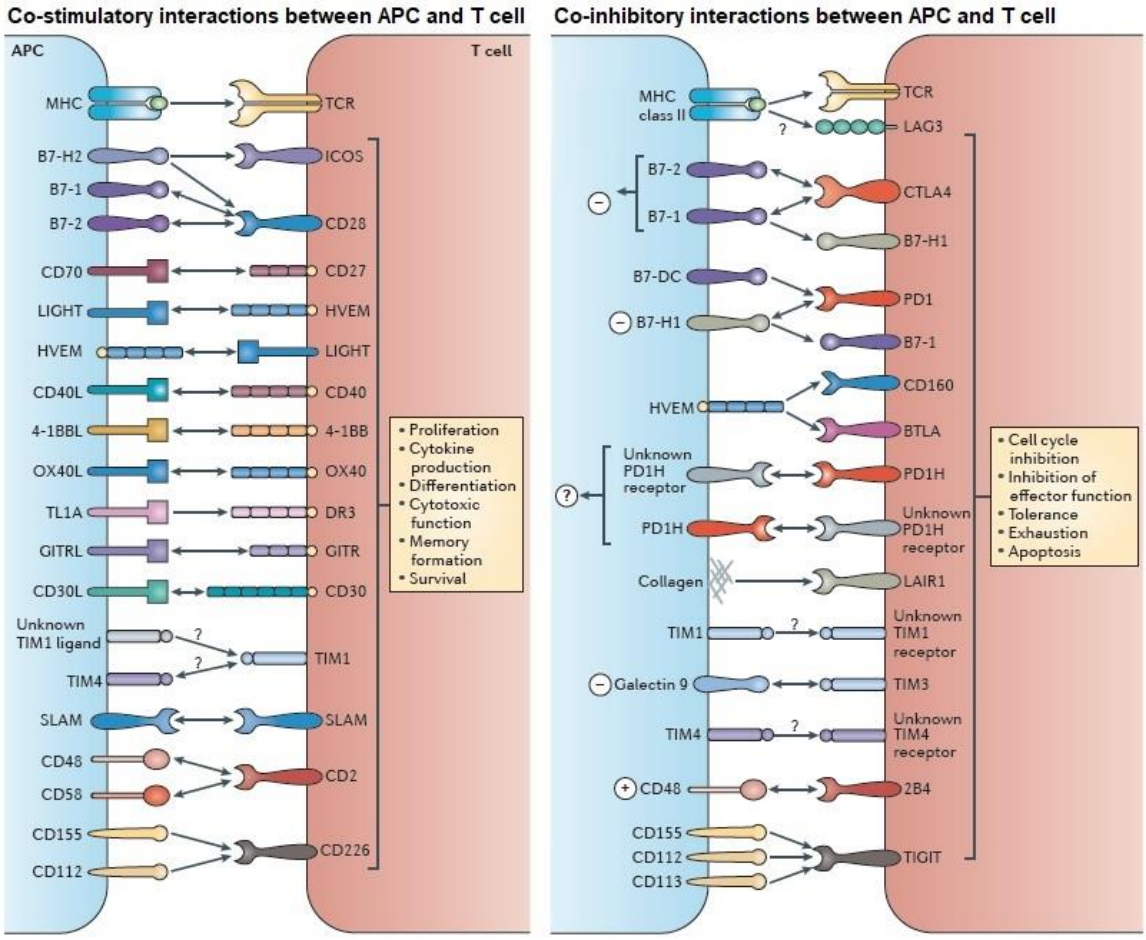


Fig. 4: Co-signalling molecules and their effect on T cells. *By binding co-stimulatory molecules, positive signals are propagated towards T cells and mostly induce proliferation, differentiation, and survival of the cell. In contrast, co-inhibitory molecules promote negative signal into T cells, thus leading to induction of apoptosis, tolerance, and inhibition of effector function. Some of these interactions are bidirectional, allowing also the flow of information from T cells to APCs (↔). 4-1BBL – 4-1BB (TNFR superfamily member 9) ligand; B7-DC – PD-L2; B7-H1 – PD-L1; BTLA – B and T lymphocyte attenuator; CD30L – CD30 ligand; CD40L – CD40 ligand; CTLA4 – cytotoxic T cell antigen 4; DR3 – death receptor 3; GITR – GITRL (glucocorticoid induced TNFR-related protein) ligand; HVEM – herpes virus mediator; ICOS – inducible T cell co-stimulator; LAG3 – lymphocyte activation gene 3 protein; LIAR1 – leukocyte-associated immunoglobulin-like receptor 1; LIGHT – TNFR superfamily member 14; OX40L – OXA40 (TNFR superfamily member 4) ligand; PD1H – PD1 homologue; SLAM - signalling lymphocyte activating molecule; TIGIT – T cell immunoreceptor with immunoglobulin and ITIM domains; TIM – type I transmembrane immunoglobulin and mucin; TL1A – TNF-like ligand A. DOI: 10.1038/nri3405*

To induce activation of naïve T cells into effector T cells, additionally to antigen presentation and interactions with co-signalling molecules, a third signal is needed which is mediated by cytokines present in the environment. Whereas $CD8^+$ T cells convert into cytotoxic T cells upon activation, $CD4^+$ cells will polarise into mainly T_H1, T_H2, T_H17 or Treg cells (DuPage M 2016) (Fig. 5). In 1989 Mosmann and Coffmann were the ones who described the first effector $CD4^+$ T cell, the T_H1 and T_H2 cells. Back then, IL-12 and IL-4, respectively were reported to be sufficient to induce these effector cells (Mosmann TR 1989). However, new studies have shown that these cytokines not only drive the polarisation into T_H1 and T_H2 cells but also favour the plasticity of these cells by making it possible to convert into one another (Panzer M 2012, Hegazy AN 2010). Nonetheless, the functionality of these two subsets is different. T_H1 cells are induced as a response to intracellular infections by sensing environmental cues such as IL-2, IL-12, and interferon gamma (IFNγ). The signal is then mediated by signal transducer and activator of transcription (STAT)1/STAT4 and T-box transcription factor TBX21 (T-bet) into the nucleus, thereby inducing T_H1 specific gene expression. Upon activation, T_H1 cells express the CXC- and CC-chemokine receptor (CXCR3 and CCR5) and produce high levels of INFγ, thus promoting e.g. macrophage activation (Fig. 5). In contrast, as a response to extracellular pathogens and cytokines like IL-2 and IL-4, T_H2 cells are induced. Signalling transduction in these cells is promoted by the transcription factors (TFs) STAT6 and GATA-binding factor 3 (GATA3), thus leading to production of CCR4, CCR6 as well as IL-4, IL-5, and IL-13. T_H2 cells may activate eosinophils and promote antibody production (Fig. 5).

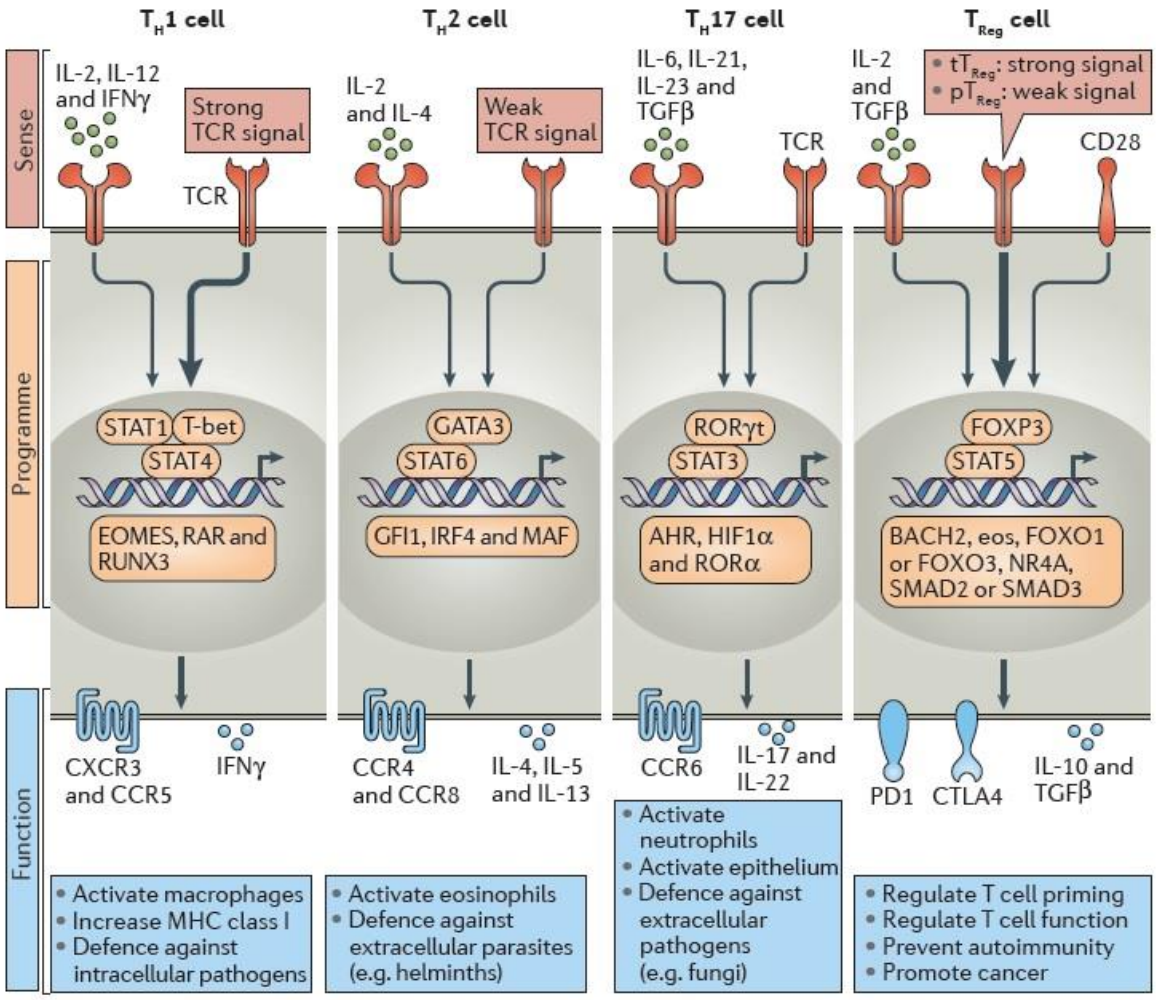


Fig. 5: Subsets of effector CD4⁺ T cells. *The different types of CD4⁺ T helper (T_H) cells are characterised by their well-defined abilities to react to external cues (sense), reprogram their gene expression (programme) and elicit effector function (function). Polarising cytokines bind to their specific receptors on the cell surface and induce signal transduction via the JAK/STAT pathway. Activated STAT proteins will translocate to the nucleus, thereby inducing transcriptional activation of specific genes. Each effector cell is programmed to induce an immune response specific for a certain type of infection. In contrast, Tregs modulate immune responses by directly inhibiting T cells effector function. AHR – aryl hydrocarbon receptor; BACH2 – BTB and CNC homolog 2; CCR – CC-chemokine receptor; CXCR – CXC-chemokine receptor; EONES – eomesodermin; eos (IKZF4) – Ikaros family zinc finger protein 4; FOXO – forkhead box; FOXP3 – forkhead box P3; GATA3 – GATA-binding factor 3; GFI1 – growth-factor independent 1; HIF1α – hypoxia- inducible factor 1α; INFγ – interferon gamma; IRF4 – interferon-regulatory factor 4; MAF – macrophage activating factor; NR4A – nuclear receptor 4; pTreg – peripherally derived Tregs; RAR – retinoic acid receptor; ROR – RAR-related orphan receptor; RUNX3 – runt-related transcription factor 3; SMAD – mother against decapentaplegic homolog; T-bet – T-box transcription factor TBX21; TGFβ – transforming growth factor beta; tTregs – thymic derived Tregs. DOI: 10.1038/nri.2015.18*

T_H17 cells are also induced as a response to extracellular pathogens but cytokines such as transforming growth factor beta (TGFβ) together with IL-6, IL-21 and IL-23 are required for their polarisation (Aggarwal S 2003, Harrington LE 2005, Park H 2005, Mangan PR 2006) (Fig. 5). T_H17 cells are characterized by the presence of the TFs RAR-related orphan receptor gamma t (RORγt) and STAT3 but also by the induction of high levels of IL-17 and IL-22 (Ivanov II 2006). However, T_H17 are capable of acquiring INFγ expression by losing RORγt and IL-17 expression by exposure to IL-12 and STAT4 (Bending D 2009, Lee YK 2009). The plasticity of effector T cells can switch even from an inflammatory programme to a regulatory one due to the presence of specific cytokines. Although TGFβ induces T_H17 cells it can only do so by co-stimulation with IL-6, otherwise the signal is transduced to Tregs (Zhou L 2008). Tregs are responsible for modulating immune responses by directly regulating the mechanism of T cell tolerance against self-antigens. These cells sense cytokines like TGFβ and IL-2 and do primarily develop in the thymus (tTregs), but can also be induced to some extent in the periphery (pTregs or iTregs). The presence of the IL-2 receptor (CD25) and the TF forkhead box P3 (Foxp3) characterises the Treg subtype (Fontenot JD 2003, Hori S 2003, Khattri R 2003) (Fig. 5).

When activated, $CD4^+$ T helper cells can regulate both, innate and adaptive immune responses, not only against pathogenic infections but also against transformed cells which might be harmful for the organism. T_H1 and T_H2 cell anti-tumour immunity may be mediated by inducing the activation of innate immune cells, such as macrophages or eosinophils which will secret factors that are directed against tumour cells (e.g. reactive oxygen species (ROS) by macrophages). Moreover, these T helper subsets are also important for maintaining cytotoxic T cell responses (Kennedy R 2008). Interestingly, although T_H17 cells are strongly associated with autoimmune diseases, it has been shown that this subpopulation might exhibit anti-tumour immunity (Zou W 2010). However, the role of these cells in tumour immunity remains debatable, as there are also reports which reveal the role of T_H17 cells in tumour progression (Martin F 2012, Bailey SR 2014). Additionally, the role of Tregs in tumour immunity is also rather controversial. Although these cells are indispensable in the regulation of immune homeostasis as well as self-tolerance, in tumour microenvironment, the immunosuppressive function that these cells exhibit proves to contribute to immune

evasion, thus sustaining tumour progression. Nonetheless, recent studies show that Treg accumulation into tumours correlates with positive prognosis in patients with certain types of cancers, the protective role being established by controlling tumour-induced inflammation (Ladoire S 2011, Chaudhary B 2016). Although $CD4^+$ T cell polarisation into different types of T_H cells is induced by immune responses to e.g. certain pathogenic infections or even transformed cells which produce unique cues that are sensed by T cells, those keep their plasticity, by modulating their function upon re-activation in a new environment (Fig. 6).

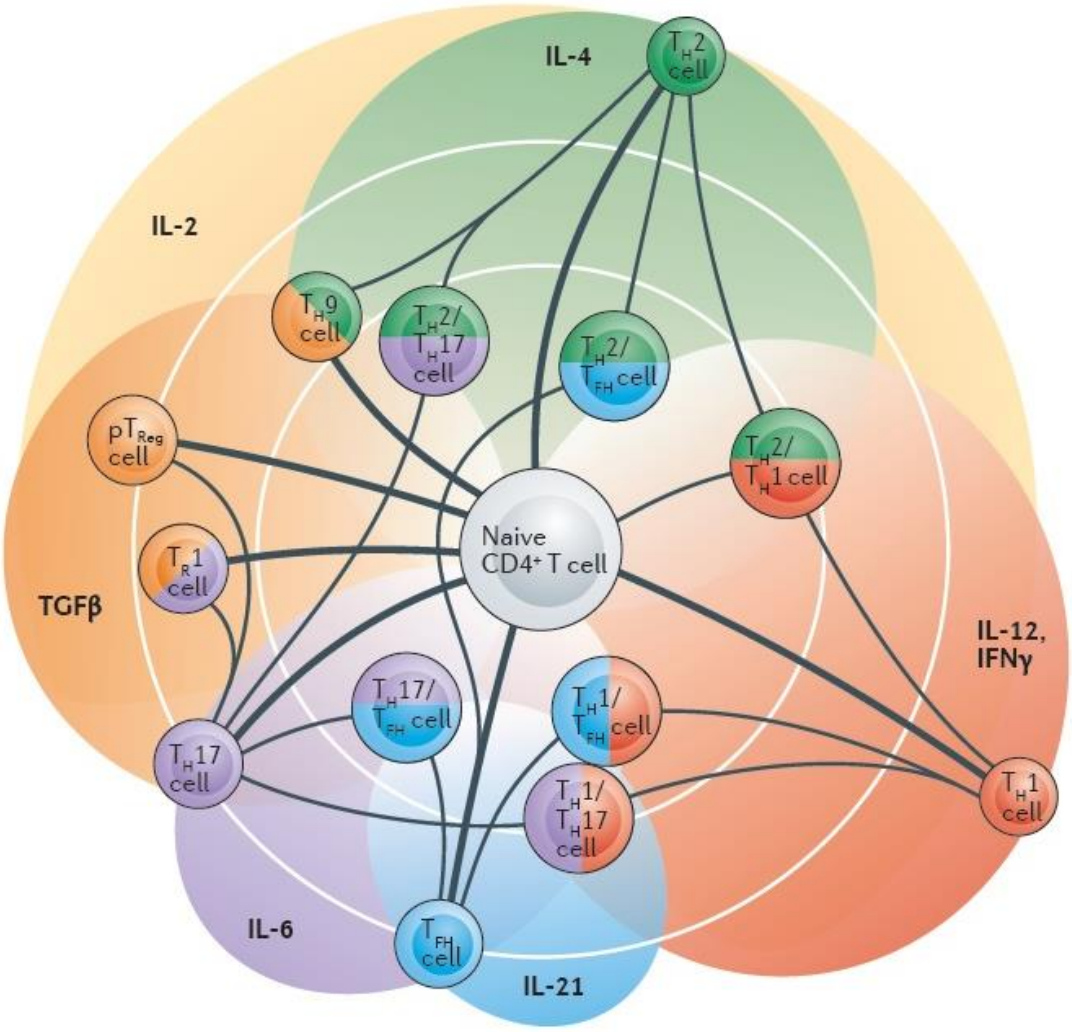


Fig. 6: Influence of cytokines on T cell plasticity. *Cytokines such as IL-12, IFNγ, IL-21, IL-6, TGFβ, IL-2, and IL-4 are known to induce the polarisation of $CD4^+$ T cells into specific T_H subtypes depicted here by their distance from the naïve $CD4^+$ T cell (T_H1, T_{FH} – follicular helper, T_H17, pTreg, and T_H2). The lines connecting the cells represent known transitions. Two coloured cells represent the plasticity between different subsets induced by environmental cytokines. DOI: 10.1038/nri.2015.18*

1.4 Macrophages

The role of macrophages in immunity was described as early as the 1983 (Elhelu MA 1983). Macrophages reside throughout the mammalian body, resembling different forms specific for the organ they are located in e.g. microglia, Kupffer cells, or osteoclasts. Nonetheless, their basic function remains the same, they engulf apoptotic cells, external pathogens or other cell debris and additionally produce effector molecules upon different environmental cues (Murray PJ 2011). Although these cells have a central role in the immunity of the host by clearing pathogens and regulating the activation and proliferation of lymphocytes, it is also known that macrophages are implicated in the pathogenesis of inflammation and some degenerative diseases such as arthritis, atherosclerosis, and fibrosis (Kawane K 2006, Woollard KJ 2010, Wynn TA 2010). Environmental stimuli which are produced by the host tissue and sensed by innate or adaptive immune cells during injuries or infections promote macrophage plasticity. As a response to those external cues, macrophages can sustain host defence, wound healing or immune regulation (Fig. 7). Classically activated (M1) macrophages are generated in response to INFγ, tumour necrosis factor (TNF) or lipopolysaccharide (LPS) and promote inflammation. They have anti-microbial activity and mediate immune responses which favour host defence. Nonetheless, the induction of such macrophages must be tightly controlled because cytokines and mediators produced by these cells can lead to tissue damage (Mosser DM 2008).

Alternatively activated (M2) macrophages promote wound healing after being induced during tissue damage by the presence of the cytokine IL-4. Regulatory macrophages are induced by various types of environmental cues (e.g. glucocorticoids, prostaglandins or apoptotic cells) and produce high levels of IL-10, therefore limiting immune responses and inflammation (Mosser DM 2008). TGFβ production by macrophages which had engulfed apoptotic cells can also contribute to their regulatory function (Fadok VA 1998). Although considered to be synonymous with M2 and regulatory macrophages, tumour-associated macrophages (TAMs) are a different type of polarised macrophages which promote immune suppressive activity. Studies have shown that in a mouse mammary cancer model, TAMs originate from circulating inflammatory blood monocytes which are recruited into the tumour (Franklin RA 2014). However, they are also thought to originate from tissue resident macrophages.

TAMs have various mechanisms by which they can promote tumour progression. These cells can secrete factors which nourish cancer cells and induce angiogenesis in the tumour microenvironment, therefore promoting tumour growth, invasion, and metastasis. Furthermore, due to their expression pattern of specific surface molecules (e.g. co-inhibitory molecules), TAMs can mediate immunosuppression at the site of tumour by induction of Tregs or inhibition of effector T cell function (Liu Y 2015).

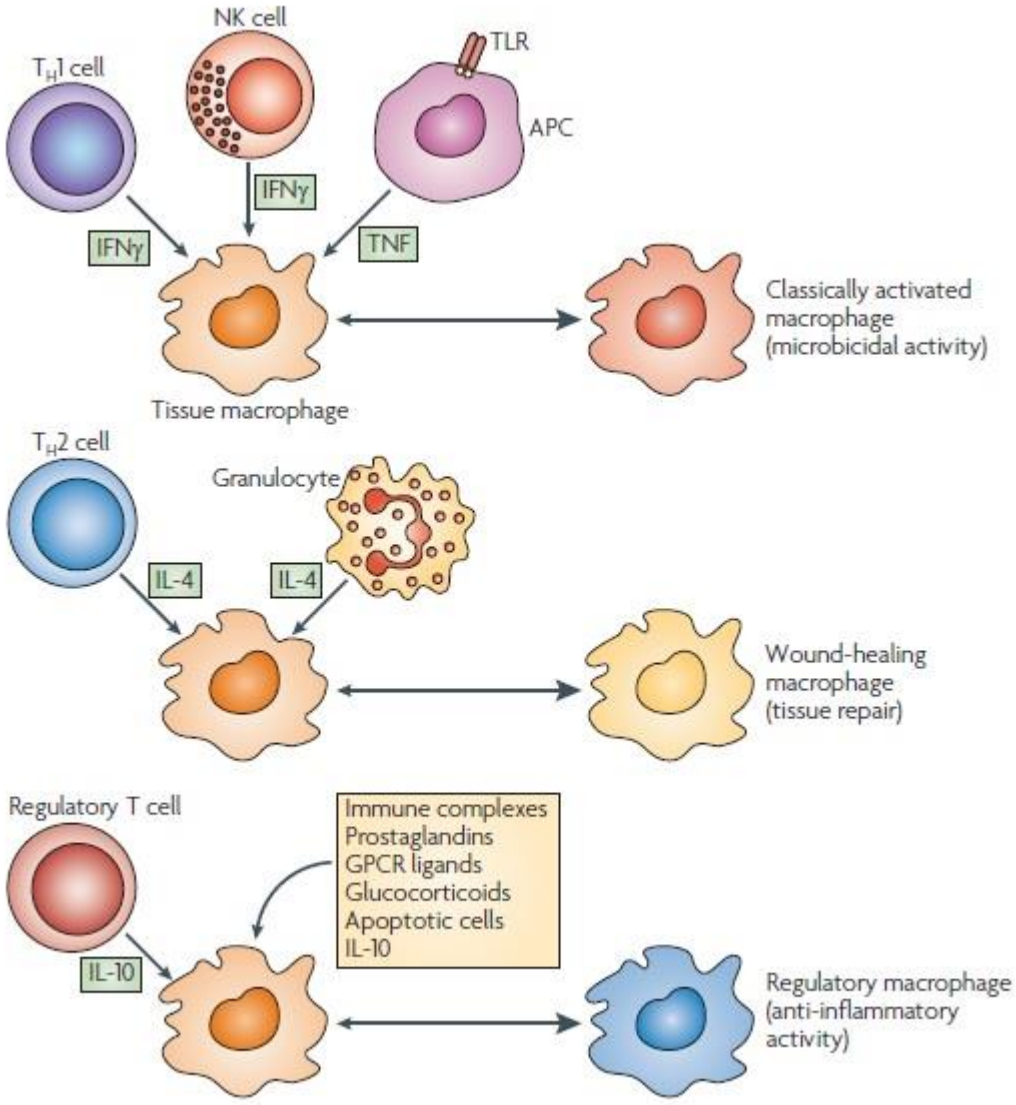


Fig. 7: Adaptive and innate immune cells can induce different types of macrophages*. Classically activated macrophages can be induced by the presence of INFγ produced by T_H1 and NK cells or by the tumour necrosis factor (TNF) derived from APCs. IL-4 produced by T_H2 cells or granulocytes, mainly basophils, mediates plasticity into alternatively activated (wound-healing) macrophages. Environmental cues such as immune complexes, prostaglandins, G-protein coupled receptor (GPCR) ligands, glucocorticoids, apoptotic cells and IL-10 generate regulatory macrophages. TLR – toll like receptor. DOI: 10.1038/nri2448*

Interestingly, the macrophage phenotypes described above are not only characterised by their different gene profiles responsible for their specific functionality, but also by the reprogramming of their central metabolic pathway during polarisation (Lawrence T 2011). Several studies have shown that LPS-induced M1 macrophages will take up more glucose, which will be used for aerobic glycolysis leading to impairment of the electron transfer chain (ETC) and increased production of ROS as well as lactate. In contrast, M2 macrophages will use the glucose to efficiently produce energy via the tricarboxylic cycle (TCA)-ETC cycle and do not exceed basal levels of ROS (Haschemi A 2012, Freemerman AJ 2014, Martinez FO 2014, Van den Bossche J 2017).

Although macrophages are part of the innate immune system, they play a tremendous role in the induction of adaptive immune responses due to their ability to process and present antigen but also to produce effector molecules. The functional activity of macrophages plays a major role in the outcome of T cell-mediated immune responses after antigen presentation (Martinez FO 2014). However, adaptive immune responses induced by macrophages might also be influenced by the genetic background of these cells. The expression of EGFR in liver macrophages was described to be coupled to the translational induction and secretion of IL-6, thus promoting HCC progression (Lanaya H 2014). Additional, tumour-associated myeloid cells which expressed the receptor mediated increased activation of STAT3 and survivin (an anti-apoptotic protein) expression by intestinal epithelial cells, consequently sustaining CRC development (Srivatsa S 2017, Hardbower DM 2017). Moreover, in a mouse model of dextran sulfate sodium (DSS)-induced colitis, Lu et. al showed that targeted deletion of the EGFR in macrophages lead to increased production of IL-10, therefore suppressing pro-inflammatory cytokines and protecting mice from colitis (Lu N 2014). On the contrary, another study had suggested that IL-4-induced EGFR down-regulation in macrophages was mediated by the production and release of the EGFR ligand, heparin-binding EGF-like growth factor (HB-EGF). Additionally, activation of the receptor induced a negative feedback mechanism which inhibited the polarisation of M2 macrophages induced by IL-4 stimulation, thus reducing gastro-intestinal tumour progression (Zhao G 2016).

1.5 Mouse models used

1.1.1 EGFR KO mice

EGFR knock-out (KO) mice ($EGFR^{-/-}$) are deficient in EGFR expression. The gene is inactivated in mouse embryonic stem (ES) cells by replacing parts of the promoter and the first exon of the *Egfr* with the *E. coli lacZ* reporter gene. EGFR had been shown to play a crucial part in the proliferation and differentiation of several epithelial cells of different organs, such as lung, liver, intestine, and skin. Therefore, most EGFR KO mice die before birth but depending on their genetic background (e.g. 129/SvxC57BL/6xMF1) they might survive until post-natal day 20 (P20). Surviving mice present severe deficiencies in growth, sight, hair development and inflammatory infiltrates (Sibilia M 1995, Sibilia M 1998). Due to the lethal phenotype of EGFR KO mice, conditional KO mice had to be generated in which *Egfr* deletion can be induced in a tissue specific but also time-dependent manner.

1.1.2 $EGFR^{\Delta Mx}$ mice

The Cre-loxP system was discovered as being part of the natural life cycle of the P1 bacteriophage. After injection of its DNA into bacteria, the DNA molecule can circularise by site-specific recombination (Sauer B 1988). This feature has been taken advantage of by scientists, who transformed it into one of the most powerful tools in mouse genetics. The system is precise in controlling the location and timing of gene expression, therefore allowing tissue-specific and/or inducible knock-out and/or knock-in. By crossing a mouse strain expressing an inducible Cre, with a mouse strain expressing a gene of interest flanked by loxP sites this will lead to an F1 generation expressing both the inducible Cre and the flanked allele (Fig. 8). Mice from the F1 generation are then crossed to generate a homozygous genotype in the F2 generation. Upon induction of the promoter and activation of the Cre recombinase, the mice will have a specific deletion of the loxP-flanked region and therefore lose the expression of the gene of interest. For our purpose, we generated $EGFR^{\Delta Mx}$ mice by crossing MxCre mice (Kühn R 1995) (JAX stock #003556) expressing an IFN-α/β (or synthetic RNA) inducible Cre recombinase to $EGFR^{fl/fl}$ mice in which the promoter and part of the first exon of the *Egfr* gene are flanked by loxP sites (Natarajan A 2007). After Cre recombinase activation parenchymal as well as bone marrow-derived cells will be deficient in EGFR expression.

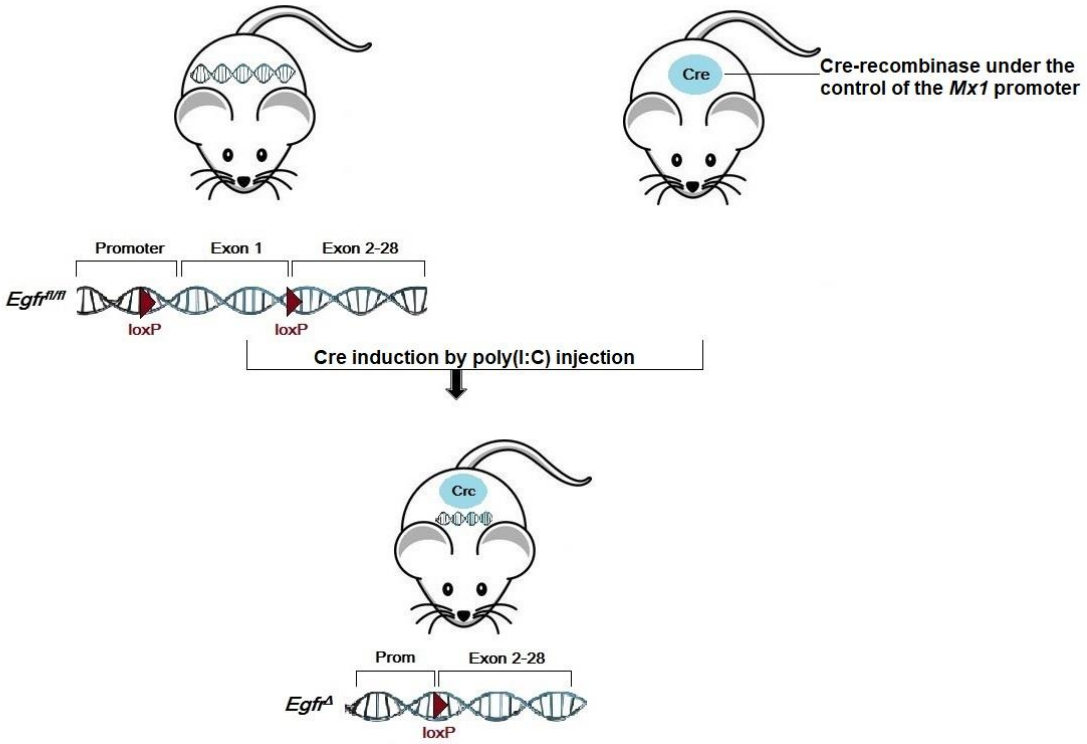


Fig. 8: Schematic representation of the EGFR$^{\Delta Mx}$ mouse model. *After crossing the mouse strain expressing the promoter-specific (Mx1 promoter) and polyinosinic:polycytidylic acid (poly I:C)-inducible Cre recombinase with the mouse strain containing the loxP-flanked Egfr gene, the F1 generation will express both, the Cre recombinase and the flanked allele. Mice from the F1 generation are then crossed to generate a homozygous genotype in the F2 generation. Poly I:C injection of mice from the F2 generation will induce Cre recombinase expression which can excise the flanked region of the Egfr, thus leading to disruptive receptor expression.*

Three-week-old mice were injected with polyinosinic:polycytidylic acid (poly I:C) to induce Cre recombinase activation and were sacrificed when mice were 6-8-week-old. Besides reduced body weight, EGFR$^{\Delta Mx}$ mice do not present any phenotypical abnormalities and can survive up to 18 months after poly I:C injection (Natarajan A 2007).

1.1.3 TCR-transgenic (ovalbumin-specific) OT-I mice

The TCR of these mice has been genetically designed by transgenic insertions for the mouse TCR Vα2 and TCR Vβ5 to recognise the ovalbumin $OVA_{257-264}$ peptide, resulting in an MHC class I-restricted $CD8^+$ T cell (OT-I) response (Hogquis KA 1994) (JAX stock #003831). Thus meaning, that isolated and purified T cells form these mice will primarily recognise the $OVA_{257-264}$ peptide presented by the MHC class I. I used these mice to inspect antigen presentation to OT-I T cells by EGFR$^{\Delta Mx}$ APCs.

1.1.4 TCR-transgenic (ovalbumin-specific) OT-II mice

In contrast to the OT-I mice, OT-II mice have a transgenic αβ TCR which can engage to the CD4 co-receptor to specifically recognise the $OVA_{323-339}$ peptide, therefore generating an MHC class II-restricted $CD4^+$ T cell (OT-II) response (Barnden MJ 1998) (JAX stock #004194). I used these mice to study OT-II T cell responses to antigen presentation and effector T cell polarisation mediated by $EGFR^{\Delta Mx}$ APCs.

Both OT-I and OT-II mice are to some extent immunodeficient. The first TCR-transgenic OVA-specific mice were developed for investigating positive selection and development of T cells in the thymus. Additionally, a better understanding of how ligands engaged to the TCR, thus leading to differentiation and activation of T cells was needed (Murphy KM 1990, Hogquis KA 1994, Barnden MJ 1998). However, endogenous TCR rearrangements in OT-I or OT-II/DO11.10 transgenic mice may lead to the development of T cells with a different TCR than the one which recognizes antigen in an $H\text{-}2K^b$ or $I\text{-}A^b/I\text{-}A^d$ complex, respectively. Consequently, for the generation of a monoclonal OVA-specific T cell population, TCR-transgenic mice should be crossed into a $RAG^{-/-}$ background. $RAG^{-/-}$ mice are deficient in the expression of the recombination-activating gene (*Rag*), therefore, V(D)J recombination cannot be induced and mice lack mature T and B cells (Mombaerts P 1992, Shinkai Y 1992).

Isolated T cells from TCR transgenic mice can be easily phenotyped by several methods. Most commonly, different allelic variants of the surface molecule such as CD45 (CD45.1/CD45.2) or CD90 (CD90.1/CD90.2) are bred onto TCR transgenic mice, thus allowing tracing of donor cells with antibodies against these molecules. More advantageous is the use of fluorochrome conjugated peptide MHC-tetramers which can detect T cells according to their antigen specificity. A third option is to stain T cells with antibodies against the TCR Vα2 or Vβ5 domain expressed by OT-I and OT-II transgenic mice (Moon JJ 2009). For analysis, all three methods can be combined with flow cytometry.

2. Aims of the Thesis

The expression of EGFR is known to be involved in a great number of human epithelial cancer, such as lung and colorectal cancer. Nonetheless, until recently it was believed that activation of the receptor and induction of downstream signalling only in tumour cells was sufficient for tumour development and progression. Nowadays it is known that not only tumour cells themselves, but also the tumour microenvironment containing immune cells, fibroblast, and endothelial cells can drive malignancies (Bremnes RM 2011, Lanaya H 2014, Wang K 2016, Srivatsa S 2017, Hardbower DM 2017). However, it is not fully understood how the cell type-specific EGFR expression in tumour or tumour stroma influences tumorigenesis.

My work is based on the findings that EGFR displayed a tumour-promoting role in liver macrophages and tumour-associated myeloid cells in an HCC and CRC mouse model, respectively. Lanaya et. al could link lL-6 production by liver macrophages to HCC progression and showed that IL-1-mediated IL-6 induction was downstream of the EGFR pathway (Lanaya H 2014). Additionally, Srivatsa et. al unveiled the relationship between EGFR expression by myeloid cells and increased activation of STAT3 and survivin expression by intestinal epithelial cells, thus correlating with CRC development (Srivatsa S 2017). These studies showed that EGFR expressing myeloid cells in tumour stroma, rather than the tumour cells themselves, promoted malignancies. A potential mechanism, by which myeloid cells in tumour microenvironment could directly regulate tumour growth, might be mediated by innate processes, such as the production of growth factors or cytokines which sustain tumour progression. Another possible regulation mechanism mediated by myeloid cells would be by indirectly sustaining tumour development through interference with the function of adaptive immune cells. Myeloid cells could induce the expression of different checkpoint molecules (e.g. PD-L1) which would inhibit the effector function of T cells, therefore limiting cancer clearance. Additionally, they might decrease antigen presentation by downregulation of MHC molecules, or even favour the activation of specific T cell subtypes (e.g. Tregs) which promote immunosuppression and survival of the tumour.

The aim of my thesis was to investigate if the expression of EGFR in macrophages or dendritic cells influences the induction of adaptive immune responses mediated by T cells, therefore favouring tumour progression.

Thus, I conducted following experiments:

- I inspected the capacity of LPS or IL-4 stimulated EGFR-deficient macrophages and dendritic cells to take up antigen and present it to T cells
- I investigated the role of EGFR in the expression pattern of co-signalling and scavenger molecules on the surface of stimulated APCs
- I investigated the potential of EGFR-deficient APCs to induce different subtypes of effector T cells such as T_H1, T_H2, T_H17, and Treg cells

3. Results and Discussion

3.1 Unstimulated EGFR-deficient BMDMs and BMDCs could efficiently cross-present antigen to OT-I T cells and induced OT-II T cell proliferation

Egfr deletion was induced by poly I:C injection in 3-week-old EGFR$^{\Delta Mx}$ (EGFR$^{fl/fl}$ x Mx1Cre) mice. 6-8-week-old mice were sacrificed for the isolation of bone marrow. Isolated cells were cultured in L929 or granulocyte-macrophage colony-stimulating factor (GM-CSF) conditioned medium for the generation of macrophages (BMDMs) or dendritic cells (BMDCs), respectively. Isolated cells lacked genomic *Egfr* (Fig. 9).

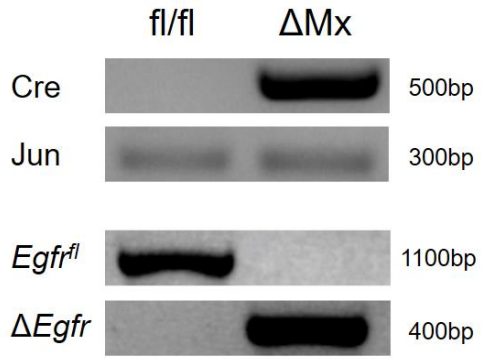


Fig. 9: Genomic Egfr deletion in bone marrow-derived cells from EGFR$^{\Delta Mx}$ mice. *EGFR$^{\Delta Mx}$ (ΔMx) mice expressed the Cre recombinase (Cre) whereas the EGFR$^{fl/fl}$ (fl/fl) control mice did not. The amplified Jun product was used as an internal control for this PCR. Isolated DNA from bone marrow-derived cells was used to amplify the Egfr alleles. fl/fl cells had both loxP-flanked alleles (Egfr^{fl}), whereas ΔMx cells expressed the deleted alleles (ΔEgfr).*

To investigate, if the EGFR signalling pathway in BMDMs and BMDCs plays a role in antigen presentation, therefore inducing T cell proliferation, I performed an antigen presentation assay in which the APCs would present OVA protein or specific OVA peptides to MHC class I or II-restricted T cells from OT-I or OT-II mice, respectively (Fig. 10 B). Before starting the assay, I inspected the phenotype of isolated T cells by flow cytometry (Fig. 10 A). Subsequently, T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE), a fluorescent dye which binds covalently to intracellular molecules. During every performed cell cycle the dye got diluted out as it was equally split between two daughter cells. Multiple cell proliferation cycles could therefore be traced by inspecting dye dilution by flow cytometry (Lyons AB 1994, Quah BJ 2007).

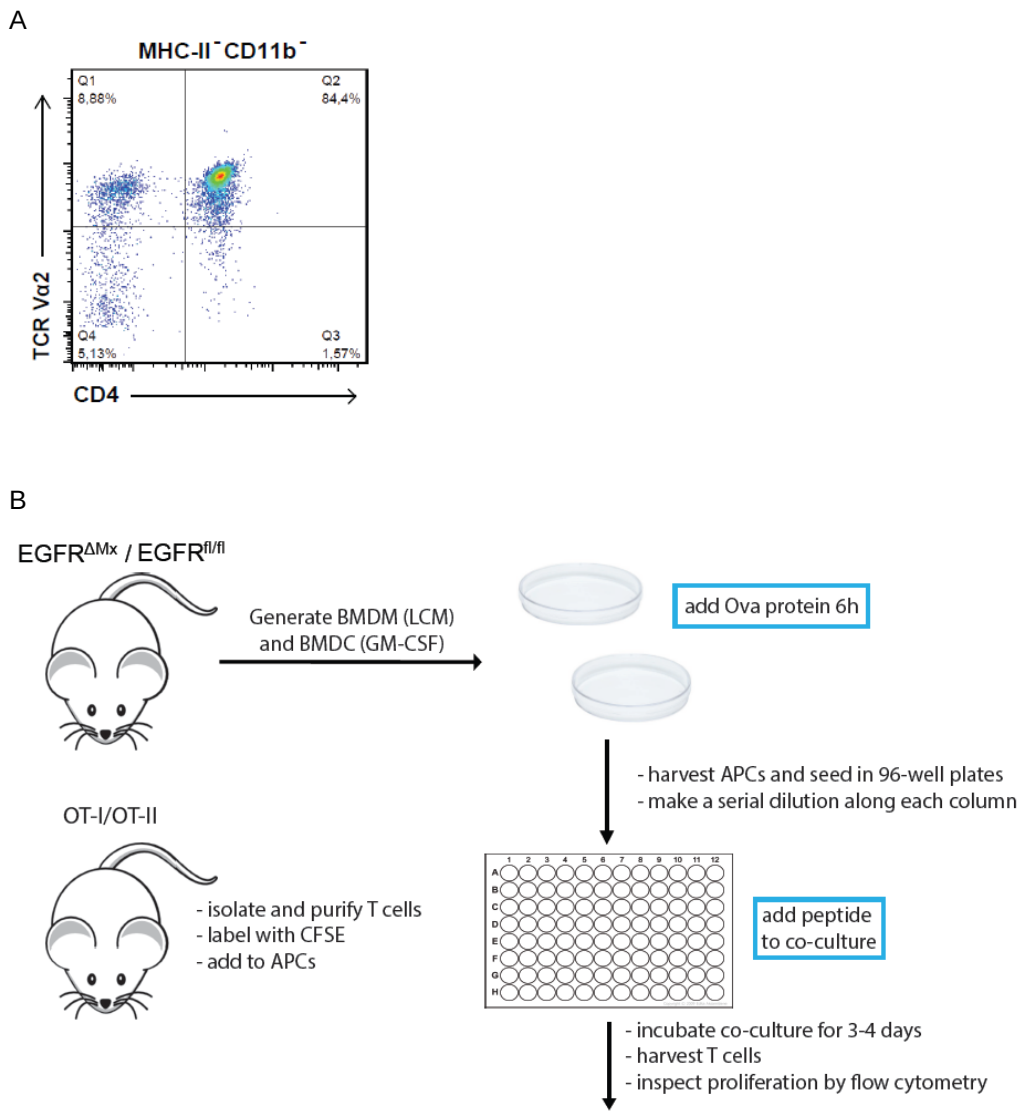


Fig. 10: Schematic representation of antigen presentation assay by unstimulated APCs. *A) Representative blot of OT-II T cell phenotyping. T cells were isolated and purified by MACS sorting, subsequently being identified as being MHC-II^{-}CD11b^{-}TCRVα2^{+}CD4^{+} by flow cytometry. B) Briefly, EGFR$^{\Delta Mx}$ and EGFR$^{fl/fl}$ BMDCs and BMDMs were incubated with OVA protein for 6h. Subsequently, APCs were washed and 2 x 10^{7} cells were seeded to one well of a 96-well plate. From this well a serial dilution of APCs was made for each column so that after adding the carboxyfluorescein succinimidyl ester (CFSE)-labelled T cells, there would be different APC : T cell ratios (e.g. 1:1, 1:2, 1:10, 1:50). OVA$_{323-339}$ or OVA$_{257-264}$ (as a positive control) peptide was added to the APC – T cell co-cultures. Co-cultures were then incubated for 3-4 days, subsequently harvesting T cells and analysing those by flow cytometry.*

Because purified OT-I and OT-II T cells were labelled with CFSE prior to the co-culture incubation, I could not only identify the percentage of T cells which did divide but also how many cell division cycles these cells performed. Harvested OT-II and OT-I T cells

were identified by flow cytometry analysis as being MHC-II$^-$CD11b$^-$CD45.1$^+$CD4$^+$ or CD8$^+$, respectively (Fig. 11 A). Cell populations were then investigated for T cell proliferation. Fig. 11 B shows that addition of OVA$_{323-339}$ peptide to the co-culture induced proliferation of 66.3% of T cells, whereas APCs which were pre-incubated with OVA protein triggered proliferation of only 2.2% of cells. Not only was there a big difference between the percentage of T cells which proliferated after peptide or protein presentation, but also the mean fluorescence intensity (MFI) of the CFSE dye of the dividing cells was very different in the two cases. Because the CFSE got diluted out with every performed cell cycle, the MFI of the dye was an output of how many times T cells divided, thus meaning that the more cell cycles T cells underwent, the lower the MFI was (Fig. 11 B).

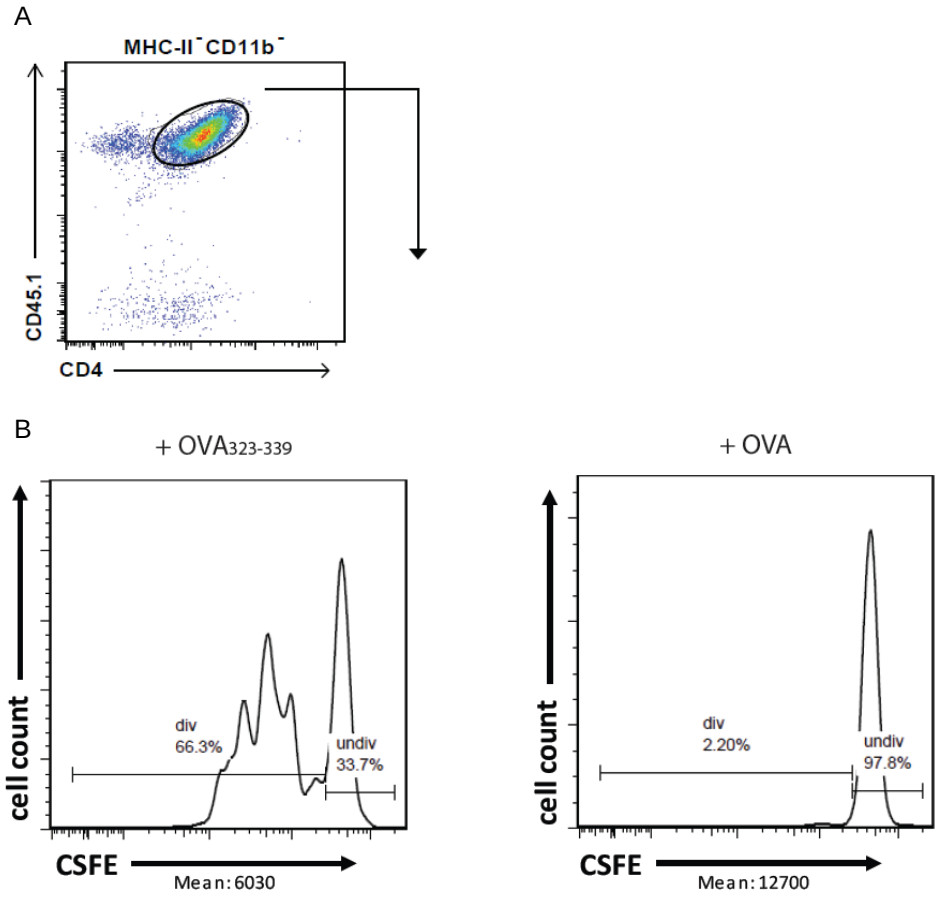


Fig. 11: Data output of flow cytometry analysis. *A) Representative blot of gating strategy for harvested OT-II T cells which were MHC-II$^-$CD11b$^-$CD45.1$^+$CD4$^+$. B) The addition of OVA$_{323-339}$ peptide (+OVA$_{323-339}$) to the APC – T cell co-culture induced proliferation of 66.3% of T cells, whereas APCs which had been pre-incubated with OVA protein (+OVA) before the assay could induce proliferation of just 2.2% of T cells. An increase in the number of performed cell cycles was associated with a low MFI value of CFSE of diving T cells (Mean).*

For the interpretation of my antigen presentation assay data, I inspected the percentage of T cell which proliferated but also the number of cell division performed after protein or peptide presentation. The number of cell division was an output measured by the MFI value of CFSE of the dividing T cells.

As a positive control, I intended to use the incubation of APC – OT I T cell co-cultures with the $OVA_{257-264}$ peptide to assess if the assay was working. As this peptide is specific for the presentation by the MHC class I molecule, present of the surface of all nucleated cells, the addition of the peptide to the co-cultures would induce antigen presentation not only by the APCs in the co-culture but also by the T cells, which can present the peptide to one another. Indeed, even at a very low APC : T cell ratio (1:50), presentation of the $OVA_{257-264}$ peptide induced high OT-I T cell proliferation (above 80% of cells divided). Interestingly, a slightly higher percentage was mediated by BMDMs at a ratio of 1:1 (Fig. 12 A). The capacity of both, dendritic cells and macrophages, to induce $CD8^+$ T cell proliferation *in vivo*, had already been shown by Pozzi et. al (Pozzi LA 2015). Although BMDMs had induced slightly higher percentage of dividing T cells after peptide presentation at a ratio of 1:1, there were no differences between the number of cell divisions induced by BMDCs or BMDMs (Fig. 12 A – lower panel). Interestingly, BMDMs cross-presented OVA protein more efficiently than BMDCs which is in contrast to what had already been published (Rodriguez A 1999). Strikingly, increased numbers of both, dividing OT-I T cells as well as cell cycles, would suggest that the cross-presentation by BMDCs and BMDMs was performed better at a ratio of one APC to two T cells. Nonetheless, EGFR expression by BMDCs or BMDMs did not affect the capacity of these APCs to present antigen to OT-I T cells.

In contrast to $OVA_{257-264}$, the $OVA_{323-339}$ peptide is presented by the MHC class II molecule, which is only expressed by APCs but not by T cells. Induction of OT-II T cell proliferation after peptide presentation dropped steadily with the decrease of the APC : T cell ratio (Fig. 12 B). When pre-incubated with OVA protein for 6h both, BMDCs and BMDMs, processed and presented equally well to OT-II T cells just at a ratio of 1:1 (Fig. 12 B). Yet again, there were no differences between the number of induced cell divisions after BMDC or BMDM protein presentation. Moreover, as described for the antigen presentation to OT-I T cells, EGFR expression did not influence antigen presentation to OT-II T cells.

To summarise, both, EGFR-deficient BMDMs and BMDCs, were able to (cross-)present antigen to OT-I and OT-II cells.

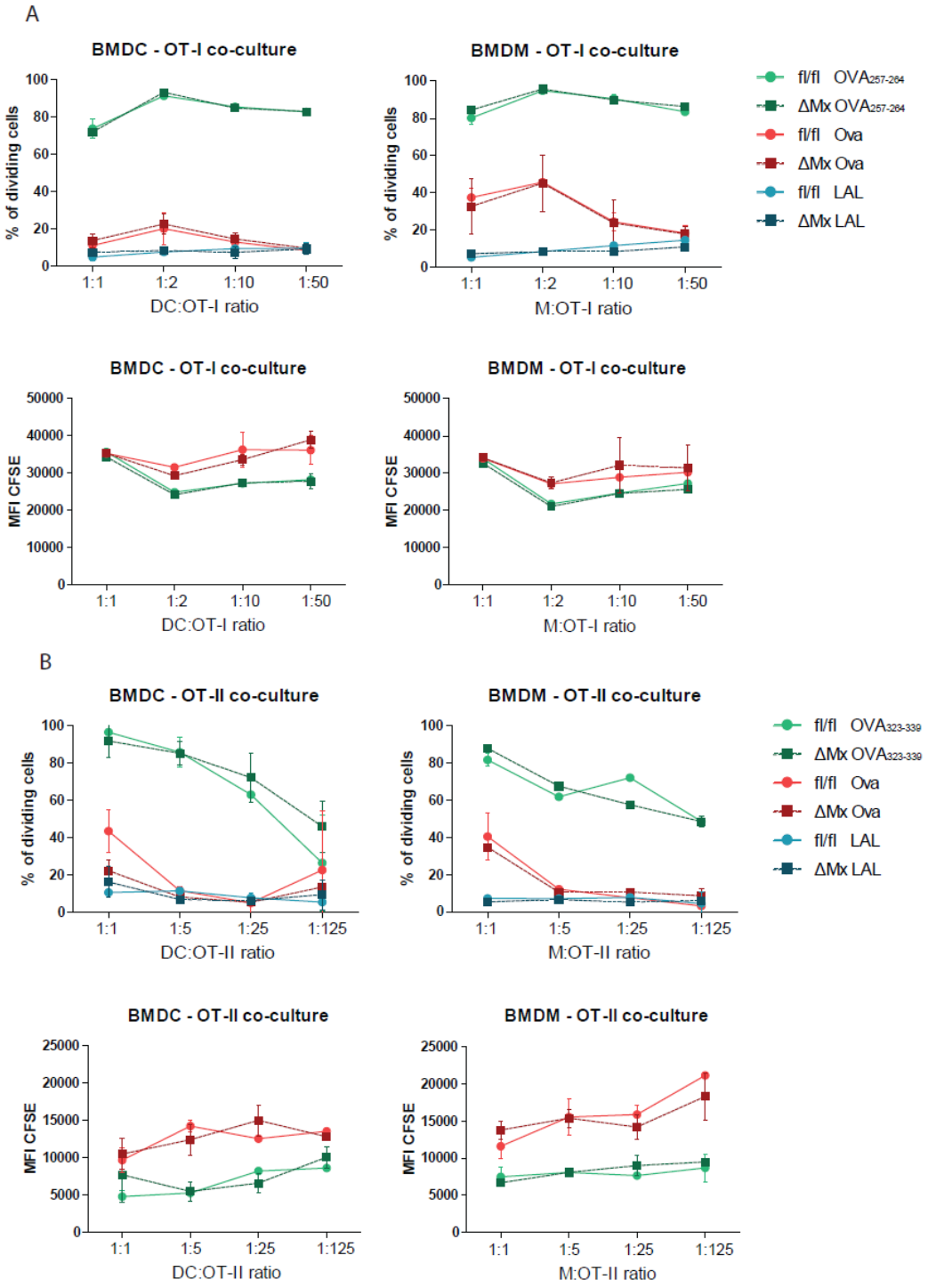


Fig. 12: Flow cytometry analysis of T cell proliferation after antigen presentation by unstimulated EGFR$^{\Delta Mx}$ BMDCs and BMDMs. *Percentage and the MFI of CFSE of dividing T cells for each of the APC : T cell ratios (1:1, 1:2, 1:5, 1:10, etc. – 1:1 = 1 x 10^4 APCs : 1 x 10^4 T cells) are depicted here. EGFR$^{\Delta Mx}$ (ΔMx) and control EGFR$^{fl/fl}$ (fl/fl) APCs were incubated with OVA protein, OVA peptide or LAL (vehicle control) and co-cultured for 3 days with A) OT-I T cells or 4 days with B) OT-II T cells – representative example (n=2).*

Studies have suggested that it is not sufficient that DCs take up antigen and present it to T cells, but that they need an additional maturation stimulus, to induce a specific effector T cell response (Mellman I 2001, Trombetta ES 2005). Several studies had emphasised the importance of activating toll-like receptor (TLR) signalling for dendritic cell maturation, thus affecting antigen uptake and presentation to T cells. When immature DCs sensed the right signal (e.g. LPS) and transformed into mature cells, those started reducing their capacity to take up antigen but elicited an exceptional efficiency to stimulate T cells proliferation by increased MHC upregulation (Delamarre L 2003, Weck MM 2007, Kool M 2011). Furthermore, DCs which had been cultured with IL-4 additional to GM-CSF proved to be more efficient in inducing OT-II T cell proliferation than DCs which were not exposed to the stimulus (Wells JW 2005). Interestingly, the same stimuli are also involved in the induction of different phenotypes of macrophages. LPS or IL-4 polarisation of macrophages into either the M1 or the M2 phenotype is a model to mimic different functional variants of effector macrophages *in vitro* which might induce different types of effector T cells after antigen presentation.

Thus, although there was no effect of EGFR expression in antigen presentation mediated by naïve BMDCs or BMDMs, we were interested to see if the capacity of EGFR-deficient APCs to present antigen is influenced by the presence of environmental stimuli, such as LPS or IL-4.

3.2 Stimulated EGFR-deficient BMDMs and BMDCs could efficiently present antigen to OT-II T cells

To be able to analyse the antigen presention capacity of LPS or IL-4 stimulated BMDCs and BMDMs, I had to change some steps in the antigen presentation protocol. APCs received in addition to the OVA protein or $OVA_{323-339}$ peptide also LPS or IL-4 for 6h prior to the addition of labelled OT-II T cells (Fig. 13). Preliminary data from our lab showed that LPS stimulation of macrophages from $EGFR^{fl/fl}$ and $EGFR^{\Delta Myl}$ (Cre-mediated *Egfr* deletion in the myeloid cell lineage) mice for 6h was sufficient to induce expression of IL-6 and iNOS (data not shown). Thus, I added the stimuli before the T cells so that during the 6h incubation time, the APCs would have had time to sense and react to the environmental cues, thus maybe influencing their capacity of processing and presenting antigen.

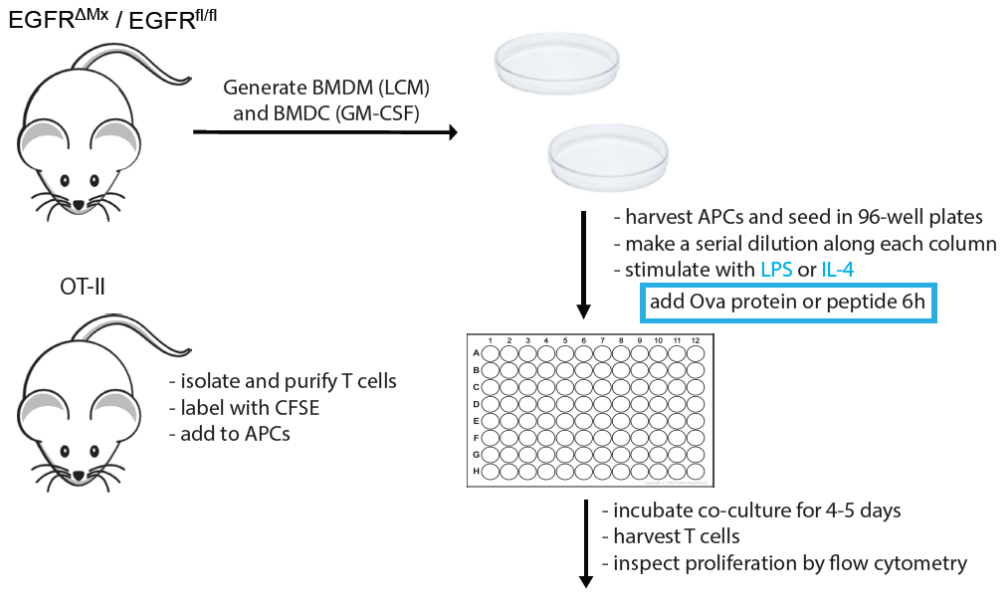


Fig. 13: Schematic representation of antigen presentation assay by stimulated APCs. *Briefly, 2 x 10^7 $EGFR^{\Delta Mx}$ and $EGFR^{fl/fl}$ BMDCs and BMDMs were seeded into one well of a 96-well plate. From this well a serial dilution of APCs was made for each column, so that after adding CFSE-labelled OT-II T cells, there would be different APC : T cell ratios (1:1, 1:2, 1:10, 1:100). OVA protein or $OVA_{323-339}$ peptide, in combination with LPS (50 ng/ml) or IL-4 (20 ng/ml), was added to the APCs and incubated for 6h. In the meantime, T cells were isolated from OT-II mice, labelled with CFSE and added to the APCs. Co-cultures were incubated for 4-5 days, subsequently harvesting T cells and analysing those by flow cytometry.*

Unstimulated (LAL) BMDCs performed equally good in protein and peptide presentation, as there were no differences in the percentage of induced proliferating T cells, whereas BMDMs induced through protein presentation a slightly higher percentage of proliferating T cells at a ratio of 1:1 and 1:2 (Fig. 14 A and B – left panel). Surprisingly, although LPS stimulated DCs should be more efficient in antigen presentation, therefore inducing higher percentage of proliferating T cells (Delamarre L 2003), in my hands LPS stimulation neither of BMDCs nor of BMDMs did have an effect on T cell proliferation, since the percentage of dividing T cells seemed to be rather lower than the ones induced by unstimulated (LAL) APCs (Fig. 14 A and B – middle panel). On the contrary, IL-4 stimulation of APCs boosted antigen presentation, therefore inducing not only proliferation of almost all OT-II T cell (%) but also increased the number of cell cycles performed by these cells (decrease of the MFI value) (Fig. 14 A and B – right panel). Wells et. al showed that BMDCs which had been cultured in the presence of IL-4 could induce T cell proliferation better than the ones that lacked the stimulus. However, presentation of $OVA_{323-339}$ peptide rather than OVA protein was more efficient in inducing proliferation (Wells JW 2005). Interestingly, in my hands both, protein and peptide presentation by IL-4 stimulated APCs, induced equally well T cell proliferation. However, independent of the stimuli available (LPS or IL-4), induction of T cell proliferation by antigen presentation was less efficient when the APC : T cell ratio decreased more than 1:2 (Fig. 14).

The fact that LPS stimulated APCs could not induce high levels of T cell proliferation might be explained by the setup of the assay. APCs were exposed to the environmental stimuli together with the antigen preliminary to the addition of the labelled T cells. There was no washing step in-between, thus the stimuli might have had a direct effect on the uptake and processing of the antigen by APCs or directly on T cell proliferation.

In conclusion, similar to unstimulated APCs, after stimulation with LPS or IL-4 I could not detect any EGFR-mediated differences in the efficiency of these cells to induce T cell proliferation after antigen presentation.

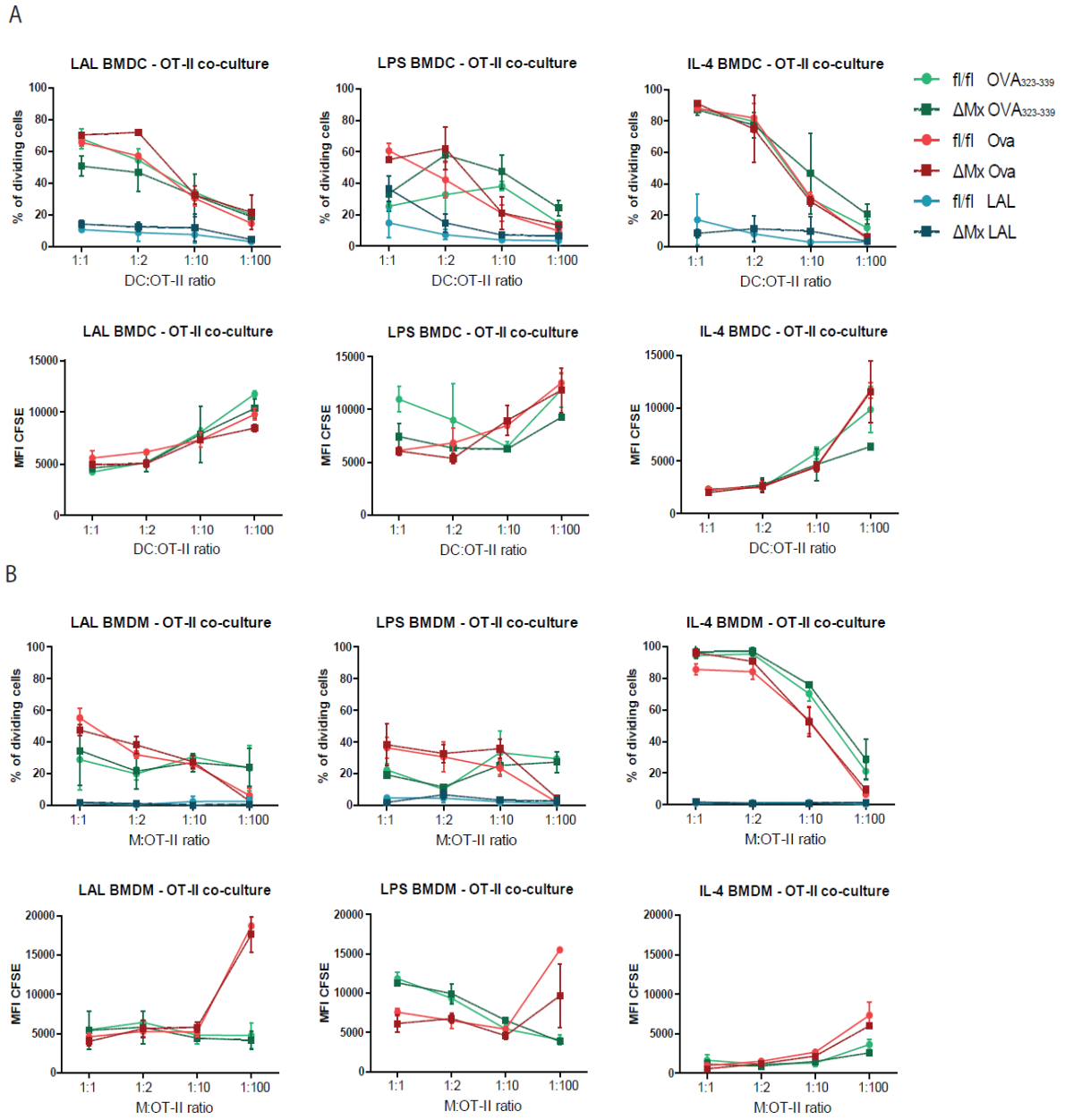


Fig. 14: Flow cytometry analysis of T cell proliferation after antigen presentation by stimulated EGFR$^{\Delta Mx}$ BMDCs and BMDMs. *Percentage and the MFI of CFSE of dividing OT-II T cells for each of the APC : T cell ratios (1:1, 1:2, 1:10, 1:100 – 1:1 = 1 x 10^4 APC : 1 x 10^4 T cells) are depicted here. A) LAL (vehicle control), LPS or IL-4 stimulated EGFR$^{\Delta Mx}$ (ΔMx) and control EGFR$^{fl/fl}$ (fl/fl) BMDCs – OT-II co-cultures were incubated for 4 days or B) stimulated ΔMx and fl/fl BMDMs – OT-II co-cultures had an additional day of incubation – representative example (n=2).*

3.3 Expression pattern of surface molecules on EGFR$^{\Delta Mx}$ APCs after LPS or IL-4 stimulation

There are always three signals needed for an effective effector T cells activation. For once, T cells need to recognize antigen which is presented by the MHC molecules, thus promoting TCR signalling into the cells. Secondly, T cells need to encounter an additional signal mediated by co-signalling molecules, which sustain TCR signalling and induce proliferation, differentiation, and survival of the cell. At last, depending on the available cues T cells sense in their environment, those will polarise into different types of effector T cells. In accordance with this, I was interested to see if EGFR plays a role in the efficiency of unstimulated and stimulated APCs to present antigen to T cells and if so, whether there is any correlation to the expression pattern of MHC class II, co-stimulatory, co-inhibitory, and integrin molecules on the surface of these APCs. Therefore, I not only inspected the efficiency of stimulated APCs to induce OT-II T cell proliferation by antigen presentation but in parallel I also investigated how EGFR might regulate the expression pattern of surface molecules in these cells.

For this purpose, aliquots of EGFR$^{fl/fl}$ and EGFR$^{\Delta Mx}$ BMDMs and BMDCs which were used for the antigen presentation assay, were investigated for the expression of surface molecule after LPS or IL-4 stimulation. APCs were stimulated for 24h and subsequently inspected by flow cytometry. BMDMs were gated as a uniform cell population and were CD11b^{high}MHC-II^{mid}, whereas BMDCs were gated after the strategy adopted by Heft et. al (Helft J 2015) into mature (CD11b^{mid}MHC-II^{high}) and immature (CD11b^{high}MHC-II^{mid}) BMDCs (Fig. 15).

Although I repeated this experiment several times, the results were not always consistent, therefore I could not pool the data. However, because the trend of the expression pattern of the surface molecules on APCs were mostly similar I will show one representative experiment for each of the cell types. In summary, there were neither EGFR-depended differences in the expression pattern of surface molecules on BMDCs or BMDMs nor could I make any correlation to the differences in the induction of T cell proliferation after antigen presentation by stimulated APCs.

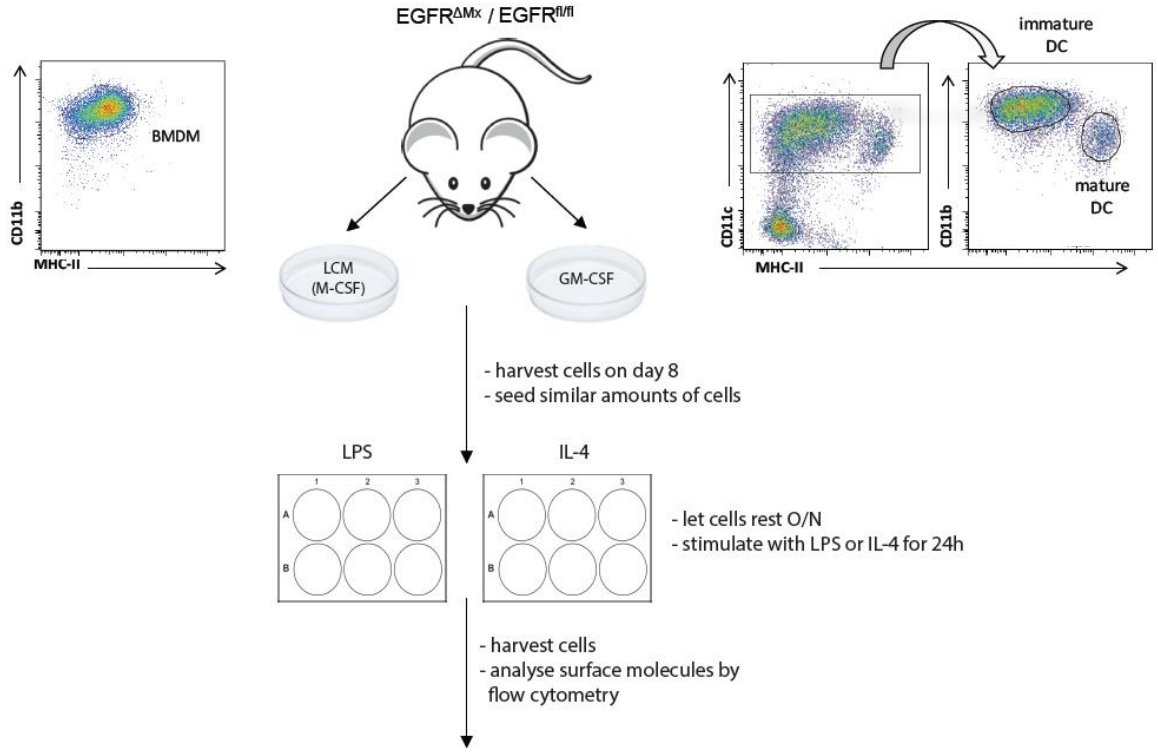


Fig. 15: Schematic representation of the protocol for the determination of surface molecules on EGFR$^{\Delta Mx}$ BMDMs and BMDCs. *Briefly, 0.8 x 10^6 cells/well (BMDMs or BMDCs) were seeded on 6-well plates and stimulated on the following day with LPS (50 ng/ml) or IL-4 (20 ng/ml) for 24h. Cells were harvested, stained for surface molecules and subsequently analysed by flow cytometry.*

Increased expression of the MHC class II molecule was descirbed to be a phenotypic characteristic of LPS-induced M1 macrophages and mature DCs (Mosser DM 2003, Mellman I 2001). Although I could show that both, EGFR$^{fl/fl}$ and EGFR$^{\Delta Mx}$, BMDMs induced MHC class II expression after LPS stimulation, in two out of three experiments the former ones tended to be more efficient (Fig. 16). Additionally, LPS stimulation was reported to induce the expression of CD86 (B7-1) and PD-L1, two structurally related molecules belonging to the B7 family of cell surface protein ligands (Mellman I 2001, Loke P 2003). The former one can mediate co-stimulatory or co-inhibitory signals into T cells depending on which receptor it binds to, CD28 or CTLA-4, respectively (Sharpe AH 2002). In contrast, PD-L1 but also PD-L2, bind the PD-1 receptor on the surface of T cells and regulate negatively TCR signalling (Sharpe AH 2002). EGFR$^{fl/fl}$ and EGFR$^{\Delta Mx}$ BMDMs induced equally well the expression of CD86 and PD-L1 in response to LPS stimulation, whereas PD-L2 expression was induced after cells had been exposed to IL-4. (Fig. 16). The differential induction of PD-L1 and PD-L2 expression in macrophages had already been shown by Loke et.

(Loke P 2003). Interestingly the expression of the integrin CD11b on BMDMs seemed to be suppressed by LPS stimulation, whereas the integrin CD11c was upregulated in response to IL-4 (Fig. 16). These two integrins are thought to be somehow involved in scavenging and tethering of opsonized material. Additionally, studies have emphasised the role of CD11b in phagocytosis by DCs (Chen J 2008).

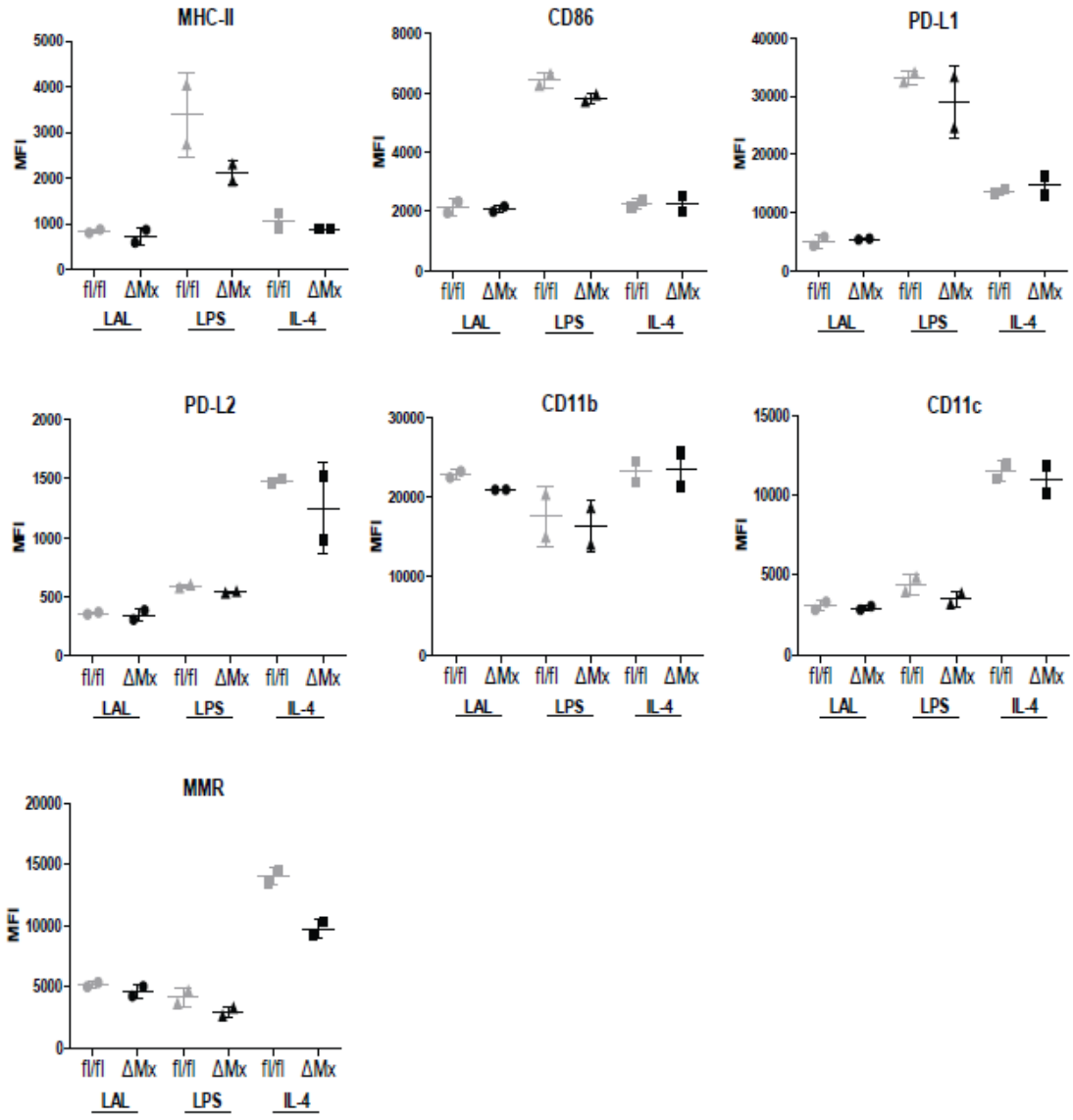


Fig. 16: Surface molecules expressed by stimulated EGFR$^{\Delta Mx}$ (ΔMx) and control EGFR$^{fl/fl}$ (fl/fl) BMDMs. *Cells had been stimulated with LAL (vehicle control), LPS or IL-4 for 24h. Expression of MHC class II (MHC-II), CD86, and PD-L1 were elevated after stimulation with LPS. IL-4 induced the expression of PD-L2, mannose receptor (MMR) and CD11c. Neither LPS nor IL-4 influenced the expression of CD11b – one representative experiment (n=2) out of three.*

Additionally, IL-4 stimulation induced the expression of the mannose receptor (MMR), a scavenger receptor which is important in phagocytosis of pathogens by recognising various types of sugars on their surface. Increased expression of MMR was shown to be upregulated in M2 macrophages (Stein M 1992). As in the case of MHC class II expression, there was a trend that $EGFR^{fl/fl}$ BMDMs were better in inducing MMR expression than $EGFR^{\Delta Mx}$ cells. However, this trend was detectable just in this particular experiment

Interestingly, although BMDMs induced MHC class II and CD86 expression after LPS stimulation (Fig. 16), this did not affect antigen presentation, where LPS stimulation of BMDMs did not increase T cell proliferation (Fig. 14 B – middle panel). Moreover, IL-4 stimulated BMDMs induced a high percentage of proliferating T cells, but only displayed increased expression of PD-L2, CD11c and MMR on their surface. Thus, meaning that the decreased capacity of BMDMs to induce T cell proliferation after LPS stimulation, but also elevated T cell proliferation after IL-4 stimulation might be regulated by other mechanism which I did not inspect. Nonetheless, there were no significant differences in the expression pattern of investigated surface molecules between $EGFR^{fl/fl}$ and $EGFR^{\Delta Mx}$ BMDMs.

Mature BMDCs expressed a different pattern of surface molecules than the one of BMDMs. Mature BMDCs did already express high levels of MHC class II (gating strategy – Fig. 15), therefore, stimulation with LPS or IL-4 did not influence the expression level of this molecule. In this representative example, $EGFR^{\Delta Mx}$ BMDCs were less efficient in inducing the expression of MHC class II (Fig. 17). However, as expected LPS induced the expression of CD86 (Mellman I 2001, Trombetta ES 2005) but additionally, PD-L1, CD11b, CD11c, as well as MMR, showed a trend to be upregulated in response to this stimulus (Fig. 17). Although mature BMDCs expressed roughly 10-fold more of the MHC class II molecules than LPS stimulated BMDMs did, there was no further induction of T cell proliferation after antigen presentation by LPS stimulated BMDCs.

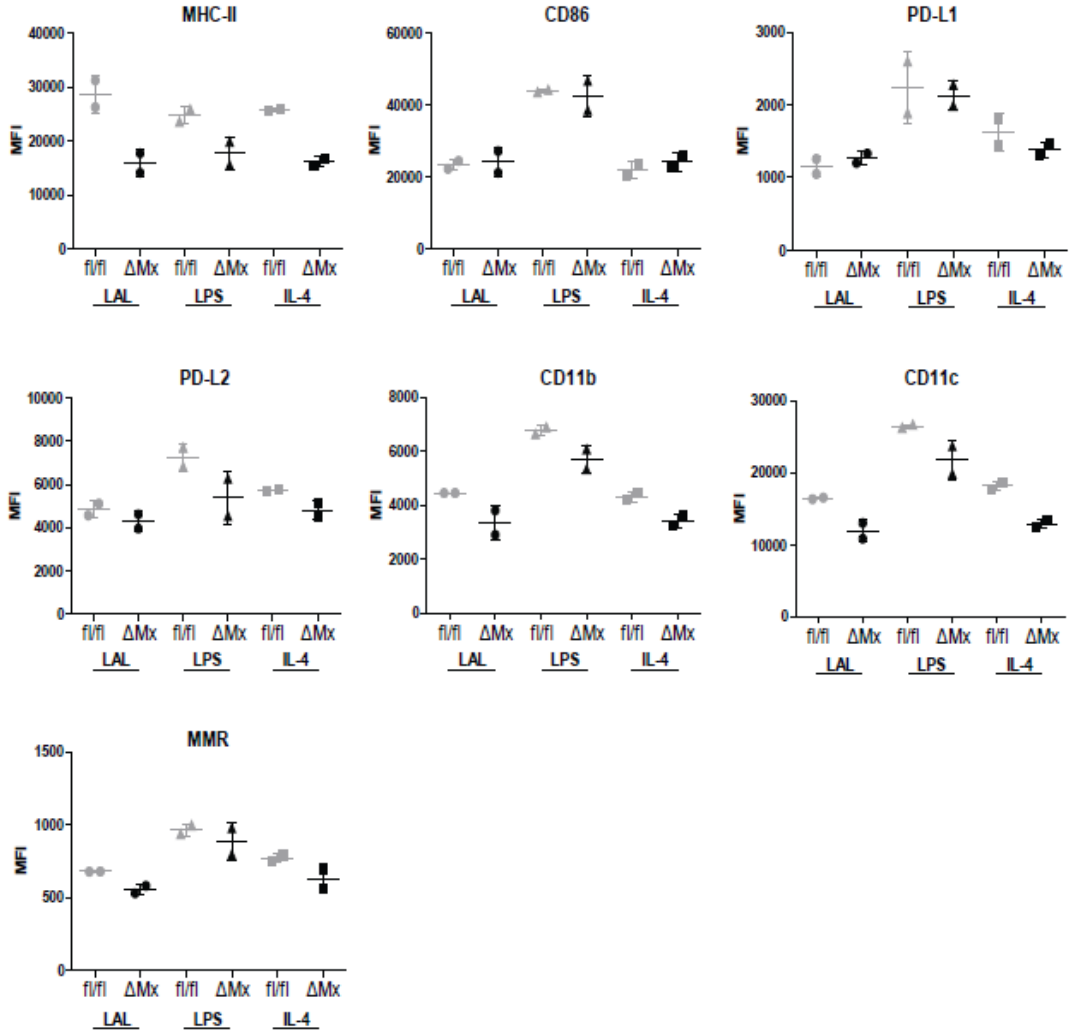


Fig. 17: Surface molecules expressed by stimulated EGFR$^{\Delta Mx}$ (ΔMx) and control EGFR$^{fl/fl}$ (fl/fl) mature BMDCs. *Cells had been stimulated with LAL (vehicle control), LPS or IL-4 for 24h. EGFR-deficient mature BMDCs seemed to be less efficient in inducing MHC class II expression. LPS induced CD86 and PD-L1 expression. There was also a trend of CD11b, CD11c, and MMR to be upregulated in response to LPS – one representative experiment (n=2) out of three.*

Strikingly, immature BMDCs from the same culture as the mature BMDCs resembled in part the expression pattern I described for the EGFR$^{\Delta Mx}$ BMDMs but also mature BMDCs (Fig. 18). Like BMDMs, IL-4 stimulation induced PD-L2 and CD11c expression. In contrast, the expression pattern of MMR was more similar to the one of mature BMDCs. Elevated expression of PD-L1 and CD86 was induced by LPS stimulation, as was it in BMDMs and mature BMDCs. This showed that immature DCs behave phenotypically rather like macrophages than mature DCs (Helft J 2015). Intriguingly, neither LPS nor IL-4 induced expression of MHC class II or CD11b on

immature BMDCs. The BMDC cultures I used for the antigen presentation assay encompassed both immature and mature DCs. However, studies have showed that only mature DCs were able induce T cell proliferation (Delamarre L 2003, Mellman I 2001, Trombetta ES 2005). Moreover, in my hands the percentage of mature DCs after LAL or IL-4 stimulation was roughly about 35% and doubled after LPS stimulation in both $EGFR^{fl/fl}$ and $EGFR^{\Delta Mx}$ cells (data not shown). Additionally, mature DCs expressed almost 50-fold more of the MHC class II molecules than the immature cells did (Fig. 17 and Fig. 18).

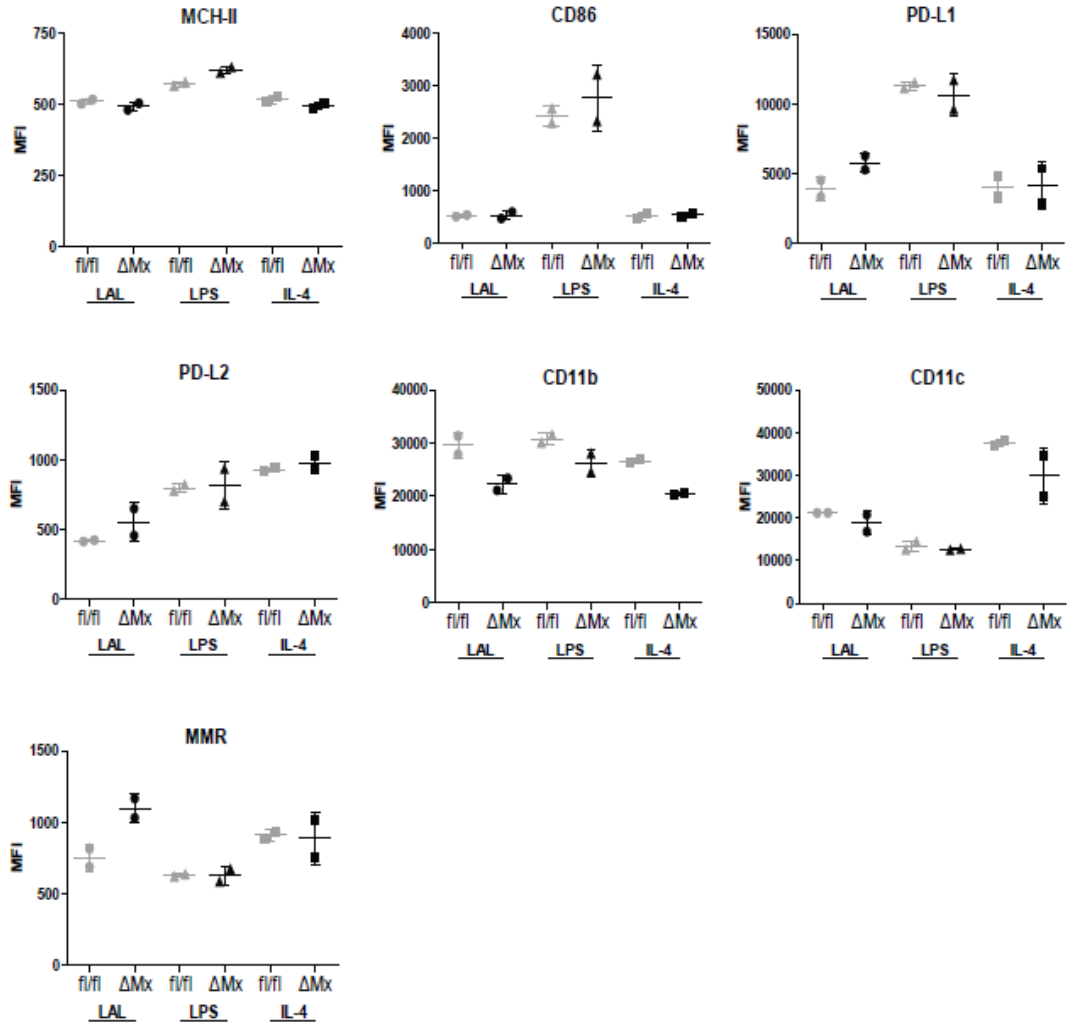


Fig. 18: Surface molecules expressed by stimulated $EGFR^{\Delta Mx}$ (ΔMx) and control $EGFR^{fl/fl}$ (fl/fl) immature BMDCs. *Cells had been stimulated with LAL (vehicle control), LPS or IL-4 for 24h. LPS induced CD86 and PD-L1 expression, whereas IL-4 stimulation induced CD11c and PD-L2 expression. Neither LPS nor IL-4 influenced the expression of MHC class II (MHC-II) and CD11b – one representative experiment (n=2) out of three.*

Nonetheless, there was no correlation between the antigen presentation mediated by BMDCs and the expression pattern of the surface molecules on mature DCs. Interestingly, although both, LPS and IL-4 stimulated mature BMDCs expressed high levels of MHC class II, IL-4 stimulated BMDCs were more efficiently in inducing T cell proliferation.

To exclude, the fact that there were only few differences observed in $EGFR^{\Delta Mx}$ APCs was due to incomplete *Egfr* deletion, I additionally inspected the expression pattern of the surface molecules in cells isolated from EGFR KO mice. The EGFR plays a crucial role in the proliferation and differentiation of various epithelial tissues, thus leading to early lethality (mostly pre-natal) in these mice. Survival of these mice had been shown to be background dependent, where 129/SvxC57BL6/6xMF1 EGFR KO mice live up to P20 (Sibilia M 1995). I had the opportunity to isolate bone marrow from P21 EGFR KO mice in two independent experiments. Isolated cells lacked genomic *Egfr* (Fig. 19).

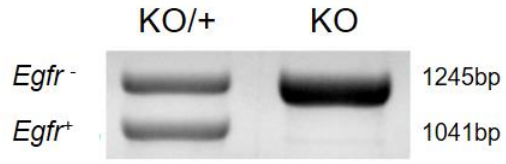


Fig. 19: Genomic Egfr deletion in bone marrow-derived cells from EGFR KO mice. *$EGFR^{KO/+}$ (KO/+) control cells have one KO ($Egfr^-$) and one wt allele ($Egfr^+$), whereas cells from EGFR KO mice have both KO alleles.*

Interestingly, I was not able to generate mature BMDCs from P21 EGFR KO mice (Fig. 20 A and B), therefore I inspected the expression pattern of surface molecules only in BMDMs from these mice. Although I pooled the data from two independent experiments, and the error bars were in some cases rather high, the expression pattern of surface molecules on EGFR KO BMDMs were very similar to the one of $EGFR^{\Delta Mx}$ BMDMs. LPS stimulation induced expression of CD86 and PD-L1 and additionally somewhat repressed the induction of CD11b expression. PD-L2, CD11c, and MMR were induced in response to IL-4 stimulation (Fig. 20 C). Thus, I could conclude that the EGFR in BMDMs did not influence the expression pattern of inspected surface molecules.

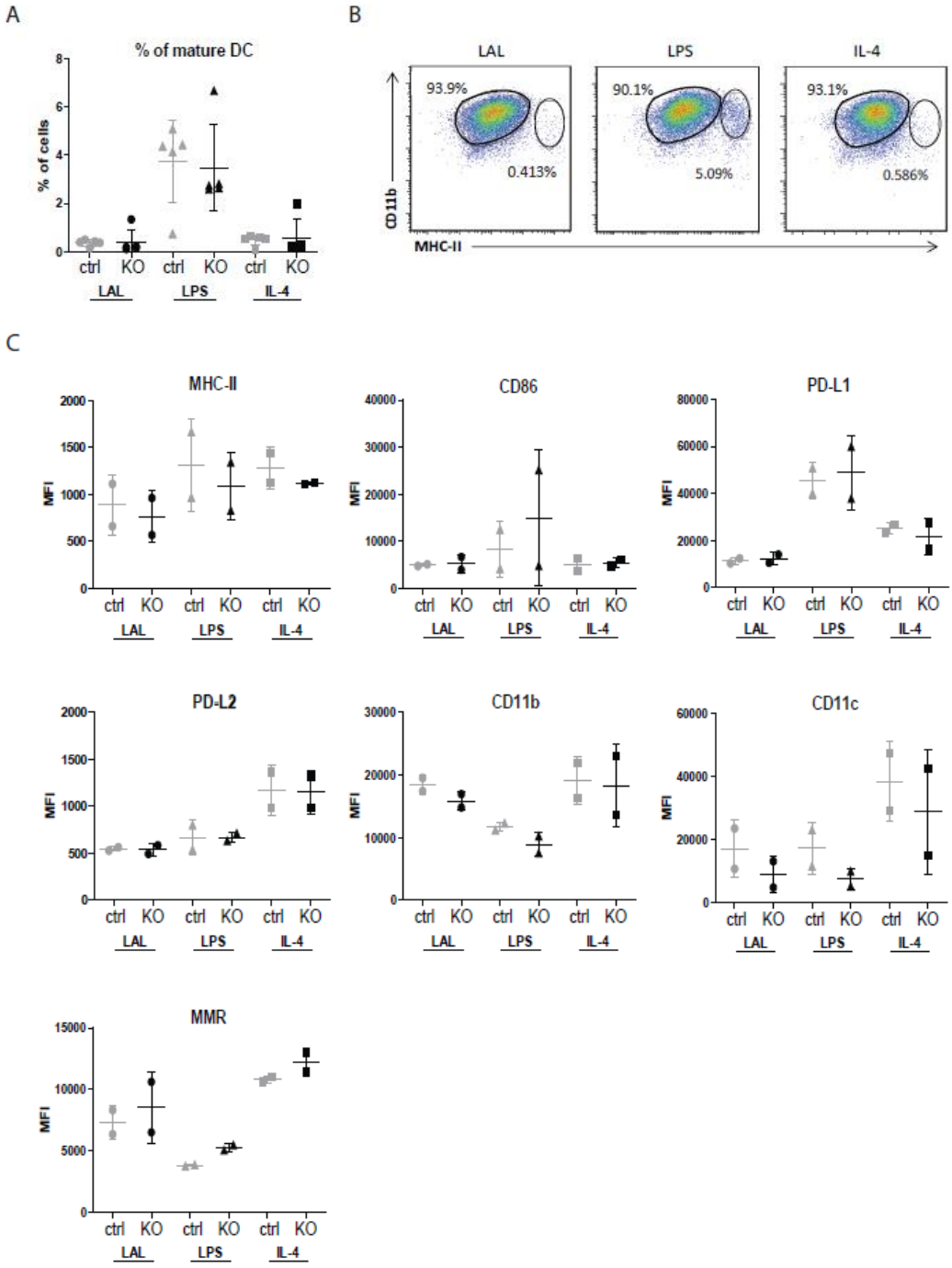


Fig. 20: Surface molecules expressed by stimulated EGFR KO (KO) and control EGFR$^{KO/+}$ (ctrl) BMDMs. *A) Percentage of mature BMDCs (DC). B) Representative blots of flow cytometry results. LPS stimulation induced significantly more mature BMDCs (CD11b^{+}MHC-II^{+}) than vehicle control (LAL) or IL-4. Two independent experiments (n=1) pooled with technical replicates. C) KO and control BMDMs increased expression of PD-L1 after LPS stimulation. LPS seemed to also have set a trend of higher expression levels for CD86. IL-4 on the other hand, increased expression of PD-L2, CD11c, and MMR. MHC class II (MHC-II) expression remained rather constant independent of the type of stimuli added – two independent experiments (n=1) pooled.*

Although all, the expression pattern of investigated surface molecules in $EGFR^{\Delta Mx}$ APCs, as well as EGFR KO BMDMs, were induced as expected and the pattern were very similar between $EGFR^{\Delta Mx}$ and EGFR KO macrophages, there were no significant EGFR-mediated differences, a fact which might explain the equal capacity of EGFR-deficient and $EGFR^{fl/fl}$ BMDCs and BMDMs to present antigen to T cells.

Not only can APCs present antigen to T cells and simulate TCR signalling by expression of co-stimulatory molecules, they can also produce different cytokines in response to pathogens or harmful self-molecules. All these features mediated by APCs allow the induction of a T cell-mediated immune response which is specific for a certain type of threat (e.g. pathogenic infections or transformed cells). Induction of such responses are required for tumour clearance, whereas induction of a Treg-mediated immunosuppression sustains tumour survival. We know that EGFR expressing liver macrophages and tumour associated myeloid cells were described to be involved in tumour progression of HCC and CRC, respectively (Lanaya H 2014, Srivatsa S 2017, Hardbower DM 2017). In these cases, tumour progression was mediated by innate mechanisms, where EGFR expression in myeloid cells mediated IL-6 production or activated STAT3 and survivin expression, thus sustaining HCC or CRC development, respectively. IL-6 might directly affect T_H cell proliferation but it is also described as being involved in polarisation of these cells into specific effector T cells (Zhou L 2008). In these co-culture experiments, I did not investigate whether APCs, especially macrophages from $EGFR^{\Delta Mx}$ mice displayed reduced levels of IL-6. However, although rather heterogenous, preliminary data from our lab showed a trend of decreased IL-6 production by BMDMs from both, $EGFR^{\Delta Myl}$ and $EGFR^{\Delta Mx}$ mice, after LPS stimulation (data not shown). Thus, I wanted to investigate whether stimulated BMDCs or BMDMs would differently affect T_H polarisation after antigen presentation.

3.4 Induction of effector CD4$^+$ T cells after antigen presentation

I investigated if both, BMDMs and BMDCs, from EGFR$^{fl/fl}$ and EGFR$^{\Delta Mx}$ mice could efficiently induce Tregs *in vitro* and addtionally inspected the capacity of BMDMs to polarise T_H1, T_H2, and T_H17 cells. I adapted the antigen presentation assay, starting with a constant APC : T cell ratio of 1:2 because in the former experiments, we had observed that there were no significant differences between the efficiency of BMDMs or BMDCs to induce T cell proliferation by antigen presentation at a ratio of 1:1 or 1:2. Additionally, after seeding APCs, those were exposed overnight (O/N) to different stimuli, thus allowing the cells to fully respond to the environmental cues. The second morning, without washing, APCs were loaded with the $OVA_{323-339}$ peptide. We chose to use only the peptide for presentation because it had proven to be as efficient as presentation of protein by stimulated APCs. In the meantime, OT-II T cells were isolated, purified and added this time without CFSE to the APCs, following incubation of co-cultures for 4 or 7 days. After the respective incubation period, I inspected both, the presence of T_H-specific TFs and cytokines. TFs control the transcription rate of genes by actively binding to their promoter or enhancer sequences in response to environmental changes mediated by intracellular signalling (Latchman DS 1997). Those are rapidly induced and favour the expression of specific genes (e.g. differential effector T cell gene expression) which are needed for the mediation of extracellular signals. Because TFs are rapidly induced but might lose expression with time, I inspected the presence of Foxp3 and RORγt after 4 days (Zheng SG 2007). Cytokines which are produced by the different subtypes of T cells are products of genes which are regulated by TFs. Therefore, I inspected the presence of INFγ, IL-4, and IL-17 after 3 more days of incubation (Fig. 21). Importantly, before harvesting T cells and inspecting the cytokine profile, a mix containing Phorbol 12-myristate 13-acetate (PMA), Ionomycin (Iono), and 1x Brefeldin A was added to the co-cultures for 4h to boost cytokine production and block protein transport so that the cytokines remain trapped in the cytoplasm (Helms JB 1992, Ai W 2013).

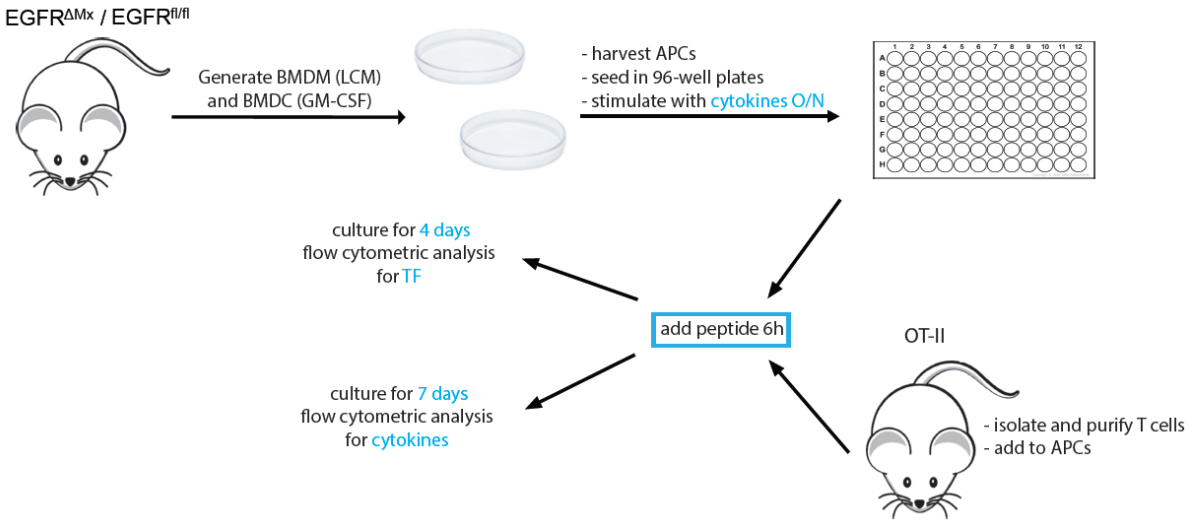
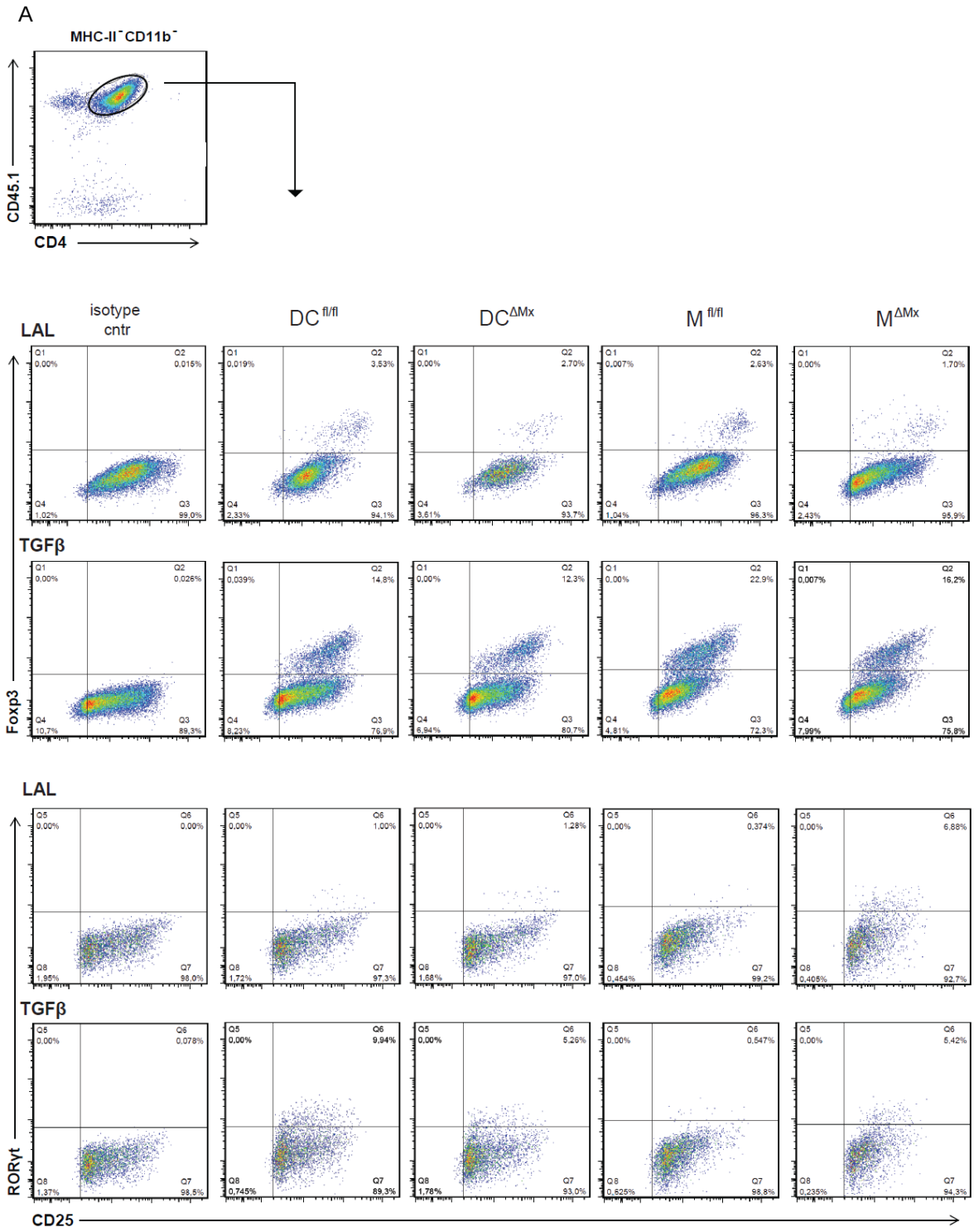


Fig. 21: Schematic representation of the T cell polarisation assay after antigen presentation by stimulated APCs. *Briefly, 1 x 10^4 APCs were seeded to 8 wells of a 96-well plate. APCs were stimulated overnight (O/N), following addition of the $OVA_{323-339}$ peptide on the second day for 5-6h. In the meantime, OT-II T cells were isolated, purified and 2 x 10^4 T cells were added to the APCs, reaching a APC : T cell ratio of 1:2. Subsequently, co-cultures were incubated for 4 (identification of transcription factors) or 7 days (detection of produced cytokines).*

Based on the study that EGFR in liver macrophages was linked to the transcriptional induction and secretion of IL-6, which led to tumour progression in a mouse model of HCC (Lanaya H 2014), we hypothesised that the decrease in IL-6 production by EGFR$^{\Delta Mx}$ BMDMs would lead to increased numbers of Treg formation and reversely less T_H17 polarisation. To study TF expression under Treg and T_H17 conditions, I stimulated EGFR$^{fl/fl}$ and EGFR$^{\Delta Mx}$ APCs O/N with TGFβ. TGFβ alone is known to induce Treg development, but in combination with IL-6 it induces T_H17 cells formation (Mangan PR 2006, Zhou L 2008). After 4 days of co-culture, OT-II T cells were harvested and the percentage of Foxp3$^+$ or RORγt$^+$ T cells was determined by flow cytometry.

As expected, antigen presentation mediated by TGFβ stimulated APCs induced polarisation of both, Tregs (iTregs) and T_H17 T cells, which were identified by flow cytometry analysis as being OT-II T cells (CD45.1$^+$CD4$^+$) positive for CD25 and Foxp3 or RORγt, respectively (Fig. 22 A).


A
MHC-II⁻CD11b⁻
CD45.1
CD4
isotype cntr
DC fl/fl
DC ΔMx
M fl/fl
M ΔMx
LAL
TGFβ
Foxp3
LAL
TGFβ
RORγt
CD25

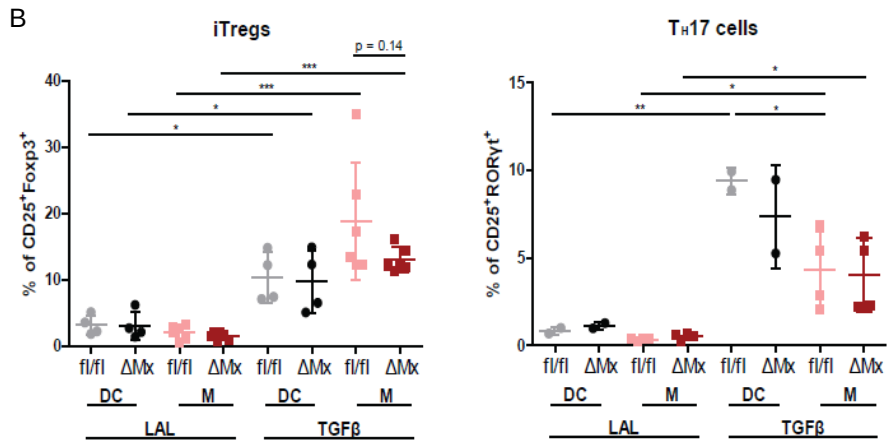


Fig. 22: Percentage of induced Tregs (iTregs) and T_H17 cells after antigen presentation by TGFβ stimulated APCs. *LAL (as a control) and TGFβ stimulated $EGFR^{fl/fl}$ (fl/fl) and $EGFR^{\Delta Mx}$ (ΔMx) BMDCs (DC) and BMDMs (M) induced OT-II T cell proliferation after $OVA_{323-339}$ peptide presentation. A) Representative blots of flow cytometry analysis. After 4 days of co-culture, T cells were harvested and the OT-II T cells population was identified as being MHC-II$^-$CD11b$^-$CD45.1$^+$CD4$^+$. Furthermore, iTregs were identified by the expression of CD25 and Foxp3, whereas T_H17 cells expressed CD25 and RORγt. Foxp3$^+$ cells were not RORγt$^+$. B) Percentage of iTregs and T_H17 cells. For the induction of iTregs mediated by BMDCs or BMDMs there were 2, respectively 3 independent experiments (n=2) which were pooled. There was one experiment (n=2) for the induction of T_H17 T cells mediated by BMDCs, but 2 independent experiments (n=2) for the induction mediated by BMDMs.*

Interestingly, stimulated BMDMs induced more iTregs than stimulated BMDCs. Moreover, although not significant $EGFR^{\Delta Mx}$ BMDMs seemed to induce fewer iTregs than the control $EGFR^{fl/fl}$ group (p =0.14). In the same co-culture where antigen presentation was mediated by TGFβ stimulated APCs not only was expression of Foxp3 but also RORγt induced, the latter one being specific for T_H17 cells. Studies have suggested that M2 macrophages are the key players in inducing Treg-T_H17 cell axis and that alternatively activated human macrophages could induce Treg-mediated regulatory immune responses by binding and re-releasing TGFβ cytokine (Haribhai D 2016, Schmidt A 2016). Although Foxp3 and RORγt are known to be co-expressed in naïve CD4$^+$ T cells (Zhou L 2008), iTregs and T_H17 T cells in this experiment were not double positive for these TFs. Interestingly, TGFβ stimulated BMDCs induced almost the same amount of iTregs and RORγt$^+$T cells (about 10%), whereas BMDMs induced rather Tregs than RORγt$^+$ T_H17 cells.

BMDMs could not only induce iTregs by antigen presentation, they were also able to induce T_H1, T_H2 and very few T_H17 cells when exposed to the right stimulus. I could show that antigen presentation by IL-4 stimulated BMDMs supressed induction of T_H1 ($CD4^+INF\gamma^+$) cells, but induced T_H2 ($CD4^+IL\text{-}4^+$) cell formation (Fig. 23 B). However, T_H1 cell differentiation could be induced by LPS stimulated BMDMs (Fig. 23 B).

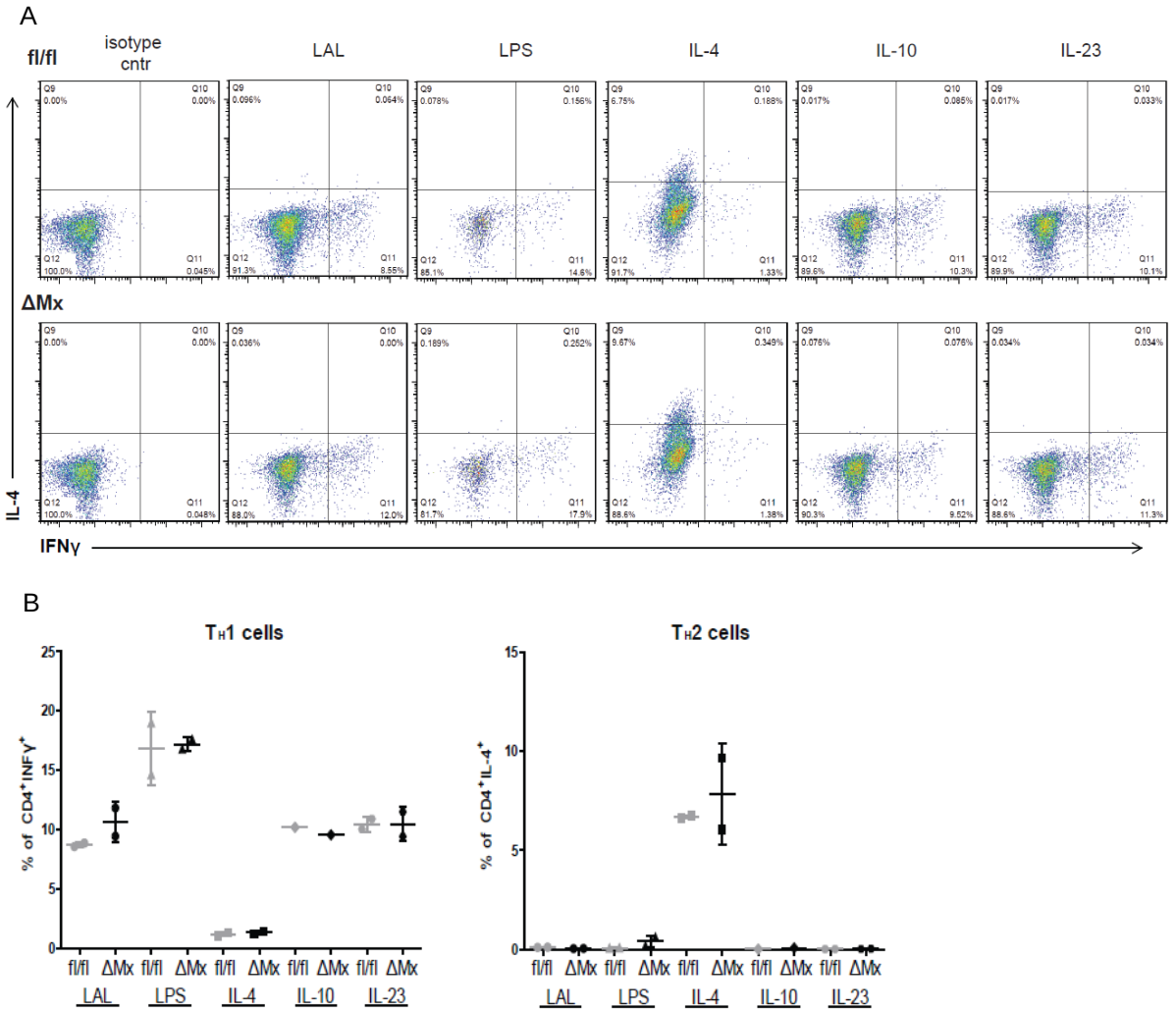
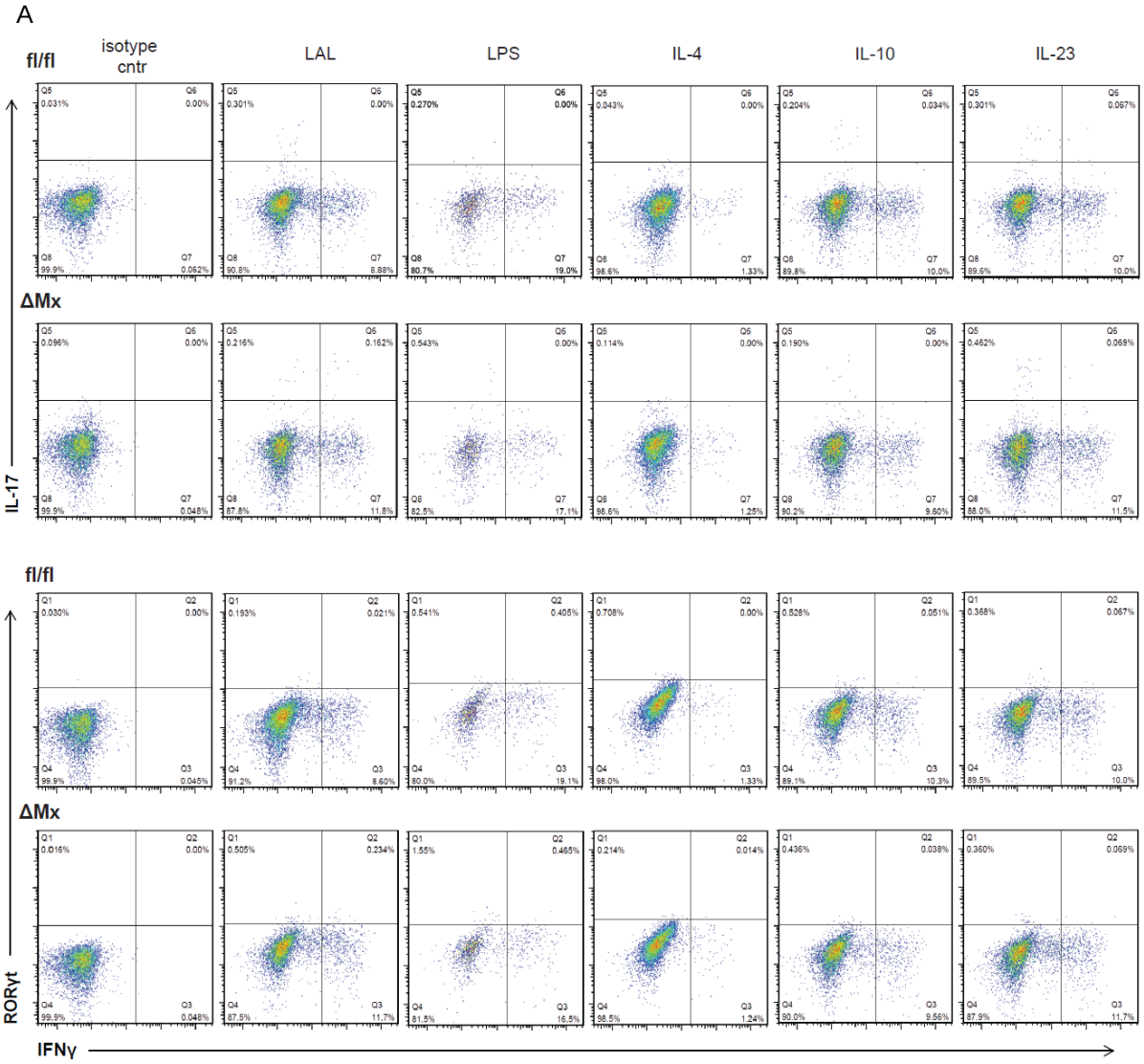


Fig. 23: Percentage of T cells which produced T_H1 and T_H2 cytokines after antigen presentation by stimulated BMDMs. *LAL (as a control), LPS, IL-4, IL-10, and IL-23 stimulated $EGFR^{fl/fl}$ (fl/fl) and $EGFR^{\Delta Mx}$ (ΔMx) BMDMs induced OT-II T cell proliferation after $OVA_{323\text{-}339}$ peptide presentation. A) Representative blots of flow cytometry analysis. After 7 days of co-culture incubation, T cells were harvested and the OT-II T cells population was identified as being $MHC\text{-}II^-CD11b^-CD45.1^+CD4^+$. Furthermore, T_H1 cells were identified by the expression of INFγ, whereas T_H2 cells expressed IL-4. B) Percentage of induced T_H1 and T_H2 cells (n=2).*

Activation of macrophages with LPS alone or in combination with INFγ has previously been shown to be a potent inducer of T_H17 or T_H1 cells, respectively (Arnold CE 2015). I could show that BMDMs which had been subjected to LPS could induce T_H17 cells (Fig. 24).



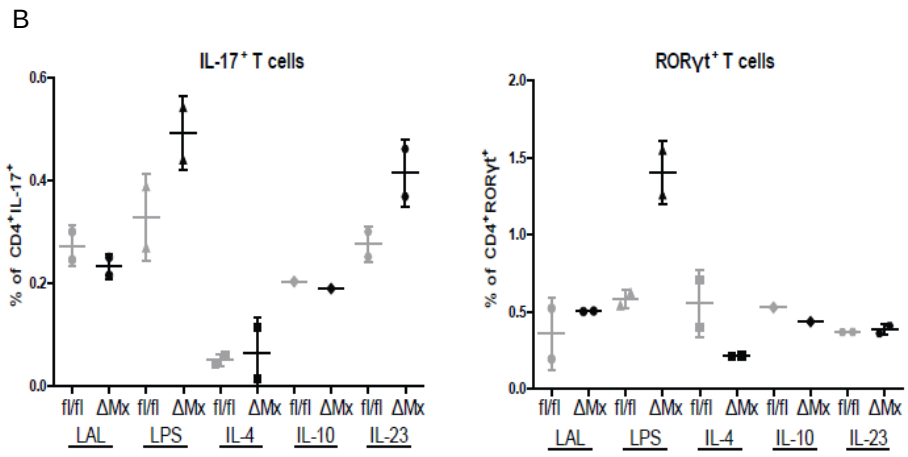


Fig. 24: Percentage of T cells which produced T_H17 markers after antigen presentation by stimulated BMDMs*. LAL (as a control), LPS, IL-4, IL-10 and IL-23 stimulated EGFR$^{fl/fl}$ (fl/fl) and EGFR$^{\Delta Mx}$ (ΔMx) BMDMs induced OT-II T cell proliferation after OVA3_{23-339} peptide presentation. A) Representative blots of flow cytometry analysis. After 7 days of co-culture incubation, T cells were harvested and the OT-II T cells population was identified as being MHC-II$^-$CD11b$^-$CD45.1$^+$CD4$^+$. T cells were investigated for the expression of the cytokine IL-17 or the TF RORγt. B) Percentage of T cells which produced IL-17 or RORγt are depicted here (n=2).*

However, after 7 days of co-culture I inspected additional to the presence of the cytokine IL-17 also the expression of the TF RORγt, to see whether there were any differences in the percentage of induced RORγt$^+$ T cells by either TGFβ or LPS stimulated BMDMs. EGFR$^{\Delta Mx}$ BMDMs which have been stimulated with LPS induced production of both, the cytokine IL-17 and the TF RORγt, by OT-II T cells after antigen presentation (Fig. 24 B). Nonetheless the percentages of induced RORγt$^+$ T cells was higher than the one of IL-17$^+$ T cells, thus meaning that not every RORγt$^+$ T cells was also positive for IL-17. Additionally, although after 7 days of co-culture EGFR$^{\Delta Mx}$ BMDMs seemed to have had an advantage in inducing RORγt$^+$ T cells, the percentage of RORγt$^+$ T cells which had been induced after antigen presentation by TGFβ stimulated BMDMs was far greater.

The cytokine IL-23 had been described as being required for the maintenance and survival of T_H17 cells, rather than the induction of this T helper subtype (Stritesky GL 2008). Moreover, IL-17 induction by T_H17 cells requires the expression of the TF RORγt (Ivanov II 2006). Surprisingly, in my hands, IL-23 stimulation of BMDMs differentially influenced the production of IL-17 and RORγt by T cells.

Although IL-23 stimulated BMDMs induced T cells to produce IL-17, there was no detectable RORγt production (Fig. 24 B).

Surprisingly, although we hypothesised that antigen presentation by TGFβ stimulated $EGFR^{\Delta Mx}$ BMDMs would induce higher numbers of Tregs and decreased numbers of T_H17 cells, my data showed that the capacity of TGFβ stimulated EGFR-deficient BMDMs to induce Tregs seemed to be impaired. In contrast, when stimulated with LPS, $EGFR^{\Delta Mx}$ BMDMs seemed to have an advantage over the $EGFR^{fl/fl}$ cells, inducing higher levels of IL-17 and RORγt by T cells. Functionally, reduced induction of Tregs by EGFR-deficient macrophages might explain why mice harbouring macrophages lacking the receptor had a decreased tumour burden (Lanaya H 2014). Thus, decreased immunosuppression and better tumour clearance might be induced.

However, because I was able to perform the experiment which included the investigation of T_H-specific cytokines only once, this experiment would need to be repeated.

4. Conclusion

Aberrant expression of EGFR or its ligands, as well as mutations of the receptor, are known to be involved in different types of epithelial cancer. Studying the signalling pathways induced by EGFR has led to the understanding of how the mechanism of signal induction and translation works. Nowadays, we know that not only tumour cells which express EGFR but also stromal cells, especially myeloid cells which express the receptor can promote malignancies and sustain tumour progression (Lanaya H 2014, Srivatsa S 2017, Hardbower DM 2017). Because most patients receiving EGFR-targeted therapies develop therapy-induced resistance in the end, understanding how the precise mechanism by which tumour progression might be induced by EGFR expression in specific cell types, could lead to the development of better therapeutic treatment for patients.

My study was based on the previous findings published by Maria Sibilia's lab in which EGFR was found to have a tumour-promoting role in liver macrophages and tumour-associated myeloid cells in a mouse model of HCC and CRC, respectively (Lanaya H 2014, Srivatsa S 2017). In the former study, they could link the EGFR signalling pathway in liver macrophages to the inflammation mediated induction and secretion of IL-6, thus promoting tumour progression. Based on this, the aim of my thesis was to investigate if the expression of EGFR in macrophages plays a role in regulating other immunological mechanisms, such as antigen presentation and induction of effector T cell.

Although I could show that both, BMDMs and BMDCs, from $EGFR^{\Delta Mx}$ and $EGFR^{fl/fl}$ mice could present antigen to T cells, I did not detect any EGFR-mediated differences in antigen presentation. Moreover, although it was previously described that LPS activated DCs promote antigen presentation (Delamarre L 2003, Mellman I 2001, Trombetta ES 2005, Weck MM 2007), I could not detect an increase in T cell proliferation after antigen presentation by LPS stimulated BMDCs or BMDMs. Instead, IL-4 stimulation of APCs boosted antigen presentation and T cell proliferation, in line with the findings that BMDCs, which were co-cultured with IL-4, showed an increased stimulatory potential (Wells JW 2005). To investigate, whether there was a correlation between surface molecules which are involved in antigen presentation and the capacity of APCs to induced T cell proliferation after antigen presentation, I also

analysed the effect of LPS and IL-4 stimulation on the expression pattern of surface molecules of the same $EGFR^{\Delta Mx}$ and $EGFR^{fl/fl}$ APCs which had been used for the antigen presentation assay. Additionally, to exclude the possibility of incomplete EGFR deletion in $EGFR^{\Delta Mx}$ cells, thus maybe masking some EGFR-mediated differences, I also analysed the expression pattern of surface molecules in EGFR KO mice. However, I could neither detect any EGFR-mediated differences in the expression pattern of the inspected surface molecules, nor could I make a correlation to the results from the antigen presentation assay.

Furthermore, although we hypothesised that possible reduced IL-6 production by $EGFR^{\Delta Mx}$ BMDMs would facilitate Treg polarisation after antigen presentation, rather than T_H17 cell formation, I could show that TGFβ stimulated $EGFR^{\Delta Mx}$ BMDMs seemed to induce fewer iTregs than the control $EGFR^{fl/fl}$ cells after 4 days of co-culture with OT-II T cell. Moreover, after 7 days of co-culture, LPS stimulated $EGFR^{\Delta Mx}$ BMDMs seemed to have an advantage over the $EGFR^{fl/fl}$ cells and induced more T_H17 cells. Functionally, the reduced efficiency of EGFR-deficient BMDMs to induce Tregs might explain why mice harbouring EGFR-deficient macrophages had a decreased tumour burden (Lanaya H 2014). A better tumour clearance might possibly be mediated by a decreased immunosuppression at the site of tumour.

Therefore, in future experiments I would inspect if the *in vitro* results could also be applicable *in vivo*, by analysing Treg cell number in $EGFR^{\Delta Myl}$ tumour bearing mice and if so, further inspect a possible interaction between TGFβ and EGFR signalling in macrophages. Additionally, exploring a possible link between EGFR and IL-6 induction in macrophages which might lead to altered antigen presentation and T cell differentiation *in vivo* should be considered. Finally, to verify my flow cytometry results, I planned on further investigating the presence of T_H-specific cytokines in the supernatants of the co-cultures.

Concluding, I think that investigation and understanding of how immune cells interact with one another and how actually innate immune cells can mediate adaptive anti-tumour immune responses, but also what they need or do not need to induce such responses, could help develop better and more effective therapies for patients.

5. Materials

5.1 Reagents, Buffers and Media

Products	Company
10% Formaldehyde	Carl Roth®
1000x Brefeldin A Solution	BioLegend®
100mM dNTP's	Promega
20% SDS Solution	BIO-RAD
2-Propanol	Emplura®
50mM 2-Mercaptoethanol	Thermo Fisher Scientific
Albumin from chicken egg white (OVA)	Sigma-Aldrich
Ammonium chloride (NH_4Cl)	Merck
BD IMag™ Streptavidin Particles Plus-DM	BD Bioscience
Carboxyfluorescein succinimidyl ester (CFSE)	Sigma-Aldrich
Deoxyribonuclease I (DNase I) from bovine pancreas	Sigma-Aldrich
DreamTaq DNA Polymerase (5 U/µl)	Thermo Fisher Scientific
Ethanol (EtOH)	Emplura®
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth®
Ionomycin calcium salt (Iono) from *Streptomyces conglobatus*	Sigma-Aldrich
LPS-EB (LPS from *E. coli* O111:B4)	InvivoGen
Mouse TGF beta 1 Recombinant Protein (TGFβ)	eBioscience™ Thermo Fisher Scientific
$OVA_{257-264}$ / $OVA_{323-339}$	InvivoGen
peqGOLD Universal-Agarose	Peqlab
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
Polyinosinic:polycytidylic acid (poly I:C)	Sigma-Aldrich
Potassium chloride (KCl)	Merck
Recombinant Human IL-10	Peprotech®
Recombinant Mouse IL-23 (carrier-free)	BioLegend®
Recombinant Murine IL-4	Peprotech®
Recombinant Murine GM-CSF	Peprotech®
Sodium chloride (NaCl)	Emsure®
SYTOX™ Blue Dead Cell Stain	Life Technologies™
Tris acetate	Carl Roth®
Tris-HCl	Carl Roth®
Tri-Sodium citrate dihydrate	Emsure®
Trypan Blue 0.4% Solution	Szabo Scandic Lonza
Trypsin	Gibco® Thermo Fisher Scientific
Zombie Aqua™ Fixable Viability Kit	BioLegend®

Media/Buffer	Company
100x MEM Non-essential amino acids (NEAA) Solution	Sigma-Aldrich
100x Penicillin-Streptomycin (p/s)	Sigma-Aldrich
1M HEPES Solution	Sigma-Aldrich
1M Sodium pyruvate	Sigma-Aldrich
1x Dulbecco's Phosphate Buffered Saline w/o $CaCl_2$ and $MgCl_2$ (PBS)	Sigma-Aldrich
200mM L-Glutamine Solution (glu)	Sigma-Aldrich
50mM 2-Mercaptoethanol	Thermo Fisher Scientific
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich
Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 Ham (DMEM F-12)	Sigma-Aldrich
Endotoxin-free water (LAL)	Sigma-Aldrich
Fetal Bovine Serum (FCS)	Sigma-Aldrich
Foxp3/Transcription Factor Staining Buffer Set	eBioscience™ Thermo Fisher Scientific
Heat inactivated (H.I) FCS	Incubate for 30 min at 56˚ C
Mouse serum	Sigma-Aldrich
Perm/Wash Buffer	BD Bioscience
RPMI 1640 Medium	Sigma-Aldrich
Water	Sigma-Aldrich

Media/Buffer	Composition
10x citric saline	ddH_2O, 135mM KCl, 150mM Tri-sodium citrate dihydrate autoclave before use
50x TAE buffer	ddH_2O, 2M Tris acetate, 50mM EDTA pH8
BMDC media	DMEM F-12, 10% FCS, 1% p/s, 1% glu, 1% Sodium pyruvate, 1% NEAA, 20 ng/ml GM-CSF
BMDM media	DMEM F-12, 10% FCS, 1% p/s, 1% glu, 20% LCM
L929 medium	DMEM, 10% FCS, 1% HEPES, 1% glu
L929 starving medium	DMEM, 1% HEPES, 1% glu
MACS buffer	PBS, 2mM EDTA pH8, 0.5% BSA
NCM	RPMI 1640, 10% H.I. FCS, 1% p/s, 1% glu, 1% Sodium pyruvate, 1% NEAA, 1% HEPES, 50µM 2-Mercaptoethanol

Red blood cell lysis (ACK) buffer	ddH_2O, 100mM NH_4Cl, 1mM $KHCO_3$, 0.1mM EDTA pH 7.2
Tail DNA preparation buffer	ddH_2O, 50mM Tris-HCl pH 8, 100mM EDTA pH 8, 100mM NaCl, 1% SDS, 0.5 mg/ml Proteinase K
TE buffer	ddH_2O, 10mM Tris-HCl pH 8, 1mM EDTA pH 8
Würzburg buffer	PBS, 5% FCS, 5mM EDTA pH 8, 20µg/ml DNase I

5.2 Cell Culture Consumables

Product	**Company**
0.3x12 mm Disposable Needles	Henke-Sass Wolf
0.5 ml/2.5 ml/10 ml Combitips advanced®	Eppendorf
1 ml/10ml Disposable syringes	BD Plastipak™
1.4 ml Matrix Storage (FACS) Tubes	Thermo Fischer Scientific
1.5 ml Micro Tubes SafeSeal	Sarstedt
10 cm Bacteriological Petri Dishes	Corning Falcon®
10 cm Cell-Culture Treated Dishes	Corning
12x75 mm Round-Bottom Polypropylene Tubes	BD Falcon™
15 ml/50 ml Centrifuge Tubes	Starlab
6-well Non-Tissue Culture Treated Plate	Corning Falcon®
6-well Nunc™ Cell-Culture Treated Plate	Thermo Fischer Scientific
96-well Nunc™ Polystyrene Round Bottom/Flat Bottom Plates	Thermo Fischer Scientific
Cell Strainer (70 µm)	BD Falcon™
T75/T175 Flasks Nunc™ Cell-Culture Treated	Thermo Fischer Scientific

5.3 Antibodies

Antibody	Conjugated	Company	Clone
B220	Biotin	BD Pharmingen™	RA3-6B2
CD4	Biotin	BD Pharming™	GK1.5
CD4	PB™	BioLegend®	GK1.5
CD4	PE	BD Pharmingen™	GK1.5
CD4	PE-Cy™7	BioLegend®	GK1.5
CD8a	Biotin	BD Pharmingen™	53-6.7
CD8	APC	BioLegend®	53-6.7
CD8	PB™	BD Pharmingen™	53-6.7
CD11b	Biotin	BioLegend®	M1/70
CD11b	BV650™	BioLegend®	M1/70
CD11b	FITC	BioLegend®	M1/70
CD11b	V450	BD Horizon™	M1/70
CD11c	Biotin	BioLegend®	N418
CD11c	PE-Cy™5	BioLegend®	N418
CD16/32 (Fc-block)	LEAF™ purified	BioLegend®	93
CD19	Biotin	BD Pharmingen™	1D3
CD25	FITC	BioLegend®	PC61
CD45	APC-Cy™7	BD Pharmingen™	30-F11
CD45	PE.Dazzle™594	BioLegend	30-F11
CD45.1	PerCP-Cy™5.5	BioLegend® eBioscience™	A20 A20
CD86	PE-Cy™7	BioLegend®	GL-1
CD206	PE	BioLegend®	C068C2
CD273	PE	BioLegend® BD Pharmingen™	TY25 TY25
CD274	PE-Cy™7	BioLegend®	10F.9G2
Foxp3	PE	eBioscience™	FJK-16s
Gr1	Biotin	BioLegend®	RB6-8C5
MHC-II	AF®700	BioLegend®	M5/114.15.2
MHC-II	ev™655	eBioscience™	M5/114.15.2
INFγ	PE	BioLegend®	XGM1.2
IL-4	PE.Dazzle™594	BioLegend®	11B11
NK1.1	Biotin	BD Pharmingen™	PK136
IL-17A	AF®647	BioLegend®	TC11-18H10.1
RORγt	PerCP-Cy™5.5	BD Pharmingen™	Q31-378
TCR Vα2	PE	BioLegend®	B20.1
TER-119/ Erythroid Cells	Biotin	BD Pharmingen™	TER-119

Isotype	Conjugated	Company	Clone
Rat IgG1κ	FITC	BioLegend®	RTK2071
Rat IgG1κ	PE	BD Pharmingen™	R3-34
Rat IgG1κ	PE.Dazzle™594	BioLegend®	RTK2071
Rat IgG1κ	AF®647	BioLegend®	RTK2071
Rat IgG2aκ	PE	BD Pharmingen™	R35-95
Rat IgG2aκ	APC.Cy™7	BioLegend®	RTK2758
Rat IgG2bκ	PE.Cy™7	BioLegend®	RTK4530
Mouse IgG2aκ	PerCP-Cy™5.5	BD Pharmingen™	G155-178

5.4 Primers

Primer Name	Sequence
R4	GCCTGTGTCCGGGTCTCGTCG
R6	CAACCAGTGCACCTAGCCTGGC
Con2_JK	CTCAGTGAAGGTCTCAGGTTGTGATGAGG
LacZ100	GGATAATGCGAACAGCGCACGGCG
K5Cre1	CATACCTGGAAAATGCTTCTGTCC
K5Cre2	CATCGCTCGACCAGTTTAGTTACC
Jun1	CCGCTAGCACTCACGTTGGTAGGC
Jun2	CTCATACCAGTTCGCACAGGCGGC

5.5 Equipment

BD LSRFortessa™	Flow Cytometry
Neubauer Haemocytometer	Cell Counting
BD IMagnet™	Cell Separation Magnet for MACS Sorting

5.6 Software

Adobe® Illustrator®/Photoshop® CS6	Picture analysis and graphic design
BD FACSDiva™	Recording FACS data
FlowJo® 7.6.4	Analysis of FACS blots
GraphPad Prism® 5	One-way ANOVA Unpaired Two-sided Student's t-test significant * $p \leq 0.05$

6. Methods

6.1 EGFR$^{\Delta Mx}$ Mice

The deletion of *Egfr* in EGFR$^{\Delta Mx}$ mice was achieved by three consecutive intraperitoneal injections of 400 µg of poly I:C into 3-week-old mice every third day.

6.2 Production of L929 conditioned medium (LCM)

The L929 cell line was a kind gift from Prof. Dr. Wilfried Ellmeier from the Medical University of Vienna. After cells were briefly thawed in a 37˚C bath, the cell suspension was suspended into L929 medium, subsequently being centrifuged for 5 min at 300x g at room temperature (RT). The pellet was resuspended in fresh medium and seeded in a T75 flask. Cells were incubated at 37˚C and 5% CO_2 until they reached confluency. When confluent, cells were first washed with pre-warmed PBS, and trypsinised by adding 1 ml Trypsin and incubating for 5 min at 37˚C. Fresh medium was added and a single cell suspension was generated by pipetting. The cell suspension was centrifuged and the pellet was resuspended in fresh medium and split to several T75 flasks. After reaching confluency, one T75 flask was split to one T175 flask and incubated until the cells reached 70-80% confluency. The medium was removed and fresh pre-warmed starving medium was added to the cells. After 10 days of incubation, supernatants were collected, centrifuged and strained into fresh centrifuge tubes. Supernatants were stored at -20˚ C until further use.

6.3 Generation of BMDMs and BMDCs

The protocol was adapted from Zhang et. al (Zhang X 2008). 6-8-week-old mice were euthanized by rapid cervical dislocation and washed with 70% EtOH. The skin and flesh were removed completely from each bone (femur and tibia). Under sterile conditions, the heads of the bones were cut off and the bones were flushed with 5 ml ice-cold PBS with 2mM EDTA using needles. The liquid was collected and strained into a fresh centrifuge tube. The cell suspension was centrifuged for 6 min at 300x g and 4˚C. The pellet was resuspended in 1 ml ACK buffer and incubated for 30-60 sec at RT. The lysis was stopped by addition of 10 ml PBS, subsequently centrifuging the cells. The pellet was resuspended into 10 ml medium (DMEM F-12, 10% FCS, 1% p/s, 1% glu).

For the generation of BMDMs, 3 x 2 ml of the cell suspension were plated on non-coated 10 cm petri dishes already containing 8 ml of BMDM medium. Cells were incubated at 37˚C and 5% CO_2. After 2 days of incubation, 5 ml fresh pre-warmed medium was added to the dishes. On day 4 and 6, the medium was changed completely. BMDMs were harvested on day 8 when the cells were mature macrophages with 1x citric saline and PBS. For harvesting macrophages from a 10 cm dish, cells were first washed with pre-warmed PBS, followed by addition of 6 ml citric saline and incubation at 37˚C for 5-6 min. After incubation, 4 ml PBS were added and the dishes were washed by pipetting and generating a single cell suspension. The cell suspension was centrifuged for 5 min at 300x g and 4˚C, subsequently resuspending the pellet in medium or Würzburg buffer (depending on the assay). Cells were counted using a Neubauer haemocytometer and used for further analysis. The same procedure was used for harvesting BMDMs from 6-well plates, just that 3 ml citric saline and 2 ml PBS were used.

For the generation of BMDCs, 2 x 2 ml of the cell suspension were plated on coated 10 cm dishes already containing 8 ml BMDCs medium and incubated at 37˚C and 5% CO_2. Medium was changed completely after 3 and 6 days. BMDCs were harvested on day 8 by pipetting carefully to not remove adherent macrophages. Cell suspension was collected and centrifuged for 5 min at 300x g and 4˚C. Pellet was resuspended in medium or Würzburg buffer (depending on the assays). Cells were counted and used for further analysis.

6.4 Identification of surface molecules

On day 8, when BMDCs and BMDMs were mature, cells were seeded 0.8 x 10^6 cells/well on 6-well plates. Cells were let to rest O/N at 37˚C and 5% CO_2, subsequently stimulating with LPS (50 ng/ml) or IL-4 (20 ng/ml) for 24h. Cells were harvested, resuspended in Würzburg buffer, stained for extracellular molecules and analysed by flow cytometry. BMDMs were identified as being $CD11b^{high}MHC\text{-}II^{mid}$, whereas BMDCs were gated into mature ($CD11c^{+}CD11b^{mid}MHC\text{-}II^{high}$) and immature ($CD11c^{+}CD11b^{high}MHC\text{-}II^{mid}$) dendritic cells.

6.5 Isolation and purification of OT-I/OT-II T cells

OT-I/ OT-II transgenic mice were euthanized by rapid cervical dislocation and washed with 70% EtOH. Mice were fixed with needles and cut into the belly along the midline. Superficial cervical, branchial, axillary, inguinal and mesenteric lymph nodes (LNs), as well as the spleen, were collected and squeezed separately through a cell strainer. The two cell suspensions were collected and centrifuged for 5 min at 300x g and 4˚C. LN pellet was resuspended in Würzburg buffer and the cell number was counted, whereas the spleen pellet had an additional step of ACK buffer lysis (see 6.3) before counting. The two cell suspensions were pooled and then suspended in an appropriate volume of Würzburg buffer so that the cell number did not exceed 4 x 10^7 cells/ml. For the purification of the OT-I or OT-II T cells, BD MACS sort protocol with Streptavidin Particles Plus – DM was used as suggested by the manufacturer. Briefly, cells were incubated for 10-15 min with Fc-block (1 µg/ml) on ice. For depletion, following biotinylated antibodies were added at a final concentration of 1 µg/ml each: B220, CD11b, CD11c, NK1.1, ER119, Gr-1, CD19 and CD4 (for OT-I enrichment) or CD8 (for OT-II enrichment) and incubated for 30 min on ice. After incubation, cells were washed with an excess of MACS buffer following centrifugation. The cell pellet was resuspended in BD IMag™ Streptavidin Particles so that 50 µl of beads were used for 1 x 10^7 cells. The cells were incubated for 30 min at 6-8˚C. MACS buffer was added to the cell suspension reaching a final concentration of 2-8 x 10^7 cells/ml. Subsequently, the cell suspension was split to 12 x 75 mm tubes (not exceeding the volume of 1 ml/tube) and placed into the BD IMagnet™ for 8 min. The suspension was transferred to a fresh tube and placed back into the magnet for 6 min. The latter step was repeated. The first tube was washed with 1 ml MACS buffer and the 8-6-6 min cycle was repeated. All cell suspensions were pooled together following centrifugation. The pellet was resuspended in NCM medium and the cell number was counted. For CFSE labelling, T cells were not resuspended in medium after centrifugation but in PBS reaching a concentration of 1 x 10^7 cells/ml. 1µM CFSE was added to the cells following incubation for 10 min at RT in the dark. Subsequently, cells were washed with an excess of medium, resuspended in medium and counted. Before using T cells in further assays, an aliquot was stained with anti-CD8 or CD4 in combination with anti-TCR Vα2 and inspected by flow cytometry for the purity of OT-I or OT-II T cells, respectively. The purity routinely reached 85%.

6.6 Antigen-presentation assay

For the antigen presentation by unstimulated APCs, APCs were incubated with OVA protein (250 µg/ml) for 6h, following a washing step with PBS and harvesting of the cells (see 6.3). After centrifugation cells were resuspended in NCM medium, counted and 2 x 10^4 cells were seeded to the first well of a 96-well flat bottom plate. From this well a serial dilution of APCs was made, so that in the end (after addition of the T cells) different APC : T cell ratios (1:1, 1:2, 1:10 etc.) were present in each well of each column. The starting ratio was 1 x 10^4 APCs to 1 x 10^4 T cells. CFSE labelled OT-I or OT-II T cells were added together with 20 µg/ml $OVA_{254-264}$ (MHC class I-restricted) or $OVA_{323-339}$ (MHC class II-restricted) peptide, respectively. Co-cultures were incubated for 3-4 days at 37˚C and 5% CO_2. After incubation, T cells were harvested and analysed by flow cytometry for their proliferation. The OT-I or OT-II T cell populations were identified as being MHC-$II^-CD11b^-CD45.1^+CD8^+$ or $CD4^+$, respectively.

For the antigen presentation by stimulated APCs, cells were harvested, counted and seeded on 96-well flat bottom plates by making a serial dilution. The starting ratio was 1 x 10^4 APCs to 1 x 10^4 T cells. LPS (50 ng/ml) or IL-4 (20 ng/ml) together with OVA protein or $OVA_{323-339}$ peptide were added to the cells and incubated for 6h before adding the CFSE labelled OT-II T cells. Co-cultures were incubated for 4-5 days, subsequently harvesting T cells and inspecting their proliferation by flow cytometry. The OT-II T cell population was identified as being MHC-$II^-CD11b^-CD45.1^+CD4^+$.

6.7 $CD4^+$ T cell polarisation assay

APCs were harvested and 1 x 10^4 cells/well were seeded to 8 wells of a 96-well round bottom plate. APCs were stimulated O/N with: LPS (50 ng/ml), IL-4 (20 ng/ml), IL-10 (10 ng/ml), IL-23 (50 ng/ml), TGFβ (10 ng/ml) or LAL at 37˚C and 5% CO_2. The second morning, the $OVA_{323-339}$ peptide was added to the APCs and incubated for 5-6h. In the meantime, OT-II T cells were isolated and purified and 2 x 10^4 cells/well were added to the APCs. For the investigation of the TFs Foxp3 and RORγt, co-cultures were incubated for 4 days. Cytokine expression was analysed after 7 days of co-culture. Note that after 7 days, additional to the cytokine staining also the expression of the TF RORγt was inspected. Before harvesting the cells, PMA (20 ng/ml), Iono (1 µg/ml) and 1x Brefeldin A were added to the co-cultures and incubated for 4h. T cells were

harvested, washed twice with PBS and stained extra- and intracellularly, subsequently analysing them by flow cytometry. The OT-II T cell population was identified as being MHC-II^{-}CD11b^{-}CD45.1^{+}CD4^{+}.

6.8 Extracellular staining

After cells were washed, the pellet was resuspended in 50 µl Würzburg buffer and 1 µg/ml Fc-block was added and incubated for 10-15 min on ice. Without washing, a mix containing the antibodies of interest at a final concentration of 1 µg/ml each, was added to the cells and incubated for another 30 min. Cells were washed with an excess of buffer and centrifuged for 5 min at 300x g and 4˚C. The cell pellet was resuspended in a residual volume of 100 µl. Right before the flow cytometric analysis, 1 µM of SYTOX™ Blue Dead Stain was added to the cells.

6.9 Intracellular staining

After washing, T cells were resuspended in 100 µl PBS and 1 µl Zombie Aqua™ was added following incubation for 15 min on ice in the dark. 1 µg/ml Fc-block and FCS to 5% were added, following a 10 min incubation period on ice. A mix containing the antibodies of interest against extracellular molecules was added to a final concertation of 1 µg/ml each and incubated for 30 min on ice.

For the identification of transcription factors, the Foxp3/Transcription Factor Staining Buffer Set was used as described by the manufacturer. Briefly, cells were washed once with PBS by centrifugation for 6 min at 400x g and 4˚C. The pellet was resuspended in a residual volume of 100 µl. 300 µl 1x Fixation buffer was added to the cells, following incubation for 45-60 min at RT in the dark. Cells were washed twice with 1x Permeabilization buffer by centrifugation at RT. The pellet was resuspended in 100 µl residual volume. To block unspecific binding of mouse antibodies to intracellular molecules, mouse serum to 2% was added following incubation for 10 min at RT in the dark. Cells were stained intracellularly by adding a mix containing antibodies of interest or isotype control antibodies at a final concentration of 1 µg/ml each following incubation for 30 min on ice. After incubation, cells were washed once with 1x Permeabilization buffer and twice with PBS by centrifugation at 4˚C.

For the identification of T cell cytokines, after extracellular staining, cells were fixed with 2% formaldehyde adjusting the volume with PBS. Cells were incubated for 20 min at RT in the dark. After centrifugation, the pellet was resuspended in 800 µl of 1x BD Perm/Wash buffer, following another centrifugation step. The pellet was resuspended in 200 µl of 1x BD Perm/Wash buffer and incubated for 20 min at RT in the dark. After incubation, cells were centrifuged and the pellet was resuspended in a residual volume of 50 µl. Mouse serum to 2% was added following incubation for 10 min at RT in the dark. An antibody mix containing antibodies against cytokines or isotype control antibodies at a final concertation of 1 µg/ml each was added to the cells and incubated for 30 min on ice. Before flow cytometric analysis, cells were washed once with 1x BD Perm/Wash buffer and twice with PBS.

6.10 Tail DNA preparation

The tip of the tail was cut off with EtOH cleaned scissors and suspended in 500 µl of Tail DNA preparation buffer, following the addition of 0.5 mg/ml Proteinase K and incubation O/N at 55˚C. The second day, tubes were placed into the Thermoshaker, shaking for 5 min at full speed. 200 µl 6M NaCl were added to the tubes, subsequently centrifuging for 10 min at full speed. The supernatants were transferred to fresh tubes, following addition of 500 µL of 2-Propanol. After another centrifugation step, supernatants were discarded and the pellets washed with 70% EtOH, following a 15 min centrifugation step. The pellets were air dried and dissolved in 500 µL TE buffer. DNA was dissolved O/N at 37˚C and kept at RT until further use.

6.11 Genotyping PCRs

ΔEGFR_JK:

Thermocycler program
2 min 95˚C
15 sec 95˚C
30 sec 70˚C
45 sec 72˚C
15 sec 95˚C
30 sec 66˚C
45 sec 72˚C
2 min 72˚C
∞ 4˚C

Mix for one sample	
16.8 µl	H_2O
2.5 µl	10x DreamTaq buffer
0.5 µl	dNTPs mix (10mM)
1.5 µl	R4 primer (5µM)
1.5 µl	R6 primer (5µM)
1 µl	Con2JK primer (5µM)
0.2 µl	Taq DNA polymerase
1 µl	DNA

The R4 and R6 primes generate the product for the wild-type (wt) (*Egfr*$^{+}$ 1041bp) and the floxed (*Egfr*^{fl} 1100bp) alleles of the *Egfr* gene, whereas the R6 and Con2_JK primers generate the product for the deletion allele (Δ*Egfr* 400bp).

EGFR LacZ100:

Thermocycler program	**Mix for one sample**	
2 min 95˚C	17.25 µl	H_2O
15 sec 95˚C	2.5 µl	10x DreamTaq buffer
30 sec 70˚C	0.5 µl	dNTPs mix (10mM)
45 sec 72˚C	1.2 µl	R4 primer (5µM)
15 sec 95˚C	1.2 µl	R6 primer (5µM)
30 sec 66˚C	1.2 µl	LacZ100 primer (5µM)
45 sec 72˚C	0.15 µl	Taq DNA polymerase
2 min 72˚C	1 µl	DNA
∞ 4˚C		

The R4 and R6 primes amplify the wt allele, whereas the KO product (*Egfr*$^{-}$ 1245bp) is generated by the R6 and LacZ100 primers.

K5Cre:

Thermocycler program:	**Mix for one sample:**	
3 min 94˚C	1.1 µl	H_2O
45 sec 94˚C	2.5 µl	10x DreamTaq buffer
1 min 60˚C	0.25 µl	dNTPs mix (10mM)
2 min 72˚C	5 µl	K5Cre1 primer (1.2µM)
5 min 72˚C	5 µl	K5Cre2 primer (1.2µM
∞ 4˚C	5 µl	Jun1 primer (1.2µM)
	5 µl	Jun2 primer (1.2µM)
	0.15 µl	TaqDNA polymerase
	1 µl	DNA

The Cre product (500bp) is generated by the K5Cre1 and K5Cre2 primers and the internal control product for Jun (300bp) is amplified by the Jun1 and Jun2 primers.

7. References

Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. 2003. "Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17." *The Journal of Biological Chemistry* no. 278 (3):1910-1914. doi: 10.1074/jbc.M207577200.

Ai W, Li H, Song N, Li L, Chen H. 2013. "Optimal Method to Stimulate Cytokine Production and Its Use in Immunotoxicity Assessment." *International Journal of Environmental Research and Public Health* no. 10 (9):3834–3842. doi: 10.3390/ijerph10093834.

Arnold CE, Gordon P, Barker RN, Wilson HM. 2015. "The activation status of human macrophages presenting antigen determines the efficiency of Th17 responses." *Immunobiology* no. 220 (1):10-19. doi: 10.1016/j.imbio.2014.09.022.

Bailey SR, Nelson MH, Himes RA, Li Z, Mehrotra S, Paulos CM. 2014. "Th17 Cells in Cancer: The Ultimate Identity Crisis." *Frontiers in Immunology* no. 5 (276). doi: 10.3389/fimmu.2014.00276.

Barnden MJ, Allison J, Heath WR, Carbone FR. 1998. "Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements." *Immunology and Cell Biology* no. 76 (1):34-40. doi: 10.1046/j.1440-1711.1998.00709.x.

Bending D, De la Peña H, Veldhoen M, Phillips JM, Uyttenhove C, Stockinger B, Cooke A. 2009. "Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice." *The Journal of Clinical Investigation* no. 119 (3):565-472. doi: 10.1172/JCI37865.

Blum JS, Wearsch PA, Cresswell P. 2013. "Pathways of antigen processing." *Annual Review of Immunology* no. 31:443-473. doi: 10.1146/annurev-immunol-032712-095910.

Bremnes RM, Dønnem T, Al-Saad S, Al-Shibli K, Andersen S, Sirera R, Camps C, Marinez I, Busund LT. 2011. "The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer." *Journal of Thoracic Oncology* no. 6 (1):209-217. doi: doi: 10.1097/JTO.0b013e3181f8a1bd.

Chalaris A, Adam N, Sina C, Rosenstiel P, Lehmann-Koch J, Schirmacher P, Hartmann D, Cichy J, Gavrilova O, Schreiber S, Jostock T, Matthews V, Häsler R, Becker C, Neurath MF, Reiss K, Saftig P, Scheller J, Rose-John S. 2010. "Critical role of the disintegrin metalloprotease ADAM17 for intestinal inflammation and regeneration in mice." *Journal of Experimental Medicine* no. 207:1617-1624. doi: 10.1084/jem.20092366.

Chaudhary B, Elkord E. 2016. "Regulatory T Cells in the Tumor Microenvironment and Cancer Progression: Role and Therapeutic Targeting." *Vaccines* no. 4 (28). doi: 10.3390/vaccines4030028.

Chen J, Namiki S, Toma-Hirano M, Miyatake S, Ishida K, Shibata Y, Arai N, Arai K, Kamogawa-Schifter Y. 2008. "The role of CD11b in phagocytosis and dendritic cell development." *Immunology Letters* no. 120(1-2):42-8. doi: 10.1016/j.imlet.2008.06.010 (1-2):42-48. doi: 10.1016/j.imlet.2008.06.010.

Chen J, Zeng F, Forrester SJ, Eguchi S, Zhang M, Harris RC. 2016. "Expression and Function of the Epidermal Growth Factor Receptor in Physiology and Disease." *Physiological Reviews* no. 96:1025-1069. doi: 10.1152/physrev.00030.2015.

Chen L, Flies DB. 2013. "Molecular mechanisms of T cell co-stimulation and co-inhibition." *Nature Reviews Immunology* no. 13 (4):227-242. doi: 10.1038/nri3405.

Chong CR, Janne PA. 2013. "The quest to overcome resistance to EGFR-targeted therapies in cancer." *Nat Med* no. 19 (11):1389-1400. doi: 10.1038/nm.3388.

Cruz FM, Colbert JD, Merino E, Kriegsman BA, Rock KL. 2017. "The Biology and Underlying Mechanisms of Cross-Presentation of Exogenous Antigens on MHC-I Molecules." *Annual Review of Immunology* no. 35:149-176. doi: 10.1146/annurev-immunol-041015-055254.

Delamarre L, Holcombe H, Mellman I. 2003. "Presentation of Exogenous Antigens on Major Histocompatibility Complex (MHC) Class I and MHC Class II Molecules Is Differentially Regulated during Dendritic Cell Maturation." *The Journal of Experimental Medicine* no. 198 (1): 111–122. doi: 10.1084/jem.20021542.

Dobrzanski MJ. 2013. "Expanding roles for CD4T cells and their subpopulations in tumor immunity and therapy." *Frontiers in Oncology* no. 3:1-19. doi: 10.3389/fonc.2013.00063.

DuPage M, Bluestone JA. 2016. "Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease." *Nature Reviews Immunology* no. 16 (3):149-163. doi: 10.1038/nri.2015.18.

Elhelu MA. 1983. "The Role of Macrophages in Immunology." *National Medical Association* no. 75:314–317.

Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. 1998. "Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF." *The Journal of Clinical Investigation* no. 101(4):890-8. (4):890-898. doi: 10.1172/JCI1112.

Fontenot JD, Gavin MA, Rudensky AY. 2003. "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells." *Nature Immunology* no. 4 (4):330-336. doi: 10.1038/ni904.

Franklin RA, Liao W, Sarkar A, Kim MV, Bivona MR, Liu K, Pamer EG, Li MO. 2014. "The Cellular and Molecular Origin of Tumor-associated Macrophages." *Science* no. 344 (6186):921-925. doi: 10.1126/science.1252510.

Freemerman AJ, Johnson AR, Sacks GN, Milner JJ, Kirk EL, Troester MA, Macintyre AN, Goraksha-Hicks P, Rathmell JC, Makowski L. 2014. "Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype." *The Journal of Biological Chemistry* no. 289 (1):7884-796. doi: 10.1074/jbc.M113.522037.

Fregni G, Perier A, Avril MF, Caignard A. 2012. "NK cells sense tumors, course of disease and treatments." *Oncoimmunology* no. 1 (1):38–47. doi: 10.4161/onci.1.1.18312.

Grotzke JE, Sengupta D, Lu Q, Cresswell P. 2017. "The ongoing saga of the mechanism(s) of MHC class I-restricted cross-presentation." *Current Opinion in Immunology* no. 46:89-96. doi: 10.1016/j.coi.2017.03.015.

Hanahan D1, Weinberg RA. 2011. "Hallmarks of cancer: the next generation." *Cell* no. 144 (5):646-674. doi: 10.1016/j.cell.2011.02.013.

Hardbower DM, Coburn LA, Asim M, Singh K, Sierra JC, Barry DP, Gobert AP, Piazuelo MB, Washington MK, Wilson KT. 2017. "EGFR-mediated macrophage activation promotes colitis-associated tumorigenesis." *Oncogene* no. 36 (27):3807-3819. doi: 10.1038/onc.2017.23.

Harding J, Burtness B. 2005. "Cetuximab: an epidermal growth factor receptor chemeric human-murine monoclonal antibody." *Drugs of Today* no. 41 (2):107-127. doi: 10.1358/dot.2005.41.2.882662.

Haribhai D, Ziegelbauer J, Jia S, Upchurch K, Yan K, Schmitt EG, Salzman NH, Simpson P, Hessner MJ, Chatila TA, Williams* CB. 2016. "Alternatively activated macrophages boost iTreg and Th17 cell responses during immunotherapy for colitis." *The Journal of Immunology* no. 196 (8):3305-3317. doi: 10.4049/jimmunol.1501956.

Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. 2005. "Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages." *Nature Immunology* no. 6 (11):1123-1132. doi: 10.1038/ni1254.

Haschemi A, Kosma P, Gille L, Evans CR, Burant CF, Starkl P, Knapp B, Haas R, Schmid JA, Jandl C, Amir S, Lubec G, Park J, Esterbauer H, Bilban M, Brizuela L, Pospisilik JA, Otterbein LE, Wagner O. 2012. "The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism." *Cell Metabolism* no. 15 (6):813-826. doi: 10.1016/j.cmet.2012.04.023.

Hegazy AN, Peine M, Helmstetter C, Panse I, Fröhlich A, Bergthaler A, Flatz L, Pinschewer DD, Radbruch A, Löhning M. 2010. "Interferons Direct Th2 Cell Reprogramming to Generate a Stable GATA-3+ T-bet+ Cell Subset with Combined Th2 and Th1 Cell Functions." *Cell Immunity* no. 31 (1):116-128. doi: 10.1016/j.immuni.2009.12.004.

Helft J, Böttcher J, Chakravarty P, Zelenay S, Huotari J, Schraml BU, Goubau D1, Reis e Sousa C. 2015. "GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells." *Immunity* no. 42 (6):1197-1211. doi: 10.1016/j.immuni.2015.05.018.

Helms JB, Rothman JE. 1992. "Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF." *Nature* no. 360 (6402):352-354. doi: 10.1038/360352a0.

Hogquis KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone Fr. 1994. "T cell receptor antagonist peptides induce positive selection." *Cell* no. 75 (1):17-27. doi: http://dx.doi.org/10.1016/0092-8674(94)90169-4.

Hori S, Nomura T, Sakaguchi S. 2003. "Control of regulatory T cell development by the transcription factor Foxp3." *Science* no. 299 (5609):1057-1061. doi: 10.1126/science.1079490.

Inukai M, Toyooka S, Ito S, Asano H, Ichihara S, Soh J, Suehisa H, Ouchida M, Aoe K, Aoe M, Kiura K, Shimizu N, Date H. 2006. "Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer." *Cancer Research* no. 66 (16):7854-7858. doi: 10.1158/0008-5472.CAN-06-1951.

Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. 2006. "The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells." *Cell* no. 126 (6):1121-1133. doi: 10.1016/j.cell.2006.07.035.

Kawane K, Ohtani M, Miwa K, Kizawa T, Kanbara Y, Yoshioka Y, Yoshikawa H, Nagata S. 2006. "Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages." *Nature* no. 443 (7114):998-1002. doi: 10.1038/nature05245.

Kennedy R, Celis E. 2008. "Multiple roles for CD4+ T cells in anti-tumor immune responses." *Immunological Reviews* no. 222 (129-44). doi: 10.1111/j.1600-065X.2008.00616.x.

Khattri R, Cox T, Yasayko SA, Ramsdell F. 2003. "An essential role for Scurfin in CD4+CD25+ T regulatory cells." *Nature Immunology* no. 4 (4):337-342. doi: 10.1038/ni909.

Kool M, Geurtsvankessel C, Muskens F, Madeira FB, van Nimwegen M, Kuipers H, Thielemans K, Hoogsteden HC, Hammad H, Lambrecht BN. 2011. "Facilitated antigen uptake and timed exposure to TLR ligands dictate the antigen-presenting potential of plasmacytoid DCs." *Journal of Leukocyte Biology* no. 90 (6):1177-1190. doi: 10.1189/jlb.0610342.

Kühn R, Schwenk F, Aguet M, Rajewsky K. 1995. "Inducible gene targeting in mice." *Science* no. 269 (5229):1427-1429. doi: 10.1126/science.7660125.

Ladoire S, Martin F, Ghiringhelli F. 2011. "Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer." *Cancer Immunology, Immunotherapy* no. 60 (7):909-918. doi: 10.1007/s00262-011-1046-y.

Lanaya H, Natarajan A, Karin Komposch K, Li L, Amberg N, Lei Chen L, Wculek SK, Hammer M, Zenz R, Peck-Radosavljevic M, Wolfgang Sieghart W, Trauner M, Wang H, Sibilia M. 2014. "EGFR has a tumor-promoting role in liver macrophages during hepatocellular carcinoma formation." *Nature Cell Biology* no. 16 (10):972–977. doi: 10.1038/ncb3031.

Latchman DS. 1997. "Transcription Factors: An Overview." *The International Journal of Biochemistry & Cell Biology* no. 29 (12):1305-1312. doi: https://doi.org/10.1016/S1357-2725(97)00085-X.

Lawrence T, Natoli G. 2011. "Transcriptional regulation of macrophage polarization: enabling diversity with identity." *Nature Reviews Immunology* no. 11:750-761. doi: 10.1038/nri3088.

Lee YK, Turner H, Maynard CL, Oliver JR, Chen D, Elson CO, Weaver CT. 2009. "Late developmental plasticity in the T helper 17 lineage." *Cell Immunity* no. 30 (1):92-107. doi: 10.1016/j.immuni.2008.11.005.

Liu Y, Cao X. 2015. "The origin and function of tumor-associated macrophages." *Cellular and Molecular Immunology* no. 12:1-4. doi: doi:10.1038/cmi.2014.83.

Loke P, Allison JP. 2003. "PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells." *PNAS* no. 100 (9):5336–5341. doi: 10.1073/pnas.0931259100.

Lu N, Wang L, Cao H, Liu L, Van Kaer L, Washington MK, Rosen MJ, Dubé PE, Wilson KT, Ren X, Hao X, Polk DB, Yan F. 2014. "Activation of the epidermal growth factor receptor in macrophages regulates cytokine production and experimental colitis." *The Journal of Immunology* no. 192 (3):1013-1023. doi: 10.4049/jimmunol.1300133.

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA. 2004. "Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to

gefitinib." *The New England Journal of Medicine* no. 50 (21):2129-2139. doi: 10.1056/NEJMoa040938.

Lyons AB, Parish CR. 1994. "Determination of lymphocyte division by flow cytometry." *Journal of immunological methods* no. 171 (1):131-137. doi: https://doi.org/10.1016/0022-1759(94)90236-4.

Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT. 2006. "Transforming growth factor-beta induces development of the T(H)17 lineage." *Nature* no. 441 (7090):231-234. doi: 10.1038/nature04754.

Martin F, Apetoh L, Ghiringhelli F. 2012. "Controversies on the role of Th17 in cancer: a TGF-β-dependent immunosuppressive activity?" *Trends in Molecular Medicine* no. 18 (12):742-749. doi: 10.1016/j.molmed.2012.09.007.

Martinez FO, Gordon S. 2014. "The M1 and M2 paradigm of macrophage activation: time for reassessment." *F1000 Prime Reports* no. 6:1-13. doi: 10.12703/P6-13.

McFarland BJ, Sant AJ, Lybrand TP, Beeson C. 1999. "Ovalbumin(323-339) peptide binds to the major histocompatibility complex class II I-A(d) protein using two functionally distinct registers." *Biochemisrty* no. 38 (50):16663-16670. doi: 10.1021/bi991393l.

Mellman I, Coukos G, Dranoff G. 2011. "Cancer immunotherapy comes of age." *Nature* no. 480 (7378):480–489. doi: 10.1038/nature10673.

Mellman I, Steinman RM. 2001. "Dendritic cells: specialized and regulated antigen processing machines." *Cell* no. 106 (3):255-258.

Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z, Derynck R. 1995. "Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor." *Nature* no. 376 (6538):337-341. doi: 10.1038/376337a0.

Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. 1992. "RAG-1-deficient mice have no mature B and T lymphocytes." *Cell* no. 68 (5):869-877. doi: https://doi.org/10.1016/0092-8674(92)90030-G.

Moon JJ, Chu HH, Jason Hataye J, Pagán AJ, Pepper M, McLachlan JB, Zell T, Jenkins MK. 2009. "Tracking epitope-specific T cells." *Nature Protocols* no. 4:565–581. doi: doi:10.1038/nprot.2009.9.

Mosmann TR, Coffman RL. 1989. "TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties." *Annual Review of Immunology* no. 7:145-173. doi: 10.1146/annurev.iy.07.040189.001045.

Mosser DM. 2003. "The many faces of macrophage activation." *Journal of Leukocyte Biology* no. 73:209-212. doi: 10.1189/jlb.0602325.

Mosser DM, Edwards JP. 2008. "Exploring the full spectrum of macrophage activation." *Nature Reviews Immunology* no. 8 (12):958–969. doi: 10.1038/nri2448.

Mueller DL, Jenkins MK, Schwartz RH. 1989. "Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy." *Annual Review of Immunology* no. 7:445-480. doi: 10.1146/annurev.iy.07.040189.002305.

Murphy KM, Heimberger AB, Loh DY. 1990. "Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo." *Science* no. 250 (4988):1720-1723. doi: 10.1126/science.2125367.

Murray PJ, Wynn TA. 2011. "Protective and pathogenic functions of macrophage subsets." *Nature Reviews Immunology* no. 11 (11):723-737. doi: 10.1038/nri3073.

Natarajan A, Wagner B, Sibilia M. 2007. "The EGF receptor is required for efficient liver regeneration." *PNAS* no. 104 (49):17081–17086. doi: 10.1073/pnas.0704126104.

Panzer M, Sitte S, Wirth S, Drexler I, Sparwasser T, Voehringer D. 2012. "Rapid in vivo conversion of effector T cells into Th2 cells during helminth infection." *The Journal of Immunology* no. 188 (2):615-223. doi: 10.4049/jimmunol.1101164.

Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. 2005. "A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17." *Nature Immunology* no. 6 (11):1133-1141. doi: 10.1038/ni1261.

Parker DC. 1993. "T cell-dependent B cell activation." *Annual Review of Immunology* no. 11:331-360. doi: 10.1146/annurev.iy.11.040193.001555.

Pozzi LA, Maciaszek JW, Rock KL. 2015. "Both dendritic cells and macrophages can stimulate naive CD8 T cells in vivo to proliferate, develop effector function,

and differentiate into memory cells." *Journal of Immunology* no. 175 (4):2071-2081. doi: 10.4049/jimmunol.175.4.2071.

Quah BJ, Warren HS, Parish CR. 2007. "Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester." *Nature Protocols* no. 2 (9):2049-2056. doi: 10.1038/nprot.2007.296.

Robertson JM, Jensen PE, Evavold BD. 2000. "DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope." *Journal of Immunology* no. 16 (9):4706-4712. doi: 10.4049/jimmunol.164.9.4706.

Rodriguez A, Regnault A, Kleijmeer M, Ricciardi-Castagnoli P, Amigorena S. 1999. "Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells." *Nature Cell Biology* no. 1 (6):362-368. doi: 10.1038/14058.

Rötzschke O, Falk K, Stevanović S, Jung G, Walden P, Rammensee HG. 1991. "Exact prediction of a natural T cell epitope." *European Journal of Immunology* no. 21 (11):2891-2894. doi: 10.1002/eji.1830211136.

Sauer B, Henderson N. 1988. "Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1." *PNAS* no. 85:5166-5170,. doi: 10.1073/pnas.85.14.5166.

Scaltriti M, Baselga J. 2006. "The Epidermal Growth Factor Receptor Pathway: A Model for Targeted Therapy." *Clinical Cancer Research* no. 12 (18):5268-5272. doi: 10.1158/1078-0432.CCR-05-1554.

Schmidt A, Zhang XM, Joshi RN, Iqbal S, Wahlund C, Gabrielsson S, Harris RA, Tegnér J. 2016. "Human macrophages induce CD4(+)Foxp3(+) regulatory T cells via binding and re-release of TGF-β." *Immunology and Cell Biology* no. 94 (8):747-762. doi: 10.1038/icb.2016.34.

Sharpe AH, Freeman GJ. 2002. "The B7–CD28 superfamily." *Nature Reviews Immunology* no. 2:116–126. doi: 10.1038/nri727.

Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, Charron J, Datta M, Young F, Stall AM, Alt FW. 1992. "RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement." *Cell* no. 68 (5):855-867. doi: https://doi.org/10.1016/0092-8674(92)90029-C.

Sibilia M, Steinbach JP, Stingl L, Aguzzi A, Wagner EF. 1998. "A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor." *The EMBO Journal* no. 17:719–731. doi: 10.1093/emboj/17.3.719.

Sibilia M, Wagner EF. 1995. "Strain-Dependent Epithelial Defects in Mice Lacking the EGF Receptor." *Science* no. 269 (5221):234-238. doi: 10.1126/science.7618085.

Srivatsa S, Paul MC, Cardone C, Holcmann M, Amberg N, Pathria P, Diamanti MA, Linder M, Timelthaler G, Dienes HP, Kenner L, Wrba F, Prager GW, Rose-John S, Eferl R, Liguori G, Botti G, Martinelli E, Greten FR, Ciardiello F, Sibilia M. 2017. "EGFR in Tumor-Associated Myeloid Cells Promotes Development of Colorectal Cancer in Mice and Associates With Outcomes of Patients." *Gastroenterology* no. 153 (1):178-190. doi: 10.1053/j.gastro.2017.03.053.

Steinman RM, Hawiger D, Nussenzweig MC. 2003. "Tolerogenic dendritic cells." *Annual Review of Immunology* no. 21:685-711. doi: 10.1146/annurev.immunol.21.120601.141040.

Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. 2012. "PAMPs and DAMPs: Signal 0s that Spur Autophagy and Immunity." *Immunological Reviews* no. 249 (1):158-175. doi: 10.1111/j.1600-065X.2012.01146.x.

Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC, Barnard JA, Yuspa SH, Coffey RJ, Magnuson T. 1995. "Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype." *Science* no. 269 (5221):230-234. doi: 10.1126/science.7618084.

Titu LV, Monson JRT, Greenman J. 2002. "The role of CD8+ T cells in immune responses to colorectal cancer." *Cancer Immunology, Immunotherapy* no. 51 (5):235-247. doi: 10.1007/s00262-002-0276-4.

Toes REM, Ossendorp F, Offringa R, Melief CJM. 1999. "CD4 T Cells and Their Role in Antitumor Immune Responses." *Journal of Experimental Medicine* no. 189: 753–75.

Tosi MF. 2005. "Innate immune responses to infection." *The Journal of Allergy and Clinical Immunology* no. 116 (2):241-249. doi: 10.1016/j.jaci.2005.05.036.

Trombetta ES, Mellman. 2005. "Cell biology of antigen processing in vitro and in vivo." *Annual Review of Immunology* no. 23:975–1028. doi: 10.1146/annurev.immunol.22.012703.104538.

Van den Bossche J, O'Neill LA, Menon D. 2017. "Macrophage Immunometabolism: Where Are We (Going)?" *Trends in Immunology* no. 38 (6):395-406. doi: 10.1016/j.it.2017.03.001.

Wakeling AE, Guy SP, Woodburn JR, Ashton SE, Curry BJ, Barker AJ, Gibson KH. 2002. "ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy." *Cancer Research* no. 62 (20):5749-5754.

Wang K, Li D, Sun L. 2016. "High levels of EGFR expression in tumor stroma are associated with aggressive clinical features in epithelial ovarian cancer." *Oncotargets and Therapies* no. 9:377–386. doi: 10.2147/OTT.S96309.

Wang K, Yamamoto H, Chin JR, Werb Z, Vu TH. 2004. "Epidermal Growth Factor Receptor-deficient Mice Have Delayed Primary Endochondral Ossification Because of Defective Osteoclast Recruitment." *Journal of Biological Chemestry* no. 279 (51):53848 –53856. doi: 10.1074/jbc.M403114200.

Weck MM, Grünebach F, Werth D, Sinzger C, Bringmann A, Brossart P. 2007. "TLR ligands differentially affect uptake and presentation of cellular antigens." *Blood* no. 109 (9):3890-3894. doi: 10.1182/blood-2006-04-015719.

Wells JW, Darling D, Farzaneh F, Galea-Lauri J. 2005. "Influence of interleukin-4 on the phenotype and function of bone marrow-derived murine dendritic cells generated under serum-free conditions." *Scandinavian Journal of Immunology* no. 61 (3):251-259. doi: 10.1111/j.1365-3083.2005.01556.x.

Wieduwilt MJ, Moasser MM. 2008. "The epidermal growth factor receptor family: Biology driving targeted therapeutics." *Cellular and Molecular Life Sciences* no. 65 (10):1566–1584. doi: 10.1007/s00018-008-7440-8.

Wong RW, Guillaud L. 2004. "The role of epidermal growth factor and its receptors in mammalian CNS." *Cytokine and Growth Factor Reviews* no. 15 (2-3):147-156. doi: 10.1016/j.cytogfr.2004.01.004.

Woollard KJ, Geissmann F. 2010. "Monocytes in atherosclerosis: subsets and functions." *Nature Reviews Cardiology* no. 7:77-86. doi: doi:10.1038/nrcardio.2009.228.

Wynn TA, Luke Barron L. 2010. "Macrophages: Master Regulators of Inflammation and Fibrosis." *Seminars in Liver Disease* no. 30 (3):245–257. doi: 10.1055/s-0030-1255354.

Yewale C, Baradia D, Vhora I, Patil S, Misra A. 2013. "Epidermal growth factor receptor targeting in cancer: A review of trends and strategies." *Biomaterials* no. 34 (34):8690-8707. doi: 0.1016/j.biomaterials.2013.07.100.

Zhang X, Goncalves R, Mosser DM. 2008. "The Isolation and Characterization of Murine Macrophages." In *Current Protocol in Immunology*, edited by John E. Coligan [et al.].

Zhao G, Liu L, Peek RM Jr., Hao X, Polk DB, Li H, Yan F. 2016. "Activation of Epidermal Growth Factor Receptor in Macrophages Mediates Feedback Inhibition of M2 Polarization and Gastrointestinal Tumor Cell Growth." *Journal of Biological Chemestry* no. 291 (39):20462–20472. doi: 10.1074/jbc.M116.750182.

Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA. 2007. "IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells." *Journal of Immunology* no. 178 (4):2018-2027. doi: https://doi.org/10.4049/jimmunol.178.4.2018.

Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF, Littman DR. 2008. "TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function." *Nature* no. 453 (7192):236-240. doi: 10.1038/nature06878.

Zou W, Restifo NP. 2010. "TH17 cells in tumour immunity and immunotherapy." *Nature Reviews Immunology* no. 10 (4):248–256. doi: 10.1038/nri2742.

8. Appendix

8.1 List of abbreviations

APC	Antigen-presenting cell
BMDCs	Bone marrow-derived dendritic cells
BMDMs	Bone marrow-derived macrophages
CFSE	Carboxyfluorescein succinimidyl ester
CRC	Colorectal cancer
EGFR	Epidermal growth factor receptor
Foxp3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCC	Hepatocellular carcinoma
IL	Interleukin
INFγ	Interferon gamma
KO	Knock-out
LPS	Lipipolysaccharide
O/N	Over-night
PD-1	Programmed cell death 1
PD-L	Programmed cell death 1 ligand
Poly I:C	Polyinosinic:polycytidylic acid
RORγt	RAR-related orphan receptor γ t
RT	Room temperature
RTK	Receptor tyrosine kinase
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TF	transcription factor
TGFβ	Transforming growth factor beta
T_H cell	T helper cell
wt	Wild-type