# The role of T cell help in shaping Dendritic Cell function

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# ABBREVIATIONS

| | |
|---|---|
| **A** | |
| ABIN | A20-Binding Inhibitor of NF-κB |
| ANOVA | Analysis of Variance |
| AP-1 | Activator protein-1 |
| APC | Antigen Presenting Cell |
| **B** | |
| B6 | C57Bl/6 mice |
| BATF3 | Basic leucine zipper transcription factor ATF-like 3 |
| BM | Bone Marrow |
| BSS | Buffered Saline Solution |
| **C** | |
| CBA | Cytometric Bead Array |
| CCL | C-C Chemokine Ligand |
| CCR | C-C Chemokine Receptor |
| CD | Cluster of Differentiation |
| CD40L | CD40 Ligand |
| cDC | Conventional Dendritic Cell |
| CDP | Common DC Progenitor |
| cIAP | Cellular Inhibitor of Apoptosis |
| CLP | Common Lymphoid Progenitor |
| CLR | C-type Lectin Receptor |
| CMP | Common Myeloid Progenitor |
| CpG | Cytosine-phosphate-Guanine |
| CPM | Counts per Million |
| CTL | Cytotoxic T Lymphocytes |
| CTLA-4 | Cytotoxic T-Lymphocyte Antigen-4 |
| **D** | |
| DAMPS | Danger-Associated Molecular Pattern |
| DC | Dendritic Cell |
| DE | Differentially Expressed |
| dLN | Draining Lymph Node |
| DMSO | Dimethyl Sulphoxide |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxynucleotide |
| dsRNA | Double-stranded RNA |
| **E** | |
| eCD8$^+$ DC | Equivalent of CD8$^+$ DC |
| EDTA | Ethylenediaminetetraacetate |
| eIF4B | Eukaryotic Initiation Factor 4B |
| Ell | Eleven-nineteen Lysine-rich Leukemia |
| ERK | Extracellular signal-Regulated Kinase |
| ESAM | Endothelial cell-Specific Adhesion Molecule |
| **F** | |
| FasL | Fas Ligand |
| FC | Fold Change |

| | |
|---|---|
| FCS | Foetal Calf Serum |
| FDR | False Discovery Rate |
| Flt3 | Fms-related tyrosine kinase 3 |
| Flt3L | Fms-related tyrosine kinase 3 (Flt3) ligand |
| FMO | Fluorescence Minus One |
| **G** | |
| GAS | Gamma interferon Activation Site |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| GO | Gene Ontology |
| **H** | |
| H3Ac | Histone 3 acetylation |
| HEV | High Endothelial Venule |
| HIV | Human Immunodeficiency Virus |
| HMGB | High-Mobility Group Box Protein |
| Hr(s) | Hour(s) |
| HSV | Herpes Simplex Virus |
| **I** | |
| ID | Inhibitor of DNA binding |
| IE | Immediate Early |
| IFN | Interferon |
| IFNAR | IFN Receptor |
| IFNARKO mice | B6/Ifnar2 mice |
| IL | Interleukin |
| IκB | NF-κB inhibitor |
| IKK | Inhibitor of κB kinase |
| Ikbkb | Inhibitor of NF-κB kinase subunit beta |
| IRE | IRF-Responsive Element |
| IRF | Interferon-Regulatory Factor |
| ISG | Interferon-Stimulated-Gene |
| ISGF3 | Interferon-Stimulated Gene Factor 3 |
| ISRE | Interferon-Stimulated Response Element |
| **J** | |
| Jak | Janus kinases |
| JNK | c-Jun N-terminal kinase |
| **K** | |
| K63Ub | Lysine 63 ubiquitination |
| **L** | |
| LCMV | Lymphocytic Choriomeningitic Virus |
| LN | Lymph Node |
| LPS | Lipopolysaccharide |
| LRR | Leucine-Rich-Repeat |
| **M** | |
| MDA5 | Melanoma Differentiation-Associated gene 5 |
| MAb | Monoclonal antibody |
| MAPK | Mitogen-Activated Protein Kinase |
| MAP3K | MAPKinase Kinase Kinase |
| MDP | Macrophage DC Progenitor |
| MEM | Minimal Essential Medium |

| | |
|---|---|
| MHC | Major Histocompatibility Complex |
| Min | Minute |
| MKK | MAPKinase Kinase |
| MoDC | Mococyte-derived Dendritic Cell |
| MyD88 | Myeloid Differentiation factor 88 |
| **N** | |
| NEMO | NF-κB Essential Modulator |
| NES | Nuclear-Export Signal |
| NES | Normalized Enrichment Score |
| NF-κB | Nuclear Factor kappa B |
| NF-κBi | NF-κB-inhibitor |
| NIK | NF-κB-Inducing Kinase |
| NLS | Nuclear Localization Sequence |
| NLR | NOD-Like Receptor |
| NOD | Nucleotide Oligomerization Domain |
| NTS | Nuclear Translocation Signal |
| **O** | |
| OT-II mice | OT-II x B6.Ly5.1 mice |
| OVA | Ovalbumin |
| **P** | |
| PAMP | Pathogen-Associated Molecular Pattern |
| PBP | Promoter Binding Prediction |
| PBS | Phosphate Buffered Saline |
| PCA | Principal Component Analysis |
| pDC | Plasmacytoid Dendritic Cell |
| PGS | Partek Genomics Suite |
| PI | Propidium Iodide |
| PI3K | Phosphoinositide 3-kinase |
| PKC | Protein Kinase C |
| Poly(I:C) | Polyinosinic-polycytidylic acid |
| PRR | Pattern-Recognition Receptor |
| **R** | |
| RANK | Receptor Activator of Nuclear factor |
| RBCL | Red Blood Cell Lysis |
| RE | Relative Expression |
| RHD | Rel-Homology Domain |
| RIG-I | Retinoic acid Inducible Gene I |
| RIP | Receptor-Interacting Protein 1 |
| RLR | Retinoic acid-inducible gene I (RIG-I)-Like Receptor |
| rRNA | Ribosomal RNA |
| RT | Reverse Transcription |
| **S** | |
| SC | Supplementum Completum |
| Ser | Serine |
| STAR | Spliced Transcripts Alignment to a Reference |
| STAT | Signal Transducers and Activation of Transcription |
| **T** | |
| TAB | TAK1-Binding protein |

| | |
|---|---|
| TAK1 | TGF-β-Activated-Kinase 1 |
| TCR | T Cell Receptor |
| TF(s) | Transcription Factor(s) |
| TGF | Transforming Growth Factor |
| Th | T helper cell |
| TiOx | Titanium dioxide |
| TIR domain | Toll/Interleukin-1 receptor domain |
| TLR | Toll-Like Receptor |
| TNF | Tumor Necrosis Factor |
| TNFAIP3 | TNF-α-Induced protein 3 |
| TNFR | TNF Receptor |
| Tpl | Tumour Progression Locus |
| Treg | Regulatory T cell |
| TRAF | TNF Receptor-Associated Factor |
| TRAIL | TNF-Related Apoptosis-Inducing Ligand |
| TRAM | TRIF-Related Adaptor Molecule |
| TRIF | TIR-domain-containing adaptor inducing IFN-β |
| TSS | Transcription Start Site |
| Tyk | Tyrosine kinase |
| Tyr | Tyrosine |
| **U** | |
| UBC13 | Ubiquitin-Conjugating enzyme 13 |
| **V** | |
| VACV | Vaccinia Virus |
| VSV | Vesicular Stomatitis Virus |
| **X** | |
| XCL1 | Chemokine C motif Ligand 1 |
| XCR1 | Chemokine XC Receptor 1 |

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# ABSTRACT

CD8$^+$ T cell priming depends on antigen presentation by dendritic cells (DCs) and their capacity to communicate contextual cues associated with antigen acquisition. DCs often also require additional signals from helper CD4$^+$ T cells, which upon mediation via CD40-CD40L further modulate the communication of contextual cue to the responding CD8$^+$T cells. The present study was designed to explore the kinetics and molecular mechanisms underpinning this helper-dependent modulation of DC function.

To address this, we employed an *in vitro* system of bone marrow (BM)-derived equivalents of CD8$^+$ DCs (eCD8$^+$ DCs) and we assessed the role of different CD40 signalling components in driving their IFN-αA-induced cytokine and chemokine responses by using flow cytometry, mass spectrometry-based proteomics, real time PCR and RNA sequencing. This brought to light remarkable and distinct patterns of gene regulation through which CD4$^+$ T cells triggered CD40 and thereby amplified the capacity of IFN-αA to induce or downregulate a broad range of genes. We also observed an unexpected pattern of gene regulation: some genes required both T cell help and IFN-αA stimulations but could not be induced by 'help' or IFN-α alone. By varying the exposure time, we further discovered that eCD8$^+$ DCs required 1-2 hours of IFN-αA to become responsive to CD40 triggering. Once this pre-activated state was achieved, CD40 stimulation rapidly amplified responses with remarkably fast kinetics. Combining proteomics and RNA sequencing data presented in this thesis suggests a complex interplay between the IFN-αA signalling pathway involving IRFs transcription factors and the NF-κB signalling pathway.

These findings not only reveal new insights into how T cell help adjusts the responsiveness of DC to innate stimuli, but also reveal that this can occur with remarkable speed, which aligns with in vivo imaging studies describing very brief interactions between eCD8$^+$ DCs and CD4$^+$ T cells during CD8$^+$ T cell priming.

# DECLARATION

The work presented in this thesis was conducted at The University of Melbourne, in the laboratory of A/Prof Sammy Bedoui and at The Rheinische Friedrich-Wilhelms-Universität Bonn, in the laboratory of Prof Eicke Latz. Elise Gressier was supported by the Melbourne International Research Scholarship and the Melbourne International Fee Remission Scholarship.

This is to certify that,

(i) the thesis comprises only my original work towards the PhD except where indicated in the preface;

(ii) due acknowledgement has been made in the text to all other material used;

(iii) the thesis is less than 100,000 word limit in length, exclusive of tables, maps, bibliographies and appendices as approved by the Research Higher Degrees Committee.

Elise Gressier

# PREFACE

My contribution to the experiments within each chapter was as follows:

**Chapter 3:** 100%

**Chapter 4:** 67%

I acknowledge the important contributions of others to experiments presented herein:

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# Chapter 1

## Literature review

## 1.1 Introduction

Immune responses involve the orchestration of innate signals and adaptive responses. Following the infection with microorganisms, the first line of defence is the innate immune system constituted in particular by macrophages and neutrophils capable of engulfing and killing extracellular pathogens. This first phase is based on inflammatory mediators released within the microenvironment to increase local blood flow and therefore cell influx and recruitment. This inflammatory response also induces an increased flow of lymph and enhances the migration of tissue-resident antigen-presenting cells (APCs), such as macrophages or dendritic cells (DCs), from the infected tissue to the lymphoid tissue where the adaptive response is initiated. The adaptive immunity provides long-lasting, antigen-specific responses and therefore takes time to develop. Two arms constitute the adaptive immune system: humoral immunity, relying on B lymphocytes responses; and cell-mediated immunity orchestrated by T lymphocytes such as $CD8^+$ T lymphocytes, also called cytotoxic T cells (CTLs), or $CD4^+$ T lymphocytes, known as helper T cells (Dempsey et al. 2003).

Upon infection, innate and adaptive immune systems communicate to provide an efficient protection. The subsequent literature review will focus on DCs mediating this communication between the two arms of the immune system. The present study was performed using a murine model, and, unless otherwise stipulated, the information and publications discussed relate to the murine model.

## 1.2 Dendritic cells

DCs are APCs and important players in the immune system. They are found in most tissues, recognize pathogens and are able to initiate and

modulate the immune responses following their migration to the lymphoid organs.

Being the link between its two main branches, the innate and the adaptive immunity, DCs are the sentinels of the immune system. First, they can recognize non-self molecules and produce cytokines and chemokines required for innate immune responses. Moreover, by processing and presenting antigens they can activate effector cells involved in the adaptive immune response. For instance, they transfer antigen to naïve B cells, and therefore are involved in the antibody secretion, or they stimulate T lymphocytes which can differentiate into CTLs or helper T cells via their interaction with an APC (Banchereau and Steinman 1998).

### 1.2.1 Differentiation and subsets of dendritic cells

DCs are a heterogeneous population leaving the bone marrow during their development to give rise to different subtypes differing in phenotype and function. They express molecules such as CD11c and Major Histocompatibility Complex class II (MHC II) molecules. The main subsets of DCs that can be distinguished are the monocyte-derived DCs, (MoDCs), the plasmacytoid DCs (pDCs) and the conventional DCs (cDCs) all sharing a Common Myeloid Progenitor (CMP). This CMP differentiates into a bi-potent Macrophage DC Progenitor (MDP) giving rise to the MoDCs or to a Common DC Progenitor (CDP) supporting the generation of cDCs (Heath and Carbone 2009; Merad et al. 2013; Poltorak and Schraml 2015), while pDCs are thought to raise from Common Lymphoid Progenitor (CLP)-like progenitors (Dress et al. 2019).

The MoDCs develop from circulating monocytes that are recruited at the site of inflammation. Thus, they are not found in steady state and belong to the monocytic lineage (Naik et al. 2006). These cells can produce high quantity of the cytokine called Tumor Necrosis Factor α (TNF-α), and capture antigens, but are thought as barely able to migrate to the lymph nodes (LNs) (Langlet et al.

2012). The pDCs are mainly found in lymphoid organs and in non-lymphoid tissues only under inflammatory conditions (Dalod et al. 2014). They respond to foreign nucleic acids by producing high amount of type I interferon (IFN). pDCs are also capable of capturing, processing and presenting antigens to lymphocytes T cells, but do so with much reduced efficacy when compared to cDCs (Villadangos and Young 2008).

Until recently, cDCs and pDC were thought to share a similar developmental pathway, strongly depending on the growth factor *Fms*-related tyrosine kinase 3 ligand (Flt3L) (Merad et al. 2013). However, in 2019, Dress et al. demonstrated the differentiation of pDCs from a CLP-like progenitor called “pro-pDCs. Their study highlighted that the CDP does not give rise to pDCs but to pre-cDCs only (Dress et al. 2019). Those pre-cDCs exit the bone marrow (BM), relocate into lymphoid and non-lymphoid tissues and can differentiate into various populations of cDCs. Non-lymphoid-tissue-resident DCs can migrate to the LNs and are called “migratory DCs” while the DCs distributed in the lymphoid organs are called “resident DCs”. cDCs can be sorted into two major subsets distinguished by their expression of various molecules such as CD8α and CD4 in the LN and CD11b or CD103 in the tissues (Guilliams et al. 2010; Dalod et al. 2014). $CD8\alpha^+$ cDCs within lymphoid tissues share their functions with non-lymphoid tissue $CD103^+$ cDCs and represent the cDC lineage cDC1. Their differentiation is controlled by transcription factors such as Interferon regulatory factor 8 (Irf8), Inhibitor of DNA binding 2 (Id2), Basic leucine zipper transcription factor ATF-like 3 (Batf3) (Merad et al. 2013; Schlitzer et al. 2015). Moreover, the chemokine receptor XCR1 (chemokine XC receptor 1) is a surface marker expressed by cDC1 (Crozat et al. 2011; Bachem et al. 2012).
High expression of CD11b is common to macrophages and to some migratory and resident cDCs. The latter are also characterized by their expression of CD4 and the endothelial cell-specific adhesion molecule (ESAM). These cDCs constitute the second main lineage of cDCs, cDC2 and their development requires the transcription factors Irf4, RelB and PU.1 (Merad et al. 2013; Schlitzer et al. 2015). Interestingly, human cDC1s specifically express XCR1 as

well and are referred to as CD141 (or BDCA3)$^+$ while human cDC2s are CD1c (or BDCA1)$^+$ (Dalod et al. 2014).
The lifespan of conventional DCs is short and therefore these cells are regularly replenished from their bone marrow derived precursors (Ardouin et al. 2016).

### 1.2.2 Antigen presentation

In order to stimulate the T cells, the APCs such as the DCs are capable of capturing antigen and migrating to the lymphoid organs where they process and present the antigen to induce the clonal expansion of specific T lymphocytes. This interaction between DC that have received peripheral cues, and T cells recognizing its specific antigen, is required for the initiation of adaptive immune responses. This presentation involves the binding of the antigen to molecules of the MHC class I, for the stimulation of CTLs, or class II for the priming of helper T cells (Banchereau and Steinman 1998).

The antigens presented via MHC I molecules drive CD8$^+$ T cell activation. Typically, these antigens are endogenously derived, synthesized within the cells. Consequently, this direct presentation occurs when the APC itself is infected or transfected. In other cases, antigens are captured exogenously and shunted into the MHC I pathway. This is the cross-presentation. Therefore, the presentation of the antigen can subsequently lead to the activation of naïve CD8$^+$ T cells without requiring the APC to be infected. In contrast, the MHC class II molecules have been firstly described to present mainly peptides that derive from exogenous proteins entering cells through endocytosis and play an important role in the activation of CD4$^+$ T cells (Heath et al. 2004). However, endogenous antigens can also access MHC class II presentation via for instance autophagy allowing the presentation of cytosolic and nuclear proteins (Münz 2012). Although immune cells such as macrophages and B cells are APCs as well, DCs are particularly efficient in their function of antigen presentation (de Jong et al. 2006).

While APCs express MHC II molecules, MHC I molecules is expressed at the surface of all nucleated cells. Therefore, virtually all cells of the organism can present antigen at their surface via MHC molecules. However, in addition of being the only APC stimulating naïve T cells, DCs are the cell type recognized as the most competent cross-presenting cells *in vivo* (Segura and Villadangos 2009; Embgenbroich and Burgdorf 2018). They efficiently uptake and process antigens, and they respond to inflammatory cues leading to their differentiation into APC (Brode and Macary 2004).

Furthermore, not all DC subtypes are equally efficient at antigen presentation and their respective contribution seems to depend on the infection, on the antigen and the DC respective location.

In various immunogenic models, such as vaccinia virus (VACV) infection or herpes simplex virus (HSV) skin infections or tumour challenges, migratory DCs mainly play the role of carriers collecting the antigen at the site of infection and bringing it to the draining LN (dLN). $CD8\alpha^+$ cDCs can then process the antigen and cross-present it to $CD8^+$ T cells (Villadangos and Schnorrer 2007; Segura and Villadangos 2009; Whitney et al. 2018). Non infected migratory DCs are, on the other hand, able to present the antigen to $CD4^+$ T cells (Villadangos and Schnorrer 2007). However, other means of infections show different scenarios. For instance, during subcutaneous influenza infection dermal DCs can present the antigen to both $CD8^+$ and $CD4^+$ T cells (Segura and Villadangos 2009) while during lung flu infection both migratory and resident $CD8^+$ DCs contribute to the cross-presentation of the antigen (Belz et al. 2004).

Interestingly, only $CD8\alpha^+$ and $CD103^+$ DCs are uniquely equipped for cross-presentation, possessing a specialized intracellular machinery for this mechanism. Moreover these cells exclusively express XCR1, the receptor of the chemokine XCL1 (chemokine C motif ligand 1) produced by various immune cells such as T cells and involved on $CD8^+$ T cells expansion and survival (Bevan 1976; Haan et al. 2000; Kroczek and Henn 2012). In contrast, MHC II presentation to $CD4^+$ T cells is thought to be highly dependent on $CD11b^+$ DCs (Mount et al. 2008).

## 1.3 Stimulation of dendritic cells

DCs promote the priming of T cells by providing the various signals they require in addition to the antigen presentation. To fulfil efficiently their functions, they undergo maturation, up-regulating co-stimulatory molecules and secreting cytokine and chemokine responses (Prilliman et al. 2002).

### 1.3.1 DC maturation

When immature, DCs are able to capture antigens but their presentation and priming abilities are limited as they express lower level of MHC II and co-stimulatory molecules or cytokines (Banchereau et al. 2000). Resident DCs in the LNs or spleen stay in an immature state until they receive pathogenic or inflammatory signals. In contrast, migratory DCs are only immature in peripheral tissues and undergo maturation when migrating to their dLN. Interestingly, these events can occur without pathogenic challenge (Villadangos and Schnorrer 2007). Various phenotypic and functional changes occur when DCs undergo their maturation. The DC maturation allows the conversion from antigen-capturing cell to antigen-presenting cell. DCs lose endocytic and phagocytic receptors and up-regulate co-stimulatory molecules such as CD40, CD80 and CD86. Their ability to form functional peptide-MHC II complexes is enhanced and produce cytokines and chemokines in response to microbial challenge (Banchereau et al. 2000; Mellman and Steinman 2001; Dalod et al. 2014). They also acquire higher motility thanks to cytoskeleton reorganization, down-regulation of homing receptors and up-regulation of C-C chemokine receptor (CCR) 7. LN stromal cells produce C-C chemokine ligand (CCL) 19 and CCL21, ligands of CCR7 and thus participate to the strategic location of DCs facilitating their encounter with antigen-specific T lymphocytes (Banchereau et al. 2000; Bajénoff et al. 2003; Worbs et al. 2017).

While DC maturation has been for long associated only with the acquisition of immunogenicity, there are two types of DC maturations. During an infection or in an inflammatory context, DCs mature following their pattern-recognition receptors (PRRs) engagement. This maturation is called immunogenic or induced, leads to an increase of DC migration to the dLN and induces their production of pro-inflammatory cytokines. Therefore, those DCs become able to promote the priming of antigen-specific T cells. Without infection or inflammatory signals, some tissue resident DCs can still undergo spontaneous migration, at a lower rate than during the induced maturation, bringing tissue antigens to the dLN. These DCs have been described as undergoing an homeostatic or tolerogenic maturation, they up-regulate co-stimulatory molecules but do not produce inflammatory cytokines (Lutz and Schuler 2002). A proportion of thymic DCs has been shown to undergo this homeostatic maturation as well (Ardouin et al. 2016). Presenting self-antigens without the ability of producing inflammatory signals, these DCs tolerize self-reactive T cells that left the thymus without being eliminated (Spörri and Reis e Sousa 2005). Paracrine inflammatory cytokines such IFN-α/β or TNF-α or microbiota signals could be driving the homeostatic maturation of the periphery resident DCs (Ardouin et al. 2016).

Contrasting with the theory that homeostatic maturation corresponds to an incomplete maturation or semi-maturation (Lutz and Schuler 2002), it requires large transcriptomic changes comparable to those leading to the immunogenic maturation. These two types of maturation depend on gene expression changes that are partially overlapping suggesting that the maturation pathways are overlapping as well (Ardouin et al. 2016). Interestingly, the non-canonical nuclear factor κB (NF-κB) transcription factor has been shown to be required for immunogenic DC maturation as well as tolerogenic DC maturation (Rescigno et al. 1998; Dev et al. 2010; Baratin et al. 2015). The blockade of NF-κB pathway leads to a default in DCs maturation and survival. For instance, the knockout of NIK (NF-κB-inducing kinase), part of non-canonical pathway allows lipopolysaccharide (LPS)-induced DC maturation but those DCs quickly undergo apoptosis (Quezada et al. 2004).

During immunogenic DC maturation the nature of stimuli DCs receive dictates their cytokine responses and influences the T-cell polarization (Dalod et al. 2014). With that purpose DCs express a large range of receptors able to sense pathogenic and danger signals.

### 1.3.2 Pathogen recognition receptors engagement

APC are characterized by their ability of sensing a large range of pathogens and host-derived danger signals due to their expression of PRRs. These PRRs can be extracellular or intracellular and can detect micro-organisms signals or pathogen-associated molecular patterns (PAMPs) as well as endogenous “danger” signals or damage-associated molecular patterns (DAMPs) (Dalod et al. 2014). Among various classes of PRR are the Toll-like receptors (TLRs), the C-type lectin receptors (CLRs), the Retinoic acid-inducible gene I-like receptors (RLRs) and the NOD-like receptors (NLRs). A broad range of pathogens can be detected owing to their specificity and the distinct cellular localization of these various PRRs (**Table 1.1**) (Takeuchi and Akira 2010; Broz and Monack 2013). Moreover, the expression of some PRRs is limited to cell types. This is the case for the CLRs DC-SIGN, involved in the recognition of various viruses including HIV, dengue virus and ebola virus, and Clec9A (or DNGR-1) binding damaged or dead cells (Dunnen et al. 2009; Zhang et al. 2012).

The PRR engagement induces various signalling pathways activation mainly leading to the recruitment of major transcription factors such as IRF3 or 7, activator protein-1 (AP-1), and the NF-κB transcription factors. The outcome of these pathways is the production of pro-inflammatory cytokines such as TNF-α, Interleukin (IL)-6 and pro-IL-1β (Takeuchi and Akira 2010).

The best-described PRR family are the TLRs, they are expressed extracellularly or intracellularly and 12 have been identified in the mouse. Immature DCs express these receptors but their expression is also modulated

| PRRs | Localization | Ligand |
|---|---|---|
| **Toll-Like Receptors (TLRs)** | | |
| TLR1 | Plasma membrane | Triacyl lipoprotein |
| TLR2 | Plasma membrane | Lipoprotein |
| TLR3 | Endosomal compartment | dsRNA |
| TLR4 | Plasma membrane and Endosomal compartment | LPS |
| TLR5 | Plasma membrane | Flagellin |
| TLR6 | Plasma membrane | Diacylipoprotein |
| TLR7 | Endosomal compartment | GU-rich ssRNA and short dsRNA |
| TLR8 | Endosomal compartment | GU-rich ssRNA and short dsRNA |
| TLR9 | Endosomal compartment | CpG DNA |
| TLR11 | Endosomal compartment | Profilin and flagellin |
| TLR12 | Endosomal compartment | Profilin |
| TLR13 | Endosomal compartment | Bacterial 23S rRNA |
| **RIG-Like Receptors (RLRs)** | | |
| RIG-I | Cytoplasm | Short dsRNA |
| MDA5 | Cytoplasm | Long dsRNA |
| **NOD-Like Receptors (NLRs)** | | |
| NOD1 | Cytoplasm | iE-DAP |
| NOD2 | Cytoplasm | MDP |
| AIM2 | Cytoplasm | Cytosolic DNA |
| **C-type Lectin Receptors (CLRs)** | | |
| Dectin-1/2 | Plasma membrane | β-Glucan |

**Table 1.1: Main Pattern Recognition Receptors and their ligands.**
A vast variety of Pattern Recognition Receptors (PRRs) sense various Pathogen Associated Molecular Patterns (PAMPs) that are derived from bacteria, parasites and virus. These PRRs include Toll-like receptors (TLRs), found on the cell membrane, extracellular or within endosome or RIG-like receptors (RLRs) and NOD-like receptors (NLRs) in the cytosol (adapted from Takeuchi and Akira 2010 and Broz and Monack 2013).

in response to signals the cells receive (Akira et al. 2006; Vega-Ramos et al. 2014). All TLRs can be described as type I membrane glycoproteins with extracellular domains containing leucine-rich-repeat (LRR) motifs. TLRs 1, 2, 4, 5 and 6 on the cell surface can detect external features of the pathogens, mainly lipids such as LPS (TLR4) from Gram-negative bacteria. Within the various TLRs expressed in intracellular compartments, TLR3, 7, 8 and 9 can sense internalised nucleic acids such as double-stranded RNA (dsRNA) (TLR3) or unmethylated Cytosine-phosphate-Guanine (CpG) (TLR9). The cytoplasmic portion of the TLRs is responsible for the signalling and called Toll/IL-1R homology (TIR) domain. Following the encounter of TLR ligands, adaptor proteins containing TIR domain are recruited to the cytoplasmic domain of the TLR. For most TLRs this adaptor protein is the Myeloid differentiation factor 88 (MyD88) or the TIR-domain-containing adaptor inducing IFN-β (TRIF) and Trif-related adaptor molecule (TRAM). With the exception of TLR3 interacting with TRIF, all TLRs engagement induces MyD88 recruitment and TLR4 engages both MyD88 and TRIF. The pathway downstream of these adaptor proteins depend on the TLR engaged. For instance, TLR4 can induce mitogen-activated protein kinase (MAPK) or the NF-κB pathways activation while TLR3 or 9 engagement result in the phosphorylation of IRF3 or IRF7 respectively (Akira et al. 2006; Takeuchi and Akira 2010; Broz and Monack 2013). In DCs, in addition of pro-inflammatory cytokines responses, TLR stimulation triggers their maturation with, for instance, MHC II and co-stimulatory molecules up-regulation (Vega-Ramos et al. 2014).

### 1.3.3 Indirect stimulation of dendritic cells: IFN-α/β

As described above engagement of PRRs induces DC activation and innate responses, involving for instance the release of inflammatory cytokines such as IFN-α and IFN-β, TNF-α or IL-1 (Akira et al. 2006). These molecules produced by neighbour cells can activate DCs in an indirect manner (Joffre et al. 2009; McNab et al. 2015).

Three families of IFNs have been characterised, type I, type II and type III IFNs, the most commonly studied being type I IFN which includes isotypes of the well characterised IFN-α and IFN-β, as well as, among others, IFN-ε, IFN-κ and IFN-ω (Pestka et al. 2004; Prchal et al. 2009). Type I IFN is known to play a major role in anti-viral responses with the ability to activate DCs among other immune cells and to drive cell resistance towards virus infection or death of infected cells. During a viral infection, type I IFN can virtually be expressed by all cell types especially pDCs (Le Bon et al. 2003; Villadangos and Young 2008). Type I IFN is essential in inducing DC maturation and promoting functional cross-presentation and efficient $CD4^+$ and $CD8^+$ T cell immunity responses (Montoya et al. 2002; Le Bon et al. 2003; Longhi et al. 2009). While TLR signals inhibit MHC II recycling and antigen processing in favour of a long-lasting and specific antigen presentation, inflammatory signals don't inhibit these DC functions and their MHC II presentation capacity is less efficient. Indirectly stimulated DCs are however capable of cross-presentation (Vega-Ramos et al. 2014).

The IFN receptor (IFNAR) is expressed by most of cell types. This receptor is made of two chains, IFNaR1 and IFNaR2. Its engagement induces the so-called Jak-STAT signalling pathway starting with the activation of Janus family kinases Tyrosine kinase 2 (Tyk2) and Janus kinase 1 (Jak1) and the association of Signal Transducers and Activators of Transcription 1 (STAT1) and STAT2 heterodimers. They recruit the IRF9 to form the IFN-stimulated gene factor 3 (ISGF3). IRF9 containing a nuclear localization sequence (NLS) facilitates the nuclear translocation of the complex (Lau et al. 2000). This pathway leads to the expression of interferon-stimulated genes (ISGs) dependant on the binding of the STAT proteins and IRF9 to the ISRE (interferon-stimulated response element) DNA sites. Importantly, while STAT1 and STAT2 can be activated in response to IFN-γ or IFN-α/β stimulation and bind to Gamma interferon activation site (GAS) element, IRF9 seems required for ISRE recognition by ISGF3. Eventually, various anti-viral responses are promoted such as the inhibition of viral protein synthesis or viral assembly

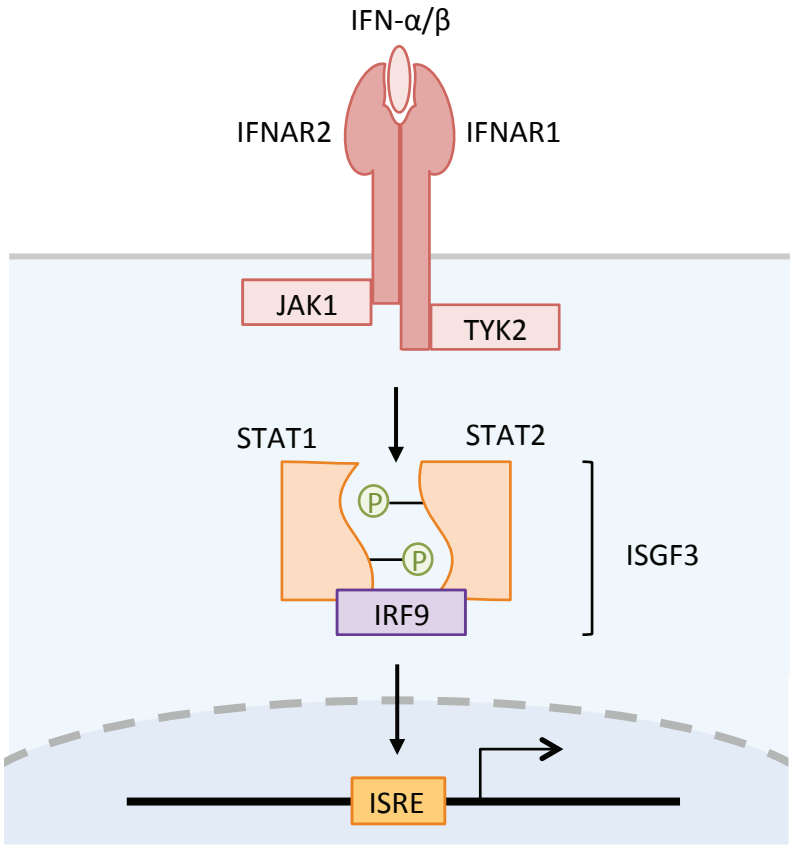
(**Figure 1.1**) (Samuel 2007; Au-Yeung et al. 2013; Schneider et al. 2014; Ivashkiv and Donlin 2014).

### 1.3.4 Synergy of stimuli

It appears that a pathogen can trigger the activation of various PRRs while a single PRR is able to recognise different ligands leading to different outcomes. For instance, while TLR4 mainly binds to LPS, inducing inflammation and tissue repair, high-mobility group box protein (HMGB1) a nucleus protein released from necrotic cells is also sensed by TLR4 but induces weaker immune responses (Iwasaki and Medzhitov 2010). Therefore, immune cells can sense various contextual cues that synergistically regulate their responses.

In 2009, De Nardo and al. showed that the pre-treatment of murine macrophages with LPS led to a greater IL-6 and TNF-$\alpha$ production following CpG stimuli showing a potential synergy between different TLR signalling (De Nardo et al. 2009). Interestingly, CpG pre-treatment on DCs amplifies their LPS-triggered IL-12 production but this effect was not occurring on LPS-pre-treated DCs subsequently activated with CpG. Moreover, the pre-treatment was required 6h prior DC stimulation for an optimal boost of cytokine response (Theiner et al. 2008). Therefore the dynamic and kinetics of TLR stimulations could modulate and control the responses induced (Tan et al. 2014). The recognition of a particular strain of HSV is induced by the sequential TLRs activation within the same DC: initially, TLR2 is engaged via the recognition of virions at the DC surface, then TLR9 is stimulated intracellularly by the internalized viral genomic DNA (Sato et al. 2006). Furthermore, this effect doesn't affect the whole gene expression but only a small portion of the genes induced by a single TLR stimulation is significantly increased by the synergy of different TLR signallings. These results imply a selective amplification of a set of genes via a combinatorial gene expression program (Napolitani et al. 2005).

This synergy phenomenon is not limited to TLR signalling. Focusing on $CD11b^+$ Flt3L-derived DCs, the enhancement of IκBα (NF-κB inhibitor α-like)




**<u>Figure 1.1</u>: Type I interferon signalling pathway.**
Type I interferon (IFN-α/β) receptor includes IFNAR1 and IFNAR2 subunits. Its engagement induces the activation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), both kinases phosphorylate IFNAR leading to the recruitment of Signal Transducer and Activator of Transcription (STAT) proteins 1 and 2. These STAT proteins dimerise and phosphorylate each other. Interacting with the IFN-Regulatory Factor 9 (IRF9), they form the Interferon-Stimulated Gene Factor 3 (ISGF3) complex. This complex translocates into the nucleus and binds to IFN-Stimulated Response Element (ISRE) sequences inducing an antiviral response program gene expression (Ivashkiv and Donlin 2014).

degradation and the increase of NF-κB nuclear translocation have been observed when the respective signalling pathways induced by curdlan, dectin-1 agonist, and by Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) were both simultaneously initiated. GM-CSF strengthens the outcome of the curdlan via a MAPK Extracellular signal-Regulated Kinases (ERK)-dependant mechanism, as phosphorylation of ERK, out of various MAPKs, was the only event that was greater under curdlan and GM-CSF co-stimulation (Min et al. 2012). Moreover, splenic $CD8^+$ DCs stimulated *in vitro* with microbial stimulus such as CpG in the presence of CD40L-transfected fibroblasts produce a higher amount of the cytokine IL-12 than with CpG alone or with CD40L-expressing fibroblasts only (Schulz et al. 2000).

## 1.4 T cell priming

Cell-mediated immune responses mainly involve T lymphocytes and rely on their ability to recognize specific antigen via the expression of particular T cell receptor (TCR) (Kappler et al. 1983). Following their thymic development, naïve T cells circulate from blood to secondary lymphoid organs such as LNs and spleen in order to scan the environment for antigens. When the naïve T cells don't encounter their specific antigen they leave the secondary lymphoid organs to reach the circulation again and travel through the lymphoid organs, blood and lymphatics within 24 hours. When stimulated at the site of infection, antigen-bearing DCs migrate to the dLN in order to precisely localise near the High Endothelial Venules (HEVs) where they can meet many non-specific T cells but also antigen-specific T cells initiating their activation. TCR stimulation triggers intracellular signalling cascades inducing cellular activation, clonal expansion and differentiation into effector cells able to migrate (Bajénoff et al. 2003; Smith-Garvin et al. 2009; Obst 2015). Naïve $CD8^+$ T cells acquire cytotoxic functions targeting tumour cells or cells that have been infected by intracellular pathogens (Zehn et al. 2012). Regarding naïve $CD4^+$ T cells, they can differentiate into various lineages and provide specific cytokine signatures,

thanks to the cues they receive from their microenvironment (O'Shea and Paul 2010).

## 1.4.1 T cell subsets differentiation and circulation

### 1.4.1.1 $CD8^+$ T cells

$CD8^+$ T lymphocytes are capable of controlling intracellular bacterial or viral infections. They specifically target infected cells as well as tumour cells, and release cytotoxic proteins inducing apoptosis. In addition, they secrete cytokines such as IFN-γ or TNF-α involved in the inhibition of viral replication and, in the recruitment and activation of macrophages (Ratner and Clark 1993; Lampe et al. 1998).

Following the encounter of their specific antigen presented via MHC I molecules at the surface of a DC, naïve $CD8^+$ T cells undergo clonal expansion, acquire effector functions and migrate to the site of infection: this is the primary response (Williams and Bevan 2007). Once the infection is cleared, the majority of the effector $CD8^+$ T cells produced during the primary response will die. However, some cells are retained and survive as long-term memory $CD8^+$ T cells. A single naïve $CD8^+$ T cells can give rise to both type of cells and the initial encounter with DCs is thought to be the means by which the immune system can modulate their differentiation (Zhang and Bevan 2011).

### 1.4.1.2 $CD4^+$ T cells

$CD4^+$ T cells themselves can have cytolytic mechanisms such as the expression of granzymes, perforin, TNF-Related-Associated-Inducing Ligand (TRAIL) or Fas Ligand (FasL) targeting tumor in a direct manner (Melssen and Slingluff 2017). However, $CD4^+$ T cells are more typically known for their ability to regulate macrophages activation and to provide help to $CD8^+$ T cells and to B

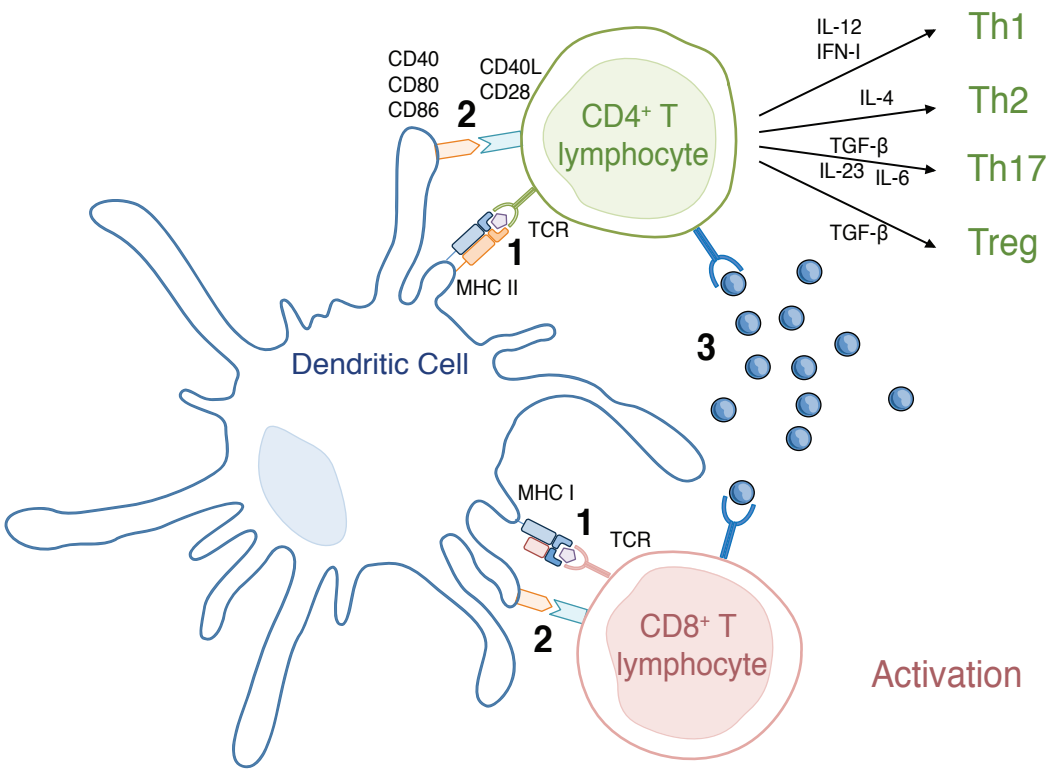
cells (Zhu et al. 2010). As for $CD8^+$ T cells, naïve $CD4^+$ T cells undergo clonal expansion and acquire their functions after the recognition of their specific antigen presented by an APC via MHC II molecules. They can differentiate into various T helper (Th) cell lineages such as Th1, Th2 for the most described but also Th17, Th9, Th22 or regulatory T cells (Tregs). All these subsets differ from each other by their cytokines production and functions. Briefly, Th1 cells are involved in intracellular pathogens responses and produce IFN-γ, IL-2 and TNF-α. Th2 cells are known to be IL-4, IL-5 and IL-13 producers and are required for humoral-mediated responses and protect hosts against extracellular pathogens such as parasites. Th17 cells produce IL-17 and are involved in host defence against bacteria and fungi. Finally, Th9 cells produce IL-9, Th22 produce IL-22 and Tregs produce IL-2 and Transforming Growth Factor β (TGF-β) (Stockinger et al. 2007; Zhu et al. 2010; Raphael et al. 2015; Li et al. 2019).

Interestingly, DCs could themselves influence Th cell lineage of $CD4^+$ T cells: for instance the cytokine IL-12 produced by $CD8^+$ DCs is required for Th1 polarization while its absence leads to Th2 cells development (Moser and Murphy 2000).

Following the clearance of the infection, most of $CD4^+$ T cells will die and only a few cells will form the memory $CD4^+$ population (MacLeod et al. 2009).

### 1.4.2 $CD8^+$ T cell priming and role of dendritic cells

Activation and differentiation of naïve T lymphocyte into effector T lymphocyte require 3 signals (**Figure 1.2**). As explained earlier, DCs have the ability to present antigens at their surface due to their MHC molecules expression. They are particularly efficient at T cells priming (Steinman and Witmer 1978). These peptides are recognized by T cells via their specific TCR and this recognition consists of the signal 1. While $CD4^+$ T cells recognize exogenous antigens presented via MHC II molecules, $CD8^+$ T cells recognize endogenous antigens presented via MHC I molecules as well as exogenous




**<u>Figure 1.2</u>: Requirement of three signals for the initiation of T lymphocytes effector responses.**
Professional APC such as dendritic cells (DCs) provide three signals that are required for naive T cell activation and polarisation. MHC-peptide complex is recognized by TCR that delivers signal 1. Co-stimulatory molecules expressed by DCs (CD40, CD80/CD86) then interact with their ligand expressed by T lymphocytes (CD40L, CD28) providing signal 2. Finally DCs secrete pro-inflammatory cytokines. This third signal functionally polarise naive $CD4^+$ T cells into T helper (Th1, Th2, Th17) or regulatory T cells (Treg).

antigens thanks to the cross-presenting ability of DCs (Banchereau and Steinman 1998; Heath et al. 2004).

DCs deliver the signal 2 through the provision of co-stimulatory molecules to co-stimulatory or co-inhibitory receptors expressed by the T lymphocyte. Co-stimulatory molecules can be classified into two main families: the CD28/B7 family with, for instance, CD28 and cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) which can both bind to CD80 and CD86 expressed by APC; and the TNF/TNF receptor (TNFR) family with members such as OX40, CD27 and CD40 ligand (CD40L) as well binding OX40L, CD70 and CD40 respectively (Elgueta et al. 2009; Magee et al. 2012). For instance, CD28 has been shown to optimize T cell responses via the remodelling of the actin cytoskeleton strengthening TCR signalling. Upon activation, the APC up-regulate their CD80/CD86 expression levels in order to transmit co-stimulatory signals (Esensten et al. 2016). The ligation of CD40 on the surface of APCs such as DCs leads to the enhancement of their functions including the up-regulation of CD80 and CD86 (Grewal and Flavell 1998).

Finally, CTL priming consistently requires a signal 3, which consists of cytokines and chemokines provided by DCs. Various cytokines and chemokines trigger various effects on $CD8^+$ T cells and the cytokines and chemokines produced seem to vary depending on the immunogenic challenge encountered. Mature DCs produce the cytokine IL-12, composed by two sub-units, p35 and p40. Following *Listeria monocytogenes* infection IL-12 secretion influences the production of IFN-γ by $CD8^+$ T cells (Henry et al. 2008). In the context of a synthetic antigen immunization, IL-12 acts on the $CD8^+$ T cells inducing clonal expansion and acquisition of cytotoxic function (Schmidt and Mescher 2002; Valenzuela et al. 2002). Moreover the activation of $CD8^+$ T cells during a graft rejection is triggered by IL-12 provided by DCs (Filatenkov et al. 2005). The cytokine IL-15 produced by DCs has been shown to play a key role in driving the priming of T cells (Verbist and Klonowski 2012) while, IL-6 promotes survival of naïve T cells and their proliferation following antigen encounter

(Takeda et al. 1998; Teague et al. 2000). In addition to influencing the divisions and functions of T cells, the DCs also produce chemokines that promote the migration of T cells. Inflammation conditions lead to the up-regulation of the receptor CCR5 on the surface of $CD8^+$ T cells, however the optimal up-regulation of this receptor occurs in a TCR-dependent manner. In addition, $CD4^+$ T cell interacting with the DCs induce their production of CCL3/CCL4 allowing the chemical guidance of the $CD8^+$ T cell towards the licensed DCs (Castellino et al. 2006; Eickhoff et al. 2015). Sharing the same receptor than CCL3/CCL4, CCL5 produced by DCs has been also shown to induce the chemoattraction of both $CD4^+$ and $CD8^+$ T cells (Son et al. 2014). In 2016, Greyer et al. demonstrated that the DC cytokine production mediating CD8 T cell immunity is dictated by the innate stimuli they receive. Indeed, if the mice are exposed to a same antigen but DCs receive different innate signals, the cytokine response is different: the priming of ovalbumin (OVA)-specific $CD8^+$ T cells requires the production of IL-12 when the OVA is presented in the presence of LPS while a stimulation with polyinosinic-polycytidic acid (poly(I:C)) at the time of antigen presentation induces an IL-15-dependent priming of CTL (Greyer et al. 2016). Interestingly, many studies conducted before implied the same theory: for instance, Edwards et al. showed that sensing CpG leads to IL-12p70 secretion while DCs stimulated by yeasts secrete IL-10 (Edwards et al. 2002). Moreover, the CD8 T cell priming in the context of an adenoviral vaccine depends on IL-15 secretion by DCs while VACV specific $CD8^+$ T lymphocytes require IFN-α/β stimulation (Oh et al. 2008; Wiesel et al. 2011).

## 1.5 $CD4^+$ T cell help

There is still much debate as to the relative importance of help, with $CD4^+$ T cell help being necessary to provide efficient memory $CD8^+$ T cells responses in viral and bacterial infections (Shedlock and Shen 2003), however its importance during a primary response has been shown as detrimental in some circumstances.

### 1.5.1 Help involvement during CD8$^+$ T cell priming

As described earlier, when DCs are activated, they produce cytokine responses and up-regulate co-stimulatory molecules including CD40, receptor of CD40 ligand (CD40L) (O'Sullivan and Thomas 2003). DCs are well known for their ability to transmit help signals derived from their interaction with CD4$^+$ T cells. They are a platform able to relay this help to CD8$^+$ T cells for them to elicit CTL responses (Mitchison and O'Malley 1987; Ridge et al. 1998). Using an antibody-based stimulation of CD40 on DCs, these help signals were quickly linked to the interaction of this receptor with CD40L expressed by the activated CD4$^+$ T cell (Ridge et al. 1998). Moreover, Schoenberger et al. demonstrated the necessity of an interaction between CD40 and CD40L for the delivery of help during the naïve T lymphocytes priming (Schoenberger et al. 1998). Furthermore, the engagement of CD40L to the CD40 receptor of DCs, promotes their characteristics involved in an efficient priming of T cells: cytokines production and co-stimulatory molecules CD80/86 up-regulation, required for the subsequent CD28 signalling. Moreover, the binding of CD40L to CD40 has been also shown to facilitate the antigen cross-presentation (O'Sullivan and Thomas 2003; Elgueta et al. 2009).

Already in 1998 evidence was provided for the requirement of a cognate CD4$^+$ T cell signal for an efficient OVA-specific CD8$^+$ T cells priming (Bennett et al. 1998). Although lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV) and human immunodeficiency virus (HIV) infections induce CD4$^+$ T cell-independent primary CD8$^+$ T cell responses, the resolution of infections of adenovirus, HSV and VACV are helper-dependent (Wiesel and Oxenius 2012). For instance, CD4$^+$ T cell-depleted mice using a murine antibody GK1.5 treatment, showed a defect in their CTL priming following HSV infection (Jennings et al. 1991).

Many studies seem to describe the requirement of a three-cell interaction between CD4$^+$ T cell, DC and CD8$^+$ T cell. Yet, other dynamic interplays have

been recently proposed. It has been observed that $CD8^+$ T cells can express CD40 molecules while DCs happen to express CD40L themselves, leading to the hypothesis that these cells could receive help from the DCs without the requirement of cognate $CD4^+$ T cells interaction. Precisely, CD40L was up-regulated by DCs during intranasal influenza challenge but not during HSV infection (Johnson et al. 2009).

### 1.5.2 Help-amplification model

It is still unclear what determines the requirement for $CD4^+$ T cell help in generating $CD8^+$ T cell priming. In 2004, Bevan suggested that the help signal is equivalent to innate signals for DC activation. An antigen challenge in a non-inflammatory context would require $CD4^+$ T cell to induce $CD8^+$ T cell responses while strong danger signals would efficiently license DCs to prime $CD8^+$ T cells in a $CD4^+$ T cell-independent manner (Bevan 2004). Since 2004, several lines of evidences point towards the magnitude of the type I IFN response as an important factor in the help dependence of $CD8^+$ T cell responses. Consistent with this, some pathogens have generated strategies to down-regulate the host responses. For instance, during VACV infection the virus expresses proteins that bind type I IFN-I thus blocking the cellular recognition of IFN-α/β and its signalling, which limits the inflammatory response. This evasion strategy is thought to be responsible for the help requirement upon this viral infection (Wiesel and Oxenius 2012). The injection of irradiated splenocytes that have been loaded with ovalbumin protein (OVA) induces a help-dependent CTL priming (Bennett et al. 1997). However, Le Bon et al. could counteract this requirement by injecting IFN-α into the immunized mice leading to a strong $CD8^+$ T cell response even in $CD4^+$ T cell-deficient mice (Le Bon et al. 2003). Upon LCMV infection, a robust amount of IFN-α/β is produced via activation of helicase family members such as RNA helicases retinoic gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (Zhou et al. 2010; Wiesel et al. 2011). Thus MDA5 deficiency impairs IFN-α/β secretion. Interestingly

Wang et al. observed that MDA5 deficiency only led to delayed $CD8^+$ T cell response to LCMV. However, this deficiency in combination with $CD4^+$ T cells depletion induced $CD8^+$ T cell exhaustion and viral persistence. Therefore, although LCMV infection is known as help-independent, the loss of an early and strong IFN-α/β production was compensated by the presence of $CD4^+$ T cells (Wang et al. 2012). In agreement with these results, induction of IFN-α/β production has been shown to compensate $CD4^+$ T cell help requirement in response to VACV infection (Wiesel et al. 2011).

While these results highlight the importance of the inflammatory stimulus strength in determining the requirement for help, they do not specify if these two signals are able to substitute for each other. In 2016, our team demonstrated that CD40-CD40L signalling amplifies weak innate signals leading to the maturation of DCs and to the efficient $CD8^+$ T cell priming, this is the amplification model (**Figure 1.3**). Using a HSV-I skin infection model, they showed that type I IFN acts directly on the $CD8^+$ DCs and with the help of $CD4^+$ T cells, induces IL-15 secretion. In turn, IL-15 participates in $CD8^+$ T cells priming. $CD4^+$ T cell help, mediated via CD40-CD40L interaction, amplifies DC IL-15 production induced by the initial innate IFN-α/β signal. In the absence of help signal, IFN-α/β signal on its own is ineffective to induce this cytokine secretion (Greyer et al. 2016). This CD40-driven enhancement of DC cytokine responses has been previously described. The CpG-triggered IL-12 production by $CD8^+$ DCs is enhanced in the presence of CD40L-transfected fibroblasts (Schulz et al. 2000; Edwards et al. 2002).

These observations allow us to envisage that innate signals, such as type I IFN, and $CD4^+$ T cell help act in a synergistic manner towards $CD8^+$ T cell priming and that the help requirement depends on the strength of the infection or immunization. Weak immunogens would thus lead to a $CD4^+$ T cell dependent maturation of the DCs.

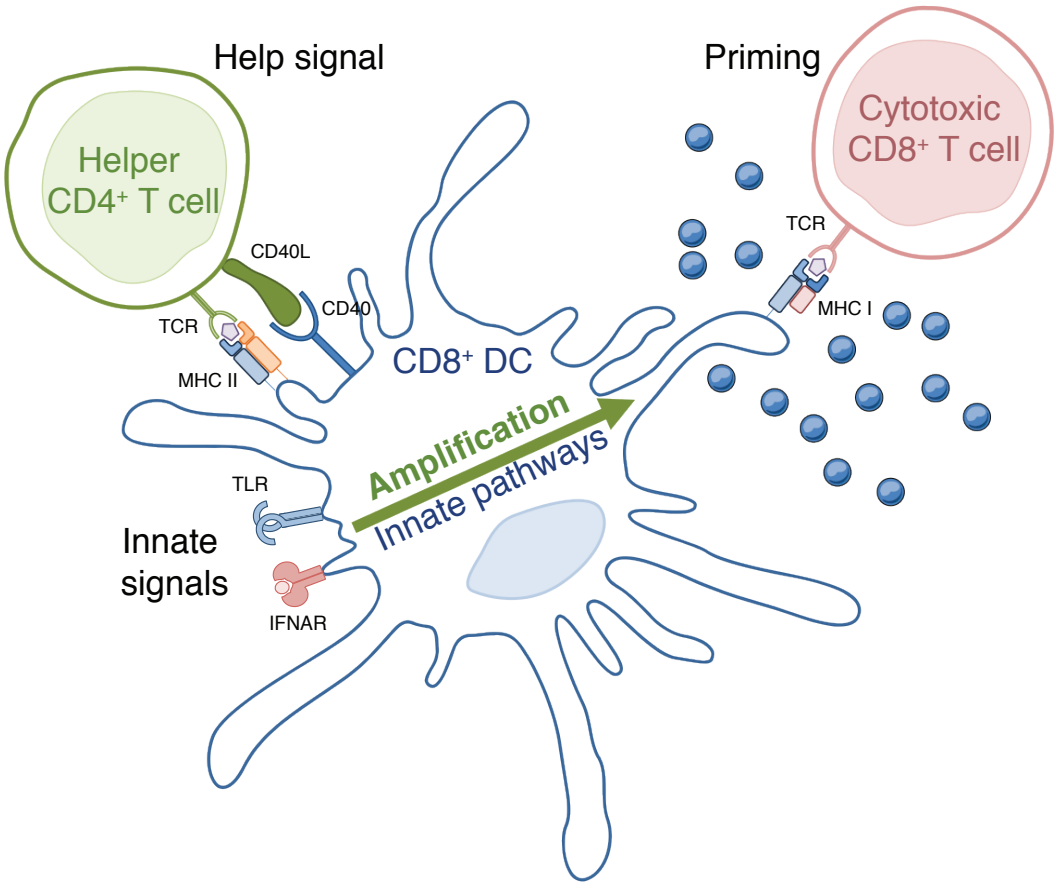



**Figure 1.3: T cell help-driven amplification of innate circuits in CD8⁺ DCs.** Activated antigen presenting DC can prime CD8⁺ T cells. DCs are stimulated by the recognition of microbial patterns and/or by inflammatory cytokines. These innate signals initiate a specific innate pathway of cytokines and chemokines production. If the innate stimuli are too weak, these innate pathways don't efficiently induce the priming of CD8⁺ T cells and the DCs require the help signal from their interaction with CD4⁺ T cell. This help signal is mediated via the binding of CD40L on CD4⁺ T cell with CD40 on DCs. The help signal initiated amplifies the innate pathways mediated by the innate stimuli and leads to the efficient priming of CD8⁺ T cells.

### 1.5.3 DC strategic localization

*In vivo*, little is known about when and where the help process is occurring. The lymphatic circulation allows antigens to be efficiently brought to the dLN by DCs, while lymphocytes travel through the LN via the blood circulation. Naïve T cells frequently migrate to LN and scan the DCs until they find their antigen-specific DC and form sustained and stable interactions. If lymphocytes cannot recognize their specific antigen, they return to the circulation (Andrian and Mempel 2003; Williams and Bevan 2007). Through the lymphatic system, lymphocytes patrol the all body within 24 hours. At the site of infection, DCs pick up the antigen and receive pathogen-derived signals instructing them to migrate to the dLN. For instance, migratory DCs up-regulated CCR7, receptor for CCL19 and CCL21 chemokines, involved in DCs entrance in lymphatic vasculature and subsequently in DCs entrance in dLNs. (Worbs et al. 2017). T cell activation by antigen-presenting DC doesn't occur randomly in the LN, but rather in precise areas of the paracortex, close to the HEVs. More precisely, antigen-presenting DCs localize in this area to meet incoming naïve T cells and therefore encounter the cells specific for their antigen (Bajénoff et al. 2003). By injecting TCR transgenic lymphocytes and fluorescent DCs, Mempel et al. showed that T cell-priming by DCs occurs in three different phases within the LN: a first phase of short interactions with DCs while T cells up-regulate their activation markers; a second phase of stable and lasting interaction with DCs inducing the T cell production of IL-2 and IFN-γ; and ultimately, a third phase, during which T cells proliferate and become able to migrate (Mempel et al. 2004). The dynamic of migration of the T lymphocytes within the dLN is precise and different between the $CD4^+$ and $CD8^+$ T cells (Mandl et al. 2012). Moreover, $CD4^+$ T cell require a prolonged phase of stable interactions with DCs compared to $CD8^+$ T cells (Kastenmüller et al. 2010).

During HSV skin infection migratory DCs allow the trafficking of antigens into the dLN but these DCs are not directly involved in the $CD8^+$ T cell priming. $CD8^+$ LN resident DCs receive the antigen from most likely dead and dying cells

and are responsible for antigen presentation and CTL priming (Allan et al. 2006). Moreover, it has been shown that, $CD4^+$ and $CD8^+$ T cells are activated by distinct DCs and these activations are temporally and spatially segregated (Eickhoff et al. 2015; Hor et al. 2015).

In particular, the help process requires a first step of recognition of cognate antigen by $CD4^+$ T cells, which induces the up-regulation of CD40L at their membrane surface. Then, $CD4^+$ T cells need to recognize their antigen on the same DC that primes $CD8^+$ T cells but these two events don't necessary occur at the same moment (Smith et al. 2004; Bedoui et al. 2016). Corroborating this observation, inflammation conditions lead to the up-regulation of the receptor CCR5 at the surface of $CD8^+$ T cells while the $CD4^+$ T cell interacting with the DCs induce their production of CCL3/CCL4 allowing the chemical guidance of the $CD8^+$ T cell towards the licensed DCs (Castellino et al. 2006).

In the case of the localized skin infection with HSV, activation of specific $CD4^+$ and $CD8^+$ T cells is performed by the encounter of different subsets of DCs, and different times throughout the infection. $CD4^+$ T cells interact early with migratory DCs and $CD8^+$ T cells interact later with the LN-resident $CD8^+$ DCs. Such a fast priming of $CD4^+$ T cells has been hypothesized as a means to give the time of the help process to occur (Hor et al. 2015). By contrast, during the intradermal VACV infection, $CD4^+$ T cells are activated by non-infected resident DCs while $CD8^+$ T cells are initially primed by infected resident DCs. It's only later during the infection that co-recognition of non-infected and cross-presenting $CD8^+$ DC allows the delivery of help signals (Eickhoff et al. 2015). These two studies demonstrate the key and central role of LN-resident cDC1 forming a platform for the delivery of $CD4^+$ T cell help and the priming of $CD8^+$ T cells.

## 1.6 CD40/CD40L interaction and molecular mechanism

### 1.6.1 CD40 receptor and CD40 ligand expression

The receptor CD40 is a co-stimulatory and transmembrane molecule part of the TNFR family that was initially characterized on B cells. DCs, monocytes and macrophages and also non-hematopoietic cells such as some fibroblasts and endothelial cells can all express membrane bound CD40 (Elgueta et al. 2009).

CD40L, also called CD154, is expressed by a large range of immune cells such as B cells, monocytes, mast cells but most predominantly, by activated $CD4^+$ T cells following TCR ligation. However, while some cytokines such as IL-4 and IL-10 down-regulate the expression of CD40L, cytokines such as IL-2, IL-12 and IL-15 have been shown to up-regulate its expression (Lee et al. 2002; Daoussis et al. 2004). CD40L is mostly expressed in its transmembrane form but can also be secreted in soluble form, following enzymatic cleavage, with similar functions (Johnson et al. 2009). Activated T cells only transiently express CD40L on their surface. $CD4^+$ T cells can up-regulate CD40L after 5-15 minutes following anti-CD3 activation without *de novo* protein synthesis required (Casamayor-Palleja et al. 1995; van Kooten and Banchereau 2000). A second wave requiring mRNA expression and protein synthesis occurs around 1-2 hours after activation, becomes maximal at 6-8 hours and CD40L is then gradually lost. Interestingly, in addition to its up-regulation, CD40L degradation is tightly regulated as well. Its interaction with CD40 receptor quickly leads to its endocytosis and lysosomal degradation and to down-regulation of its mRNA. This confers restricted time frame for the CD40L-CD40 interaction to take place (van Kooten and Banchereau 2000). Finally, a peak of surface expression has been observed at 48 hours at the surface of Th1 cells that are capable to express higher quantity of CD40L and is positively regulated by IL-12 (Lee et al. 2002).

### 1.6.2 Downstream of CD40

As part of the TNF family of ligands CD40L trimerizes to be recognized by its receptor (Daoussis et al. 2004; Johnson et al. 2009; Elgueta et al. 2009). This trimerization is induced by the binding of CD40L to CD40, as well as the subsequent recruitment of TNF Receptor Associated Factor proteins or TRAFs going from TRAF1 to TRAF6 (Bishop et al. 2007). As CD40 doesn't have kinase domain itself, these adaptor proteins interact with its cytoplasmic domain and lead to the activation of various signalling pathways such as the canonical and non-canonical NF-κB pathways (NF-κB1 and NF-κB2 respectively), the MAPKs or phosphoinositide 3-kinase (PI3K) pathways. The recruitment of a specific TRAF protein dictates the downstream signalling pathway as well as seems to regulate the involvement of other TRAF proteins. For instance, recruitment of TRAF2, TRAF5 and TRAF6 can lead to the activation of the canonical NF-κB pathway while TRAF3 negatively regulates it (Zarnegar et al. 2008; Elgueta et al. 2009; Ma and Clark 2009). The CD40 cytoplasmic tail can interact with the different adaptor proteins: TRAF2 and TRAF3 binding site are distinct but show considerable overlap. The binding of TRAF1 is weak but this adaptor protein forms an heterodimer with TRAF2 and seems to be responsible for the prolongation of TRAF2 recruitment to CD40 (Bishop et al. 2007). TRAF5 interacts indirectly with CD40, preferentially via TRAF2 (van Kooten and Banchereau 2000; Ajibade et al. 2013).
Within this complicated interplay of TRAF proteins, some TRAFs have been described as predominantly interacting with the receptor in a cell-type manner. For instance, TRAF2 and 3 are recruited following CD40 engagement in B cells while TRAF6 recruitment seems required in DCs (Ma and Clark 2009).

The binding of TRAF6 to the cytoplasmic tail of CD40 induces the recruitment of Ubiquitin-conjugating enzyme 13 (UBC13) leading to the non-degradative lysine 63 ubiquitination (K63Ub) of various proteins including TRAF6 itself and the complex NEMO (NF-κB essential modulator) or Inhibitor of κB kinase γ (IKKγ). TRAF2/5 recruitment leads to the ubiquitination of receptor-

interacting protein 1 (RIP1) which in turn recruit the IKK complex by interacting with IKKγ. Finally, these ubiquitination events induce the recruitment of TAK1 (TGF-β-activated kinase 1), member of the MAP kinase kinase kinase (MAP3K) family, which can activate NF-κB and/or MAPK pathways via phosphorylation. TAK1 constitutively interacts with TAB1 (TAK1-binding protein 1) required for TAK1 kinase activity. Following stimulation TAB2 or TAB3, ubiquitin binding proteins, interact with TRAF6 facilitating TAK1 recruitment and activation (**Figure 1.4**) (Ruland 2011; Ajibade et al. 2013; Sabio and Davis 2014).

While the activation of the non-canonical NF-κB pathway doesn't seem to depend on the recruitment of TRAF6 to CD40, TRAF2 has been shown to be able to interact with various kinases including NIK involved in NF-κB2 pathway (Malinin et al. 1997; Bishop et al. 2007). More precisely, TRAF2 constitutively interacts with cellular inhibitor of apoptosis (cIAP)1/2 and cooperates with TRAF3 and NIK. This interaction leads to the cIAP1/2-mediated degradation of NIK and thus to the basal inhibition of the pathway. When recruited to CD40 cytoplasmic tail, TRAF2 is degraded via auto-ubiquitination and TRAF3 is degraded via cIAP1/2, releasing NIK which activates NF-κB2 pathway in response (Zarnegar et al. 2008; Elgueta et al. 2009; Sanjo et al. 2010).

### 1.6.3 NF-κB signalling pathway

The NF-κB signalling pathway is highly conserved and plays critical role in various biological processes including the immune system. NF-κB is a transcription factor family consisting of five members: RelA (p65), RelB, c-Rel, NF-κB1 (p105), processed into p50 and NF-κB2 (p100), processed into p52. All NF-κB proteins contain a Rel-homology domain (RHD) bound by specific inhibitors such as IκBα, IκBβ and IκBγ. These proteins contain ankyrin repeats, displayed as well by NF-κB1/p105 and NF-κB2/p100, allowing them to retain the proteins they are bound to in the cytoplasm (Li and Verma 2002; Bonizzi and Karin 2004). The transcription factor NF-κB is known to be required for the

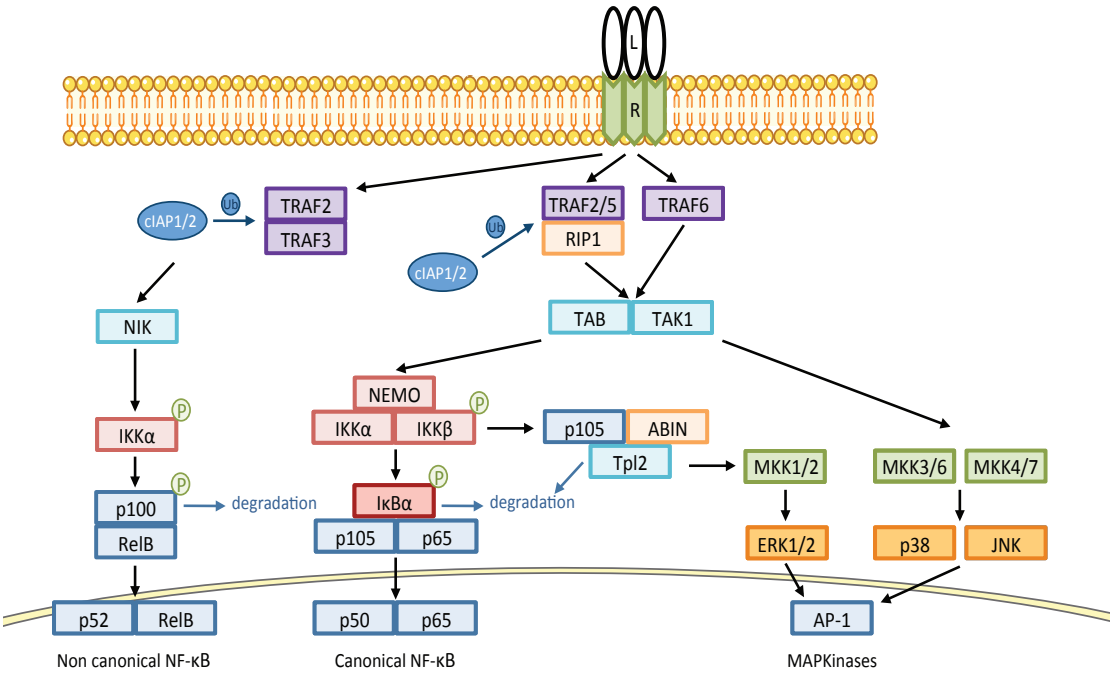



**Figure 1.4: Non canonical NF-κB, canonical NF-κB and MAPKinases pathways.** CD40 receptor stimulation triggers various pathways involving the recruitment of adaptor proteins such as TNF Receptor-Associated Factors (TRAFs). These TRAF proteins dictate the subsequent pathway engagement. Through non degradative ubiquitination events, kinases are recruited. TGF-β-activated kinase 1 (TAK1) can activate the canonical NF-κB or MAPK pathways. NF-κB-inducing kinase (NIK) recruitment leads to the non canonical NF-κB pathway activation. Eventually, transcription factors translocate in the nucleus inducing gene expression (Bishop et al. 2007; Ajibade et al. 2013).

expression of many genes induced by inflammatory stimuli, cytokines/chemokines or anti-microbial peptides. CD40 deficient and RelB deficient DCs are both inefficient in CTL priming. CD40L ligation leads to a sustained RelB signalling involved in DC functions (O'Sullivan and Thomas 2002; O'Sullivan and Thomas 2003). Moreover, the expression of CD40 itself seems to be regulated by the NF-κB transcription factors (O'Sullivan and Thomas 2003).

For the NF-κB members to fulfil their transcription factor role, the IκBs need first to be degraded via the activation of an IKK complex typically mediated by TAK1 in response to pro-inflammatory cytokines or PAMPs. Most commonly, the canonical NF-κB pathway signals through the IKK complex made up of the two catalytic subunits, IKKα and IKKβ and the regulatory subunit NEMO/IKKγ. Once activated, the IKK complex triggers the phosphorylation and poly-ubiquitination of IκBs, in this particular pathway, mainly IκBα, leading to their degradation by the 26S proteasome. Interestingly, IκBα and IκBβ have been shown to regulate NF-κB activation even further. Following its degradation, IκBα is quickly re-synthesized and able to enter the nucleus, detach the NF-κB transcription factor from the DNA and bring it back into the cytoplasm thanks to its expression of nuclear-export signal (NES). On the other hand, the newly synthesized IκBβ can enter the nucleus and interact with NF-κB transcription factors but will not bring it back to the cytoplasm as IκBβ does not express NES. In contrast, IκBβ will lead to a persistent NF-κB activation (Li and Verma 2002; Oeckinghaus et al. 2011). In the case of the alternative, or non-canonical NF-κB pathway, NIK activates an homodimer complex of IKKα which phosphorylates NF-κB2/p100 leading to poly-ubiquitination, proteosomal degradation and finally to its processing into p52. This pathway is independent of TAK1 and of the trimeric form of IKK complex (Hayden 2004; Shi and Sun 2018).

Once the NF-κB proteins are released and can translocate into the nucleus, they are able to bind specific DNA sequences. The RHD includes as

well a NLS allowing them to reach the nucleus. Gene transcription is induced by the homo- or hetero-dimerization of p50, p52, RelA, RelB and c-Rel. The dimers that these proteins form dictate the biological response they induce. Only RelA, RelB and c-Rel contain the transcription activation domain; thus, homodimers of p50 or p52 will lead to the repression of the gene transcription (Bonizzi and Karin 2004). Overall, the dimers triggered by the canonical pathway often involve RelA while the phosphorylation of the IKKα homodimer induces, in the non canonical pathway, the translocation of p52-RelB complex (Oeckinghaus et al. 2011).

Interestingly, the mutation of NIK rendering this precise kinase unable to interact with IKK complex, demonstrated the requirement of the non-canonical NF-κB pathway for the cross-presentation capacity of DCs (Ma and Clark 2009). Even if this mutation can't be directly linked to the priming ability of DCs, it has been shown to inhibit the nuclear translocation of p52, which can associate with RelA and RelB the latter being important for cross-presentation as well (Dejardin et al. 2002; Zanetti et al. 2003; Lind et al. 2008).

### 1.6.4 MAPKinase signalling pathways

Not all TRAFs functions are mediated via NF-κB pathways but can also lead to the engagement of the AP-1 transcription factor pathway, following a cascade of MAPKs activation (Oeckinghaus et al. 2011). Three different MAPKs subfamilies have been described: ERKs, c-Jun N-terminal kinases or JNKs and p38 MAPKs. As their name indicates, these pathways heavily depend on phosphorylation and dephosphorylation events to fulfil their functions. For instance, CD40 ligation is followed by phosphorylation of ERK and JNK in DCs while only the later was phosphorylated in CD40-stimulated B cells (Craxton et al. 1998; Aicher et al. 1999). p38 MAPK pathway is involved in CD40-induced production of cytokines such as IL-12p40 in DCs but interestingly, this seems to depend on the maturation state of the DCs while IL-6 production has been

shown to depend on CD40-induced p38 MAPK pathway in both immature and mature DCs (Aicher et al. 1999; Yanagawa and Onoé 2006). Moreover, the CD40-dependent IL-12p40 production by DCs might also be linked to a TRAF6-induced recruitment of JNK and p38 pathways as mutation of TRAF6 binding site impacts IL-12p40 secretion and both MAPKs activation (Mackey et al. 2003).

Briefly, the three MAPKs families follow the same complex pattern of event cascade. A MAP3K phosphorylates and activates a MAPK kinase (MKK), which in turn phosphorylates and activates a MAPK leading to the activation of transcription factors such as AP-1 (Shi and Sun 2018). The MAP3K TAK1 is involved in JNK and p38 signalling transduction while ERK activation following TLR ligation requires the MAP3K isoform Tumour Progression Locus 2 or Tpl2 that forms a complex with A20-Binding Inhibitor of NF-κB-2 (ABIN-2) and NF-κB1/p105. The activation of Tpl2 requires its release from this complex following NF-κB1/p105 degradation (Sabio and Davis 2014). Each pathway requires specific MKKs: MKK1 and MKK2 for ERK, MKK4 and MKK7 for JNK and MKK3 and MKK6 for p38 (Shi and Sun 2018). By contrast with NF-κB transcription factors which can translocate into the nucleus, activated ERK kinase translocate themselves into the nucleus to phosphorylated many transcription factors (Shapiro et al. 2014). In order to regulate this nucleus translocation, MAPKs seem to be bound to their MKK or regulatory proteins and sequestered in the cytoplasm. They required phosphorylation events to dissociate and quickly translocate by associating with nuclear transport protein via their nuclear translocation signal (NTS) motif (Yang et al. 2013). Once in the nucleus, the MAPK rapidly find their substrates. For instance, ERK has been shown to phosphorylate AP-1, composed of Fos family members (such as c-Fos, Fra-1/2) and Jun family members (c-Jun, JunB/D) (Karin and Gallagher 2009). This activation is facilitated by the localisation of c-Fos to the nuclear envelope. JNK also phosphorylates the transcription factor c-Jun. Moreover, p38 can phosphorylate these transcription factors as well as induce histone modification

and chromatin remodelling in order to facilitate gene transcription (Yang et al. 2013; Shapiro et al. 2014).

The specific inhibition of p38 MAPK pathway seems to decrease the NF-κB pathway activation, interfering with the transcriptional function of NF-κB factors (Goebeler et al. 2001; Guo et al. 2013). Thus, these two pathways, NF-κB and MAPKs can cross-interact. Moreover, while it is known that the family of transcription factors AP-1 is mediated through MAPK pathways, NF-κB can play an indirect role in its activation (Fujioka et al. 2004).

## 1.7 Thesis aims

Our team was previously able to demonstrate a functional aspect of T cell help in the context of HSV infection. While the importance of $CD4^+$ T cell help in optimizing $CD8^+$ T cell priming has been intensively studied, the mechanisms responsible for this help-driven enhancement of innate signals is yet to be unveiled. The experiments conducted and presented in this thesis aimed to investigate these unknown mechanisms. Given the possibility of using CTL immunity in a therapeutic manner with a help signal considerably amplifying the innate-induced responses in DCs, T cell-based therapeutic strategies could gain from highlighting this enhancing mechanisms. We hypothesized that CD40 stimulation induces a pathway independent from the innate signal-induced pathway. These two pathways would then act in synergy towards the optimization of DCs functions. More precisely, studies described in this thesis aim to:

1. Establish an *in vitro* model of bone marrow-derived DC specialised on the CTL priming, allowing the systematic study of CD40-mediated amplification of DC responses to various innate signals (Chapter 3).

2. Precisely describe the dynamics of CD40-signal requirement in an innate activation context, in order to methodically study the potential molecular pathways involved in this CD40-driven enhancement of innate signalling (Chapter 4).

# Chapter 2

## Materials and Methods

## 2.1 Materials

### 2.1.1 Mice

All mice used in this study were bred and maintained in specific pathogen-free conditions at the Department of Microbiology and Immunology, at The University of Melbourne, Australia animal facility.
The Animal Ethics Committee from Biochemistry & Molecular Biology, Dental Science, Medicine (RMH), Microbiology & Immunology and Surgery (RMH) approved all animal experiments (Animal ethics number 1814545).

**Table 2.1: Description of the mouse strains.**

| Strain | Description |
|---|---|
| C57Bl/6 (B6) | Inbred mice expressing MHC class II I-$A^b$ and MHC class I H-$2^b$ haplotype |
| B6/Ifnar2 (IFNARKO) | Mutation of the *Ifnar2* gene (Fenner et al. 2006) |
| IRF3KO | Mutation of the *Irf3* gene (Sato et al. 2000) |
| IRF7KO | Mutation of the *Irf7* gene (Honda et al. 2005) |
| OT-II x B6.Ly5.1 (OT-II) | F1 generation of heterozygous/homozygous OT-II and B6.Ly5.1 breeding. H-$2K^b$ restricted, TCR transgenic (Vα2/Vβ5.$1^+$), specific for the $OVA_{323-339}$ peptide (KISQAVHAAHAEINEAG) from ovalbumin (OVA) |

### 2.1.2 Cell biology reagents and materials

**Table 2.2: Reagents and materials.**

| Reagents and materials | Supplier |
|---|---|
| α-mouse CD40 pure – functional grade antibody (clone FGK45.5) | Miltenyi Biotec, USA |
| BD Cytofix™ | BD Bioscience, USA |
| BD Perm/Wash™ | BD Bioscience, USA |
| Benzylpenicillin | CSL, Australia |
| BioMag® Goat anti-rat IgG beads | QIAGEN, Germany |
| Bright-line® haemocytometer | Reichert, USA |
| Cell strainer, 70 µm | BD Biosceince, USA |
| CpG 1668 | GeneWorks, Australia |
| CD40-TRAF6 Signalling Inhibitor, Small Molecule Inhibitor 6877002 | Calbiochem, Sigma-Aldrich, USA |
| Dimethyl Sulphoxide (DMSO) | Sigma-Aldrich, USA |
| DynaMag™-5 magnet | ThermoFisher Scientific, USA |
| *E. coli* lipopolysaccharide (LPS) | Sigma-Aldrich, USA |
| EDTA-BSS | Media preparation unit, Department of Microbiology and Immunology, The University of Melbourne, Australia |
| FACS Aria™ III flow cytometry | BD Bioscience, USA |
| FACS LSR Fortessa™ flow cytometry | BD Bioscience, USA |
| Human recombinant Fms-like tyrosine kinase 3 (Flt3) ligand (Flt3L) (Flt3L-Ig (hum/hum)) | Bio X Cell, USA |

| | |
|---|---|
| Foetal Calf Serum (heat-inactivated) (FCS) | CSL, Australia |
| KDS-RPMI | Media preparation unit, Department of Microbiology and Immunology, The University of Melbourne, Australia |
| L-glutamine | Astral Scientific, Australia |
| LIVE/DEAD™ fixable Near IR dead cell stain | Life Technologies, USA |
| Minimal Essential Media (MEM) | Media preparation unit, Department of Microbiology and Immunology, The University of Melbourne, Australia |
| 2-β-mercaptoethanol | Life Technologies, USA |
| Nylon mesh, 70 μm | Madison Filter Pty Ltd., Australia |
| OVA (323-339) | Mimotopes, Australia, kindly provided by Dr Linda Wakim, Department of Microbiology and Immunology, The University of Melbourne, Australia |
| Phosphate Buffered Saline (PBS) | Media preparation unit, Department of Microbiology and Immunology, The University of Melbourne, Australia |
| Poly(I:C) | InvivoGen, USA |
| Polypropylene round-bottom FACS tubes (5 ml) | BD Bioscience, USA |
| Polystyrene round-bottom FACS tubes with cell strainer cap (5 ml) | ThermoFisher Scientific, USA |
| Propidium iodine (PI) | Sigma-Aldrich, USA |

| | |
|---|---|
| Rat IgG2a pure - functional grade antibody (clone ES26-15B7.3) | Miltenyi Biotec, USA |
| Red blood cell lysis buffer (RBCL) | Sigma-Aldrich, USA |
| RPMI 1640 | Media preparation unit, Department of Microbiology and Immunology, The University of Melbourne, Australia |
| Sphero blank calibration beads (6.0 – 6.4 µM) | BD Bioscience, USA |
| Streptomycin | Sigma-Aldrich, USA |
| Tissue Culture flask (T75, T150) | Techno Plastic Products AG (TPP), Switzerland |
| Tissue Culture plates (6/12/96 wells) | Techno Plastic Products AG (TPP), Switzerland |
| Tissue Culture petri dishes (60/90 mm) | Greiner Bio-One, Germany |
| Trypan Blue | Sigma-Aldrich, USA |
| Trypsin/EDTA (10 X, 0.5 % trypsin, 0.2 % EDTA | Gibco BRL, Australia |
| Universal Type I Interferon (IFN-αA) | PBL Assay Science, USA |

### 2.1.3 Media and solutions

**Table 2.3: Composition of media and solutions.**

| Media | Composition |
|---|---|
| EDTA-BSS – 2.5 % FCS (FACS Buffer) | Buffered Saline Solution (BSS) supplemented with 1.86 g EDTA/L and 2.5 % heat inactivated FCS |

| | |
|---|---|
| Flt3 media | KDS-RPMI supplemented with 10 % FCS, 0.2 g/L streptomycin, 100 U/ml penicillin, 90 µM 2-β-mercaptoethanol and 1.32 mM L-glutamine |
| KDS-RPMI | RPMI 1640 powder dissolved to 1 X strength in Mili Q water |
| MEM 10 % FCS | Minimal essential medium supplemented with 10 % FCS and 5 % SC |
| RPMI 1640 – 10 % FCS (RPMI-10) | Supplemented with 5 % SC and 10 % heat-inactivated FCS |
| Supplementum completum (SC) | 23.83 g/L HEPES; $2\times10^6$ U/L Benzypenicillin; 2 g/L Streptomycin; 6 g/L L-glutamine; 70 µL 2-β-mercaptoethanol (stock concentration 14.3 M); made to 1 L with RPMI 1640 |

## 2.1.4 Antibodies

### 2.1.4.1 Fluorochrome-conjugated primary antibodies

**Table 2.4: Anti-mouse antibodies used for Flow Cytometry and Cell sorting.**

| Antibody | Clone | Conjugated | Supplier |
|---|---|---|---|
| α-CD3e | 145-2C11 | BUV 395 | BD Pharmingen™ (USA) |
| α-CD4 | RM4-5 | PE | BD Pharmingen™ (USA) |
| α- CD11b | M1/70 | BV 711 | BioLegend (USA) |
| α-CD11c | N418 | PE-Cy7 | Thermo Fisher Scientific (USA) |
| α-CD24 | M1/69 | FITC | BioLegend (USA) |
| | | PE | BD Pharmingen™ (USA) |
| | | BUV 395 | BD Pharmingen™ (USA) |
| α-CD40 | HM40-3 | FITC | BD Pharmingen™ (USA) |

| | | | |
|---|---|---|---|
| α-CD45R/B220 | RA3-6B2 | PB | BD Pharmingen™ (USA) |
| α-CD86 | GL1 | FITC | BD Pharmingen™ (USA) |
| α-CD154 (CD40L) | MR1 | PE | BioLegend (USA) |
| α-CD172a (SIRP-α) | P84 | APC | eBiosciences (USA) |
| | | BV 510 | BD Pharmingen™ (USA) |
| α-IL-15Rα (CD215) | DNT15Ra | APC | eBiosciences (USA) |
| α-MHC Class II (I-A/I-E) | M5/114.15.2 | Alexa fluor 700 | Thermo Fisher Scientific (USA) |
| | | Redfluor 710 | eBiosciences (USA) |
| α-Vα2 TCR | B20.1 | Alexa fluor 700 | BD Pharmingen™ (USA) |
| Purified α-CD16/32 (mouse BD Fc Block) | 2.4G2 | | BD Pharmingen™ (USA) |

BUV = Brilliant Ultra Violet, FITC = Fluorescein isothiocyanate, PE = Phycoerythrin, APC = Allophycocyanin, Cy = Cyanin, BV = Brilliant Violet, PB = Pacific Blue.

#### 2.1.4.2 T cell enrichment cocktails

**Table 2.5: Anti-mouse antibodies used for T cell enrichment.**

| Antibody | Clone | Supplier |
|---|---|---|
| α-CD8 | 53-6.7 | Recombinant antibody facility, WEHI, Australia |
| α-F4/80 | F4/80 | Recombinant antibody facility, WEHI, Australia |
| α-Gr1 | RB6-8C5 | Recombinant antibody facility, WEHI, Australia |

| | | |
|---|---|---|
| α-I-A/E | M5/114 | Recombinant antibody facility, WEHI, Australia |
| α-Mac-1 | M1/80 | Recombinant antibody facility, WEHI, Australia |
| Erythrocytes | Ter119 | Recombinant antibody facility, WEHI, Australia |

#### 2.1.4.3 *In vitro* stimulation of T cells

**Table 2.6: Anti-mouse antibodies used for *in vitro* stimulation of T cells.**

| Antibody | Clone | Supplier |
|---|---|---|
| α-mouse CD3 functional grade | 17A2 | ThermoFisher Scientific, USA |
| α-mouse CD28 functional grade | 37.51 | ThermoFisher Scientific, USA |

### 2.1.5 Molecular biology reagents

**Table 2.7: Molecular biology reagents.**

| Reagent | Supplier |
|---|---|
| Deoxynucleotides (dNTP) Mix | Promega, USA |
| Ethanol (100 %) | Chem-Supply |
| Oligo-dT | Promega, USA |
| Omniscript reverse transcription (RT) Kit | QIAGEN |
| RNaseOUT™ Recombinant Ribonuclease Inhibitor | ThermoFisher Scientific, USA |
| Direct-zol™ RNA MicroPrep kit | Zymo Research, USA |
| TaqMan Universal PCR Master Mix | ThermoFisher Scientific, USA |
| TRIzol® Reagent | Gibco, BRL, Australia |

| | |
|---|---|
| UltraPure™ DNase/RNase-free distilled water | Invitrogen, USA |

### 2.1.6 Real-time Primers and Probes

**Table 2.8: Primers used for Taqman real-time PCR analysis.**

| Gene | Assay ID |
|---|---|
| *β2m* | Mm00437762_m1 |
| *Ccl4* | Mm00443111_m1 |
| *Ccl5* | Mm01302427_m1 |
| *Cd40* | Mm00441891_m1 |
| *Gapdh* | Mm99999915_g1 |
| *Hprt* | Mm00446968_m1 |
| *Il6* | Mm00446190_m1 |
| *Il12b* | Mm00434174_m1 |
| *Il15* | Mm00434210_m1 |
| *Il15ra* | Mm04336046_m1 |
| *Irf7* | Mm00516793_g1 |
| *Isg20* | Mm00469585_m1 |
| *Tnf* | Mm00443258_m1 |
| *Traf6* | Mm00493836_m1 |
| *18s* | Mm03928990_g1 |

All primer/probes were purchased from Life Technologies, Australia.

### 2.1.7 Cytometric Bead Array

**Table 2.9: Beads used for Cytometric Bead Array and the cytokines or chemokines targeted.**

| Cytokine/Chemokine targeted | Bead |
|---|---|
| Mouse Il-6 | Bead B4 |
| Mouse Il-12/Il-23p40 | Bead D7 |
| Mouse Mip-1β (Ccl4) | Bead C9 |
| Mouse Rantes (Ccl5) | Bead D8 |
| Mouse Tnf-α | Bead C8 |

All Flex Sets were purchased from BD™ Cytometric Bead Array, Australia. Each set contains capture beads, detection reagent and standards.

## 2.2 Methods

### 2.2.1 *In vitro* generation of dendritic cells

Murine bone marrow (BM)-derived DCs were generated *in vitro* from C57Bl/6, IFNARKO, IRF3KO or IRF7KO mice. Tibia and femur were cleaned and collected in Flt3 media: sterile KDS-RPMI medium supplemented with L-glutamine, streptomycin, 2-β-mercaptoethanol, benzylpenicillin, FCS. The bone marrow cells were flushed into fresh media using a 10 ml syringe with a 26 G needle and filtered through a 70 µm nylon mesh. Cells were centrifugated and resuspended with 1 ml of RBC lysis buffer (Sigma-Aldrich) per mouse for 60-90 seconds in order to lyse red blood cells. Following pelleting the cells were resuspended and cultured at $1.5 \times 10^6$ cells/ml of fresh media supplemented

with 150 ng/ml of Flt3L cytokine (BioXCell) for 8 days at 37 °C in 75 $cm^2$ TC flask (Naik et al. 2005).

### 2.2.2 Cell sorting and stimulation of eCD8$^+$ DCs

Following 8 days of Flt3L culture, BM precursors differentiate into myeloid cells that can be divided into pDCs and two cDCs subsets, cDC1 and cDC2. The cDC2 equivalents are identified by CD24$^{low}$ SIRPα$^{high}$ CD11b$^{high}$, whilst the cDC1 equivalents are identified by CD24$^{high}$ SIRPα$^{low}$ CD11b$^{low}$ and are also called eCD8$^+$ DCs. pDCs within the culture were uniquely identified as B220/CD45R$^+$. After 8 days of Flt3L culture, cells were harvested by collecting the supernatant from the culture flasks. Cells were then sorted on the basis of CD45R/B220, SIRPα, CD11c, CD11b, MHC II and CD24, using a FACS Aria$^{TM}$ III Cell Sorter. The dead cells were excluded by using 1 X Propidium iodide (PI) staining.

$0.25\times10^6$ CD45R/B220$^-$, SIRPα$^-$, CD11c$^+$, MHC II$^{int}$, CD11b$^{int}$ and CD24$^{hi}$ eCD8$^+$ DCs were used per condition of stimulation (Naik et al. 2010).

The eCD8$^+$ DCs were resuspended in Flt3 media at a concentration of $0.1\times10^6$ cells per condition for phenotypic analysis, $0.25\times10^6$ cells per condition for gene expression and cytokine/chemokine secretion analysis or a minimum of $2\times10^6$ cells per condition for proteomics and phosphoproteomics analysis. The eCD8$^+$ DCs were stimulated for various amounts of time, from 30 minutes to 8 hours, with IFN-αA (PBL), CpG (1668 InvivoGen), LPS (Difco, derived from *E. coli*) or poly(I:C) (InvivoGen) all at the indicated concentrations. These stimulations were conducted in the presence or absence of anti-CD40 monoclonal antibody (FGK45.5, Miltenyi Biotec) (α-CD40 antibody), or its isotype control (rat IgG2a, Miltenyi Biotec), at 10 µg/ml. All stimuli were prepared in Flt3 media. In the case of α-CD40 antibody coated plates. 96-well plates were coated with 50 µl of α-CD40 antibody at 50 µg/ml in sterile 1 X PBS and incubated overnight at 4°C. Wells were gently washed three times with 1 X PBS before use.

Moreover, various molecules have been used as specific inhibitors as described below. All the reagents were made up at the right concentration in Flt3 media. Stimulations were performed in 96 well plates, incubated at 37 °C for the time of the experiment. Following stimulation, samples were harvested, centrifugated and further processed depending on the analysis performed.

To investigate the involvement of TRAF6 in the CD40 signalling pathway, eCD8$^+$ DCs were cultured for 4 hours and treated for various amount of time with CD40-TRAF6 Signalling Inhibitor, Small Molecule Inhibitor 6877002 (3-((2,5dimethylphenyl)amino)-1-phenyl-2-propen-1-one, Calbiochem, Sigma-Aldrich), in the presence or absence of IFN-αA and/or α-CD40 antibody. All stimuli were prepared in Flt3 media and used at the indicated concentrations. Stock solutions of CD40-TRAF6 Signalling Inhibitor were reconstituted with DMSO at a concentration of 100 mM.

## 2.2.3 *In vitro* OT-II CD4$^+$ T cell – DC co-culture assay

### 2.2.3.1 CD4$^+$ T cell enrichment

CD4$^+$ T cells were enriched from naïve LNs of C57Bl/6 or OT-II transgenic female mice. Cell suspensions were prepared from LNs harvested in RPMI-10 through mechanical disruption and passaging through 70 µm mesh. Cells were resuspended in CD4$^+$ T cell negative enrichment antibody cocktail (anti-erythrocytes [Ter119], anti-I-A/E [M5/114], anti-CD8 [53-6.7], anti-Gr1 [RB6-8C5], anti-Mac-1 [M1/70], anti-F4/80 [F4/80]) for 30 minutes on ice. Cells were then washed and incubated with BioMag® sheep anti-rat IgG-coupled magnetic beads (QIAGEN) (used at 6:1 bead:cell ratio) in round bottom FACS tubes for 20 minutes on constant rotation at 4 °C. Tubes were loaded onto a DynaMag™-5 magnet (ThermoFisher Scientific) and enriched CD4$^+$ T cells were obtained via supernatant collection. Purity was determined via flow

cytometry by co-staining for anti-CD3, anti-CD4 and anti-Vα2 with an expected purity > 85 %.

#### 2.2.3.2 *In vitro* activation of $CD4^+$ T cells

6-well plate was coated with 600 µl of anti-CD3/CD28 cocktail at 5 µg/ml of each antibody in sterile 1 X PBS and incubated for 3 hours at 37 °C. Wells were gently washed three times with 1 X PBS. Enriched $CD4^+$ T cells in RPMI-10 and distributed into the coated wells at a concentration of 400,000 cells/ml, and incubated for various amount of times ranging from 6 to 48 hours. Following stimulation, cells were harvested, washed twice, enumerated and resuspended in FACS buffer for the assessment of CD40L up-regulation, or in Flt3 media at the indicated concentration for T cell-DC co-culture.

#### 2.2.3.3 T cell – DC co-culture assay

$eCD8^+$ DCs were pre-treated with IFN-αA for 1 hour (1000 U/ml, PBL). $0.5\times10^6$ enriched and *in vitro* activated $CD4^+$ T cells were resuspended with $0.25\times10^6$ $eCD8^+$ DCs (2 activated $CD4^+$ T cells to 1 $eCD8^+$ DC). The cells were also cultured with or without 1 µg/ml of OT-II specific peptide, OVA (323-339) (Mimotopes). Following 4 hours of co-culture the cell supernatant was collected for further analysis.

### 2.2.4 Quantitative real-time PCR

#### 2.2.4.1 RNA extraction

In order to analyse the gene regulation following various stimulations, the cell pellet was vigorously resuspended and lysed with TRIzol® (Life

Technologies) (400 µl for $0.25\times10^6$) for 5 minutes at RT. These samples were kept at -80 °C until further processing. For quantitative Real-Time Polymerase Chain Reaction (RT-PCR) analysis, RNA was extracted using Direct-zol™ RNA MicroPrep kit (Zymo Research) following manufacturer's instructions. Briefly, RNA from lysed cells with TRIzol® reagent was precipitated with 100 % ethanol, transferred into Zymo-Spin columns and centrifugated at 14,000 g, 10°C for 1:30 minute. Columns were then washed with 400 µl of RNA Wash buffer and centrifugated again before DNA digestion: 40 µl of DNase I cocktail (5 U of DNase I diluted in DNA Digestion Buffer (Zymo Research)) were loaded on each column and incubated for 15 minutes at 35 °C, followed by centrifugation for 1:30 minute. The columns were then sequentially washed 2 times with 400 µl of PreWash buffer and one time with 700 µl of RNA Wash buffer. The columns were centrifugated twice to entirely remove all remaining buffer. To elute the RNA, 15 µl of DNase/RNase-free water was added to the columns and incubated for 7 minutes at room temperature before centrifugation for 2 minutes. 15 µl were added, followed by another centrifugation. RNA was quantified via NanoDrop 2000.

#### 2.2.4.2 cDNA synthesis

The cDNA was synthetized from the extracted RNA with Omniscript RT kit for reverse transcription (QIAGEN) using oligo-dT primers (Promega) and RNaseOUT™ Recombinant Ribonuclease Inhibitor (ThermoFisher Scientific).

<u>cDNA synthesis Mix (final concentration per sample):</u>

3.5 µl 10 X Buffer RT

2 µl dNTP Mix (5 mM each dNTP)

1 µl Oligo$(dT)_{15}$ primer (0.5 µg/µl)

1 µl RNase inhibitor (RNaseOUT™) (10 U/µl)

1 µl Omniscript Reverse transcriptase (4 U/µl)

Total samples were < 2 µg and were entirely resuspended in 8.5 µl of cDNA synthesis Mix and incubated for 90 minutes at 37 °C. Finally, the cDNA was diluted in RNase/DNase-free water (finale concentration at 2.5 ng/µl) and used in RT-PCR.

#### 2.2.4.3 Real-time PCR

The real-time PCR (Polymerase Chain Reaction) was performed with Taqman Universal PCR Master Mix (Life Technologies) with primers for various genes of interest in a 10 µl reaction with the following cycle conditions: 95 °C for 2 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. The mRNA expression of these genes was normalized to at least 3 housekeeping genes such as *Gapdh*, *β2m*, *Hprt* or *18s* (ThermoFisher Scientific) and the relative expression (RE) was determined using unstimulated controls ($RE=2^{-(\Delta\Delta CT)}$).

PCR Master Mix (volume per reaction):

0.5 µl $H_2O$
5 µl TaqMan Universal PCR Master Mix
0.5 µl primer/probe (10 µM)
2 µl Template cDNA (2.5 µg/ml)

### 2.2.5 Cytometric Bead Array

Supernatants were harvested from the stimulated *in vitro* $eCD8^+$ DCs in order to assess the cytokines and chemokines released under stimulation, using cytometric beads array kits (CBA Flex Set assay, BD Bioscience) following manufacturer's instructions. 50 µl of cell supernatant was incubated with 50 µl of CBA bead populations in a 96 well plate for 2 hours at room temperature. 50 µl containing PE Detection was added to each well and

incubated for 1 hour at room temperature. Beads were washed with 100 µl of FACS buffer and centrifugated at 200 g, 4 °C for 5 minutes. Pelleted beads were resuspended with 200 µl of FACS buffer and assessed using a LSR Fortessa™. The cytokine and chemokine concentrations were determined relative to a standard curve. Samples were analysed using Flowjo.

### 2.2.6 Cell surface and intracellular stainings

eCD8$^+$ DCs were stained following stimulation with respective mix of cell surface fluorochrome-conjugated antibodies diluted in FACS buffer. Following 30 minutes of incubation at 4 °C, cells were washed with 100 µl of FACS buffer and resuspended with a solution of FACS buffer containing an enumerated number of blank calibration beads, and 1 X PI, to exclude non-viable cells in the case of extracellular staining only. When indicated, the measurement of an extracellular protein was compared to the same cells stained with the same fluorochrome-conjugated antibodies cocktail minus the one targeting the molecule of interest: Fluorescence Minus One (FMO).

A competition assay between the anti-CD40 stimulation antibody and the fluorescently labelled anti-CD40 was performed. Following 30 minutes to 8 hours of IFN-αA stimulation, eCD8$^+$ DCs were first stained for 20 minutes with α-CD40 monoclonal antibody FGK45. These cells were washed with 100 µl of FACS buffer and resuspended with a cocktail of fluorochrome-coupled antibodies containing the anti-CD40 (clone HM40-3) conjugated with FITC for phenotypic analysis. When necessary, cells were additionally stained intracellularly. Following antibody surface staining and the addition of a LIVE/DEAD™ fixable cell dye, the cells were fixed with BD Cytofix™ fixation Buffer (BD Bioscience). Cells were subsequently permeabilized and stained with BD Perm/Wash™ Buffer 1 X (BD Bioscience) containing the fluorochrome-conjugated antibody. All samples were assessed using the LSR Fortessa™ and results were analysed using Flowjo.

## 2.2.7 RNA sequencing handling

### 2.2.7.1 Data resources

Total mRNA was isolated from samples from 6 independent experiments and was used for RNA sequencing. Libraries were either prepared for HiSeq HT paired end (Illumina, Australian Genome Research Facility, Queensland, Australia) or for QuantSeq 3'-mRNA (Lexogen Inc., Vienna, Austria) sequencing and were generated from 100 ng total RNA by the Next Generation Sequencing Core Facility of the Institute of Human Genetics, Bonn University, Germany. Libraries were sequenced on a HiSeq2500 instrument (Illumina, Belgium) in a 50 bp single-read run. To enable a combined analysis, all raw fastq-files were aligned with Spliced Transcripts Alignment to Reference (STAR) software (v2.5.3a) against the murine genome mm10 independent of the sequencing run (**Figure 2.1**). The transcript quantification was performed on gene level in PartekFlow (v8.0.19.0707) with the E/M algorithm. The mm10 RefSeq Transcript 90 database version 2019-05-03 was chosen as annotation model. Read counts were obtained for 10,349 genes. Quantification of mRNA expression was performed as gene counts normalised as Counts per Million (CPM) in PartekFlow to ensure all samples were comparable. A batch correction of the independent sequencing runs was performed in PartekFlow to remove technical variations. The normalized gene expression values were imported into Partek Genomics Suite (PGS) software v7.18.0723. Genes that did not have mean expression values ≥1 CPM in at least one stimulatory condition were filtered out resulting in 10,222 present genes for further Analysis of Variance (ANOVA) analysis.

The handling of the RNA-Seq dataset was performed by and under Dr Susanne V. Schmidt's guidance.

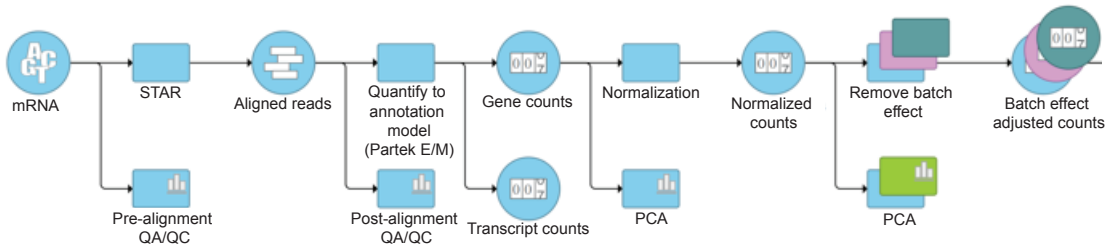



**Figure 2.1: Overview on the bioinformatics workflow for the combined analysis of RNA sequencing data.**
Raw files from three independent sequencing runs were aligned to the murine reference genome. Gene counts were quantified by the E/M algorithm and normalized as CPM (Count per Million) values. Technical variances were removed from the data set by batch effect correction. Data processing was performed in Partek Flow.

#### 2.2.7.2 Identification of differentially expressed genes and hierarchical clustering

Most variable and differentially expressed (DE) genes were identified by ANOVA in PGS according Benjamini & Hochberg. Genes with a fold change (FC) above or lower than |1.5| and a False Discovery Rate (FDR) adjusted p-value ≤ 0.05 were defined as DE between two test groups. Most variable genes were defined by a FDR-adjusted p-value ≤ 0.01.
Hierarchical clustering via Euclidean distance on gene expressions and samples dissimilarities were performed to visualize groups of DE genes or most variable genes with similar expression profiles through the conditions and time. Standardised expression values were shifted to mean zero and scaled to standard deviation of one.

Self-Organizing Map (SOM) clustering was performed on 341 DE genes (sum of the DE genes between IFN-αA 4 hours condition and all IFN-αA 4 hours with α-CD40 antibody conditions) in PGS. Expression values were z-transformed (z-scores). The DE genes were separated into 5 x 5 clusters according to similar expression patterns over the time of stimulation. In a SOM clustering, the expression values of each gene are scaled into one value representing the general expression value of the cluster it belongs to. Therefore, the clusters are influenced by the input data. The SOM clustering is visualized as a heatmap: red represents increased values; blue decreased values and green intermediate values.

Pathway enrichment analyses were performed in PGS, using default settings and providing enrichment scores and enrichment p-values (t-test), information derived from KEGG database (http://www.genome.jp/kegg/). This analysis was used on DE genes lists to highlight their involvement in biological pathways.

Gene Ontology (GO) enrichment analysis was performed in PGS, using default settings and providing enrichment scores and enrichment p-values (t-test), information derived from the Gene Ontology Consortium integrated resources (http://geneontology.org/docs/go-consortium/). This analysis was used on DE genes to group them into functional hierarchy. When mentioned, genes were compared to the Interferome v2.01 (http://interferome.org/interferome/home.jspx) (Rusinova et al. 2013).

Cystoscape software v3.5.0 was used to visualize the BiNGO, and transcription factor (TF) networks and predict master regulators of the help signal (https://cytoscape.org/). The basis structure of TF networks is based on the coexpression of the displayed TFs in the data set. TF network were generated in Biolayout Express 3D v3.3. Using a Pearson correlation value of 0.83 resulted in a network structure encompassing 505 TFs. The iRegulon plugin v1.3 for Cytoscape (Janky et al. 2014) was used to predict an identify TF candidates, which could participate in the differential expression of gene groups being regulated by the combinatorial stimulus of IFN-αA + α-CD40 and by IFN-αA stimulation at several time points of cellular programming. The BiNGO plugin v3.0.3 and the EnrichmentMap plugin v3.2.1 were used for functional enrichment analysis of groups of genes identified by SOM clustering (Maere et al. 2005) described above. The hypergeometric test was used for GO enrichment analysis with Benjamini & Hochberg FDR corrected p-value ≤ 0.1. WordCloud plugin v3.1.3 was used to visualize the most frequent annotation associated with a cluster of GO-terms (Oesper et al. 2011).

Search Tool for the Retrieval of Interacting Genes (STRING) database was used online (https://string-db.org/, (Szklarczyk et al. 2015)) to visualize known connections between proteins based on literature co-citation, experimental validation, databases, co-expression and co-occurrence. Using the default settings and a medium confidence interaction score (0.400), this analysis was used on protein identified via phosphosite in phosphoproteome

data set to analyse protein-protein interaction network and GO-Term enrichment.

## 2.2.8 Proteomics and phosphoproteomics analysis

The processing of samples dedicated for phosphoproteomics analysis as well as the analysis of results were performed by Dr Nichollas Scott from Department of Microbiology and Immunology at the Peter Doherty Institute.

### 2.2.8.1 Cell lysis and protein digestion

Cell lysis was performed as previously described (Humphrey et al. 2015). Following stimulation, cells were pooled, centrifugated and cell pellets were resuspended in guanidinium chloride (GdmCl) lysis buffer (6 M GdmCl, 100 mM Tris pH 8.5, 10 mM tris(2-carboxyethyl)phosphine (TCEP), 40 mM 2-choloacetamide (CAA)) and transferred into non-autoclaved Eppendorf Tubes®. Samples were then heated for 10 minutes at 95 °C shaking at 2000 rpm before being stored at -20 °C until all samples were collected for further processing. Protein amounts were quantified using a colorimetric BCA assay following the manufacturer's instructions. A minimum of 2 mg of protein per sample was used to perform further analysis. Samples were precipitated with cycles of acetone washes. Dried protein pellets denatured and reduced in 6 M urea, 2 M thiourea, 40 mM $NH_4HCO_3$ before being digested overnight with Lys-C and trypsin as previously described (Scott et al. 2011). Following digestion, samples were acidified with 0.5 % formic acid and desalted with tC18 Sep-Pak. 1 % of the samples are collected for proteome level input assessment.

#### 2.2.8.2 Pre-TiOx enrichment and TiOx enrichment

Pre-TiOx enrichment and TiOx enrichment were performed according to published methods from Engholm-Keller K. and Larsen M. R. (Engholm-Keller and Larsen 2016). Briefly, TiOx (titanium dioxide) resin was used to enrich the phosphorylated peptides. Following cycles of washes, phosphorylated peptides were eluted in TiOx elution buffer, dried and exposed to another round of TiOx enrichment using regenerated resin. Phosphorylated peptides were eluted again and R3/C18 clean-up for Mass Spectrometry analysis. This protocol is based on work performed by Rappsilber J. et al in 2003 and 2007 (Rappsilber et al. 2003; Rappsilber et al. 2007). Eluted phosphorylated peptides were desalted using Empire C18 tips with OLIGO R3 reverse phase resin.

#### 2.2.8.3 Identification of proteins and phosphorylated peptides

Phosphopeptides and total proteome were analysed using Liquid Chromatography-Mass Spectrometry. More precisely, samples were infused into an Orbitrap Fusion Lumos Tribid Mass Spectrometer from Bio21 at the University of Melbourne, Australia MaxQuant software (v1.5.3.1) was used for identification of proteins and phosphorylated peptides (Cox and Mann 2008). Annotation was performed against mouse proteome database (Uniprot proteome ID UP000000589 – Mus musculus, downloaded 18-05-2016). Outputs were processed with Perseus software (v1.4.0.6). Phosphosites were detected with a localization score > 0.75. Significant phosphorylation/dephosphorylation events between two comparable groups of condition were identified with a FDR (Benjamini & Hochberg) adjusted p-value ≤ 0.05 and a FC in abundance above or lower than |1.5| (Tyanova et al. 2016).

### 2.2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software. P-value, determined by the appropriate test as indicated in the figures legend, was considered as significant when $p \leq 0.05$.

# Chapter 3

*In vitro* characterization of the help signal

## 3.1 Introduction

Pathogen clearance requires the priming of naïve $CD8^+$ T cells. This complex process is induced upon TCR-mediated recognition of their specific antigen presented via MHC I molecules on the surface of a professional APC such as a DC (Williams and Bevan 2007). Precisely, $CD8^+$ DCs and their unique cross-presenting ability are critical in immune responses against some infections (Joffre et al. 2012). Danger signals derived from the pathogens themselves and associated tissue damage can activate DCs leading to their maturation and improvement of their functions. Mature DCs up-regulate co-stimulatory molecules and enhance their ability to form functional peptide-MHC complexes. In addition, DCs are critical producers of cytokines and chemokines in response to microbial challenge. Therefore, mature DCs become competent in $CD8^+$ T cell priming (Banchereau et al. 2000; Mellman and Steinman 2001; Dalod et al. 2014). One of the danger signals leading to DC maturation is type I IFN. This group of cytokines promote anti-viral mechanisms and can be produced by virtually all cell types in response to viral infection (Gallucci et al. 1999; Hensley et al. 2005; Simmons et al. 2012; Ivashkiv and Donlin 2014).

In addition to an innate stimulus, DCs often require signals provided by helper $CD4^+$ T cells that are mediated through the interaction between CD40 receptor on the surface of the DC and CD40 ligand (CD40L) on the surface of the $CD4^+$ T cell. In response to these signals DCs become competent for subsequent $CD8^+$ T cell priming (Ridge et al. 1998). $CD8^+$ T cells undergo clonal expansion and differentiation into effector cells. This is the primary response to infection. Once the pathogen is cleared, a few cognate T cells remain, the memory cells and respond more efficiently in case of a secondary exposure to the same antigen. (Williams and Bevan 2007). Interestingly, while $CD4^+$ T cells promote efficient memory $CD8^+$ T cell responses (Shedlock and Shen 2003; Sun and Bevan 2003), there is still much debate as to the relative importance of help during the generation of primary $CD8^+$ T cell responses against some infections. Early work of Jennings et al. focused on the effect of

$CD4^+$ T cell depletion in the context of HSV infection. They used a CD4-specific monoclonal antibody (mAb) GK1.5 treatment which eliminates $CD4^+$ T cells without impacting the pool of $CD8^+$ T cells, highlighting the essential role of $CD4^+$ T cells in promoting $CD8^+$ T cell cytolytic functions during the primary response to HSV infection (Jennings et al. 1991). In HSV-1 infected $CD4^+$ T cell-deficient mice, and MHC II deficient mice, unable to promote cognate activation of $CD4^+$ T cells, the $CD8^+$ T cell priming is impaired (Smith et al. 2004; Rajasagi et al. 2009). Likewise the survival of activated antigen-specific $CD8^+$ T cells during the primary response to VACV infection also depends on the presence of $CD4^+$ T cells (Wiesel et al. 2010). Altogether, these studies linked the requirement of cognate interactions between DC and helper $CD4^+$ T cells to enhance the DCs ability to induce CTL priming and memory. However, the dependence on $CD4^+$ T cell help appears not to be a general principle as several infections induce efficient $CD8^+$ T cell primary responses without requiring T cell help. This is the case for instance of LCMV or VSV infections (Wiesel and Oxenius 2012).

The disparate requirement for $CD4^+$ T cell help in generating $CD8^+$ T cell responses has been linked to the strength of the initial inflammatory response. Many infections provide strong inflammatory and danger signals inducing $CD4^+$ T cell-independent responses. However, in other cases, the infection or tissue damage associated with an antigenic challenge leads to more moderate levels of danger signal and this is where help appears to be more consistently required for efficient priming of $CD8^+$ T cells (Bevan 2004). Such danger signals can be type I IFNs, cytokines produced by most cells during virus infection providing a good local source of stimuli (Randall and Goodbourn 2008). Interestingly, the helper requirement following immunization of mice with OVA coated splenocytes could be counteracted by injecting IFN-α, which induced a strong $CD8^+$ T cell responses even in $CD4^+$ T cell-deficient mice (Le Bon et al. 2003). Moreover, during helper-dependent HSV infection, type I IFN has been shown to not be required for the direct stimulation of the antigen-specific $CD8^+$ T cells themselves but rather involved in DC stimulation. In response, DCs

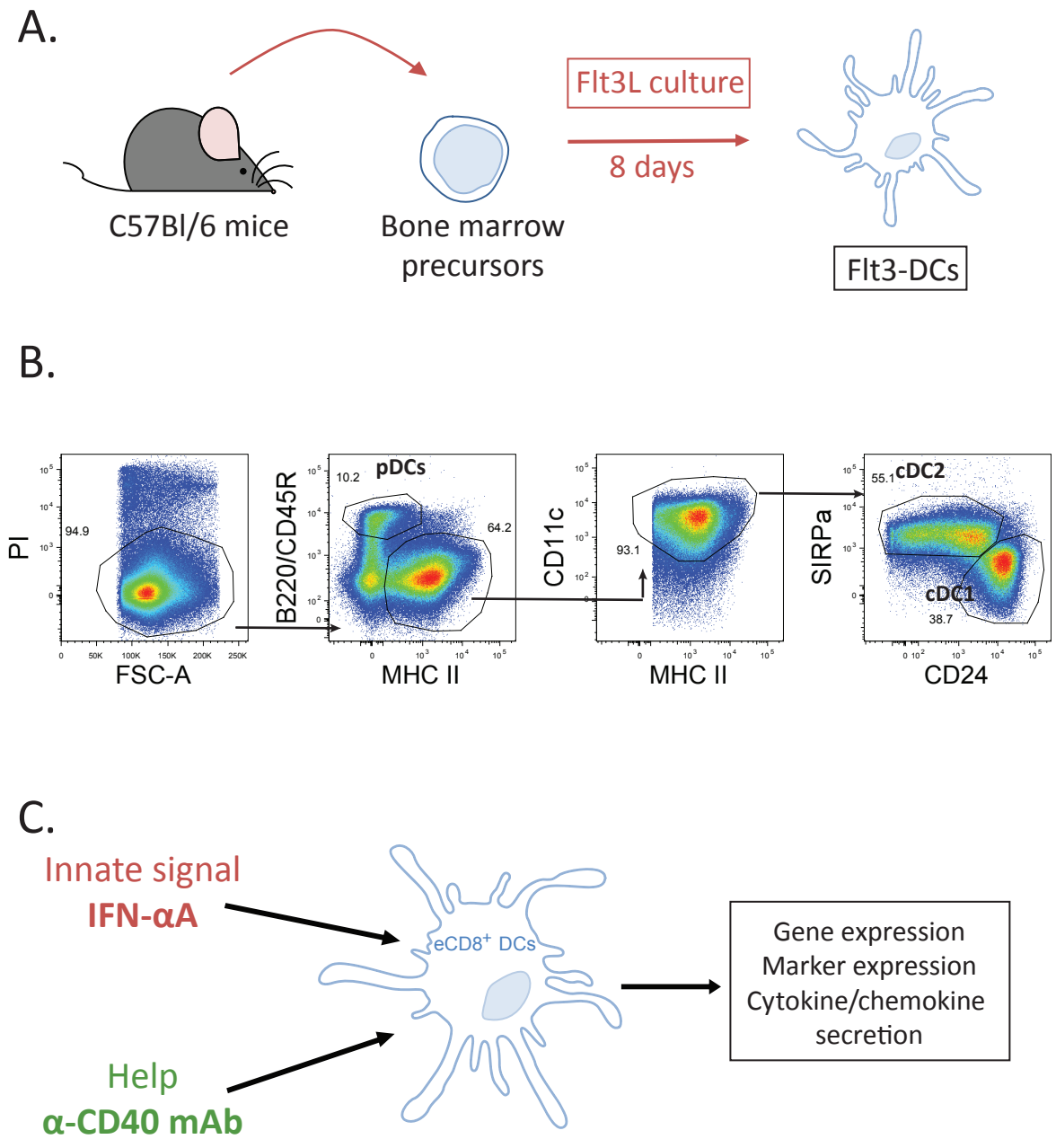



**Figure 3.1: Differentiation, sort and *in vitro* stimulation of bone marrow derived equivalent of CD8⁺ DCs.**
**(A.)** Bone marrow precursors from C57Bl/6 mice were cultured for 8 days in the presence of the growth factor FMS-like tyrosine kinase 3 ligand (Flt3L). **(B.)** The equivalent of CD8⁺ DCs (eCD8⁺ DCs) were sorted within the living cells (PI-) based on the expression of CD11c⁺ MHC II⁺ CD24⁺. **(C.)** eCD8⁺ DCs were stimulated with recombinant murine IFN-αA, with anti-CD40 monoclonal antibody (α-CD40 mAb) or with IFN-αA and α-CD40 mAb together.

secrete cytokines and chemokines critical for CTL priming. Importantly this work highlighted the synergy of $CD4^+$ T cell help with the initial type I IFN signal, together leading to the amplification of IL-15 production by the DCs (Greyer et al. 2016). Thus, $CD4^+$ T cell help through CD40L-CD40 interaction promotes the improvement of DC priming ability. However, the underlying mechanisms and dynamics of the synergy of help signal and innate stimuli for the efficient $CD8^+$ T cell responses are unclear.

The aim of the following chapter was to establish a model that would allow the study of mechanisms contributing to the help-driven amplification of innate signals. $CD8^+$ DCs being specialised on CTL cross-priming, their equivalent (equivalent of $CD8^+$ DCs, $eCD8^+$ DCs) were generated *in vitro* from bone marrow (BM) precursors cultured in the presence of Flt3L. Despite differences in their extracellular molecules expression, DCs generated in the presence of this cytokine show functional and phenotypic characteristics equivalent to the steady-state DCs (Naik et al. 2007; Naik 2010). This methods gives rise to sufficient numbers of cells to precisely characterize and examine the CD40-driven amplification of various innate responses *in vitro* using a monoclonal antibody anti-CD40 (α-CD40 mAb), known to mimic the “help” signal (Greyer et al. 2016).

## 3.2 Results

### 3.2.1 Survival and activation profiles of $eCD8^+$ DCs

BM cells cultured for 8 days in the presence of Flt3L, ligand of the receptor *fms*-like tyrosine kinase 3 (Flt3), resulted in the generation of myeloid cells (**Figure 3.1.A**). The myeloid cells could be identified by unique expression of surface markers and separated into pDCs and two cDCs subsets (cDC1 and cDC2 splenic equivalents). As shown in Figure 3.1.B, $CD24^{low}$ $SIRP\alpha^{high}$

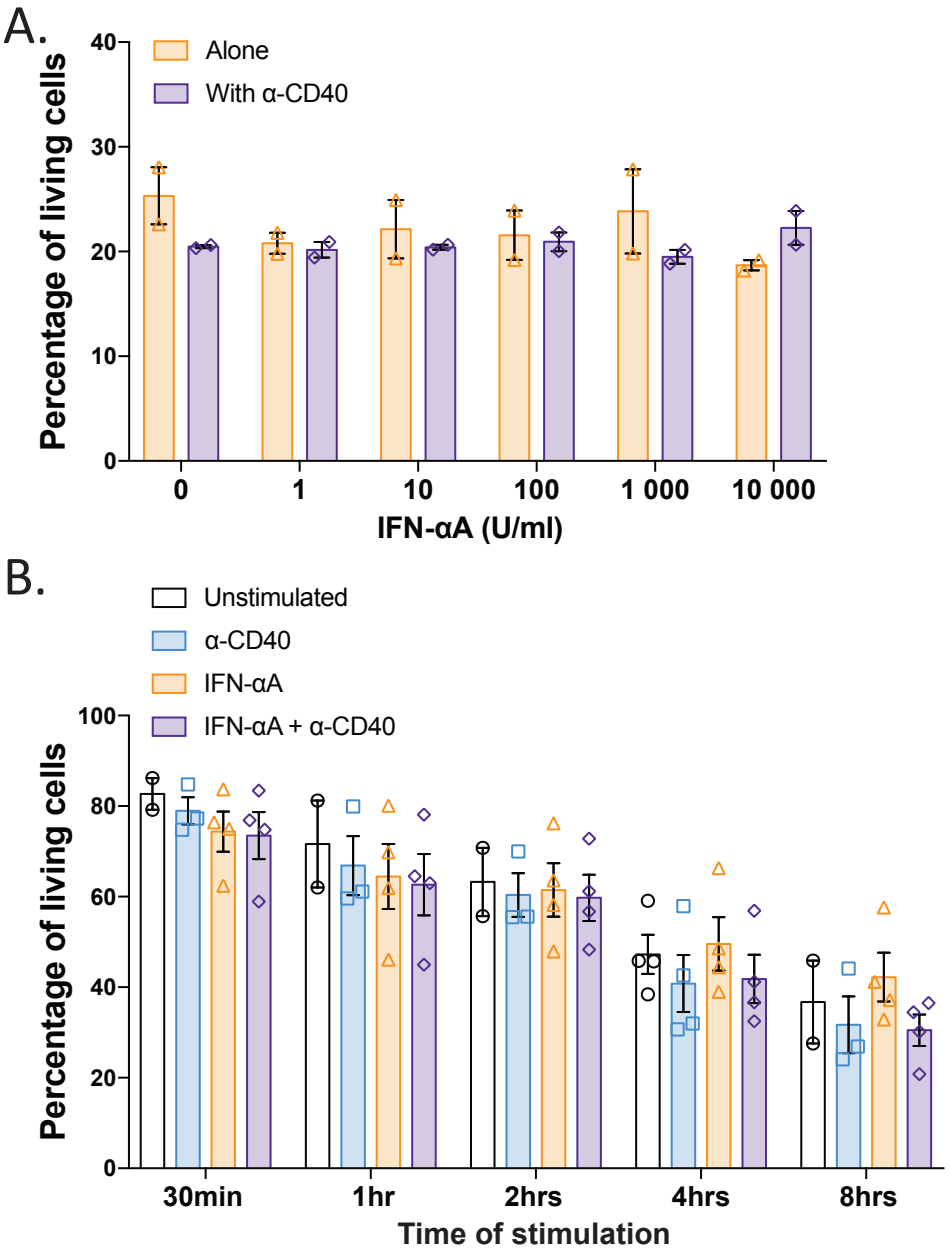



**Figure 3.2: Survival of eCD8⁺ DCs following IFN-αA dose response and following time course of stimulations from 30 minutes to 8 hours.**
**(A.)** Percentage of living eCD8⁺ DCs following 4 hours of stimulation with escalating concentrations of IFN-αA (from 1 to 10 000 U/ml), in the presence (purple) or absence (orange) of α-CD40 mAb (Pool of 2 independent experiments).
**(B.)** Percentage of living eCD8⁺ DCs measured following time course from 30 minutes to 8 hours of stimulation with media only (Unstimulated, white), α-CD40 mAb (10 µg/ml) (α-CD40, blue), IFN-αA (1000 U/ml) (IFNαA, orange) or IFN-αA (1000 U/ml) with α-CD40 mAb (10 µg/ml) (IFN-αA + α-CD40, purple) (Pool from 2 to 4 independent experiments). Error bars represent mean +/- SEM.

CD11b$^{high}$, equivalents of cDC2 and CD24$^{high}$ SIRPα$^{low}$ CD11b$^{low}$ equivalents of cDC1, also called eCD8$^{+}$ DCs, can be distinguished. The third DC subtype generated is the pDCs identified as B220/CD45R$^{+}$. Using propidium iodide (PI) to exclude dead/dying cells, eCD8$^{+}$ DCs were sorted from Flt3L cultures on day 8 on the basis of MHC II$^{+}$ CD11c$^{+}$ CD24$^{+}$ and SIRPα$^{-}$ (**Figure 3.1.B**).

IFN-αA was used to mimic endogenous type I IFN in the activation of eCD8$^{+}$ DCs (Le Bon et al. 2003). In order to investigate the help-driven amplification of IFN-α/β-initiated innate pathways, eCD8$^{+}$ DCs were additionally stimulated with an anti-CD40 monoclonal antibody (FGK45.5, α-CD40 antibody) described as mimicking T cell help (Bennett et al. 1998; Schoenberger et al. 1998; Feau et al. 2011) (**Figure 3.1.C**). eCD8$^{+}$ DCs survival and phenotypic changes in response to stimuli were assessed: the cells were stimulated for 4 hours with increasing doses of IFN-αA in the presence or absence of α-CD40 antibody (used at a concentration determined by previous work conducted by Dr Marie Greyer (Greyer et al. 2016)) (**Figure 3.2.A**). The proportion of living cells was determined using PI exclusion and flow cytometric analysis following stimulation. Compared to the unstimulated cells, IFN-αA-stimulated eCD8$^{+}$ DCs showed a constant proportion of living cells across the range of 1 U/ml to 10,000 U/ml of IFN-αA. Treatment of cells for 4 hours with α-CD40 antibody alone showed no difference in cell survival compared to unstimulated cells. Moreover, addition of α-CD40 antibody to cells treated with IFN-αA didn't induce any significant decrease or increase in the proportion of living cells. To investigate the effect of these various stimuli on the cell survival over time, eCD8$^{+}$ DCs were treated with α-CD40 antibody or IFN-αA alone or with the combination of these two stimuli from 30 minutes to 8 hours of stimulation following sort (**Figure 3.2.B**). A control group of eCD8$^{+}$ DCs were cultured unstimulated and harvested at each time point to allow comparison between pre- and post- stimulation and culture alone-induced effects. Following 30 minutes of culture at 37 °C without stimulus, there was only a minimal 20 % loss of cell viability. These unstimulated cells showed a progressive loss of cell viability with only 40 % of living cells at 8 hours of culture post sort. Therefore,

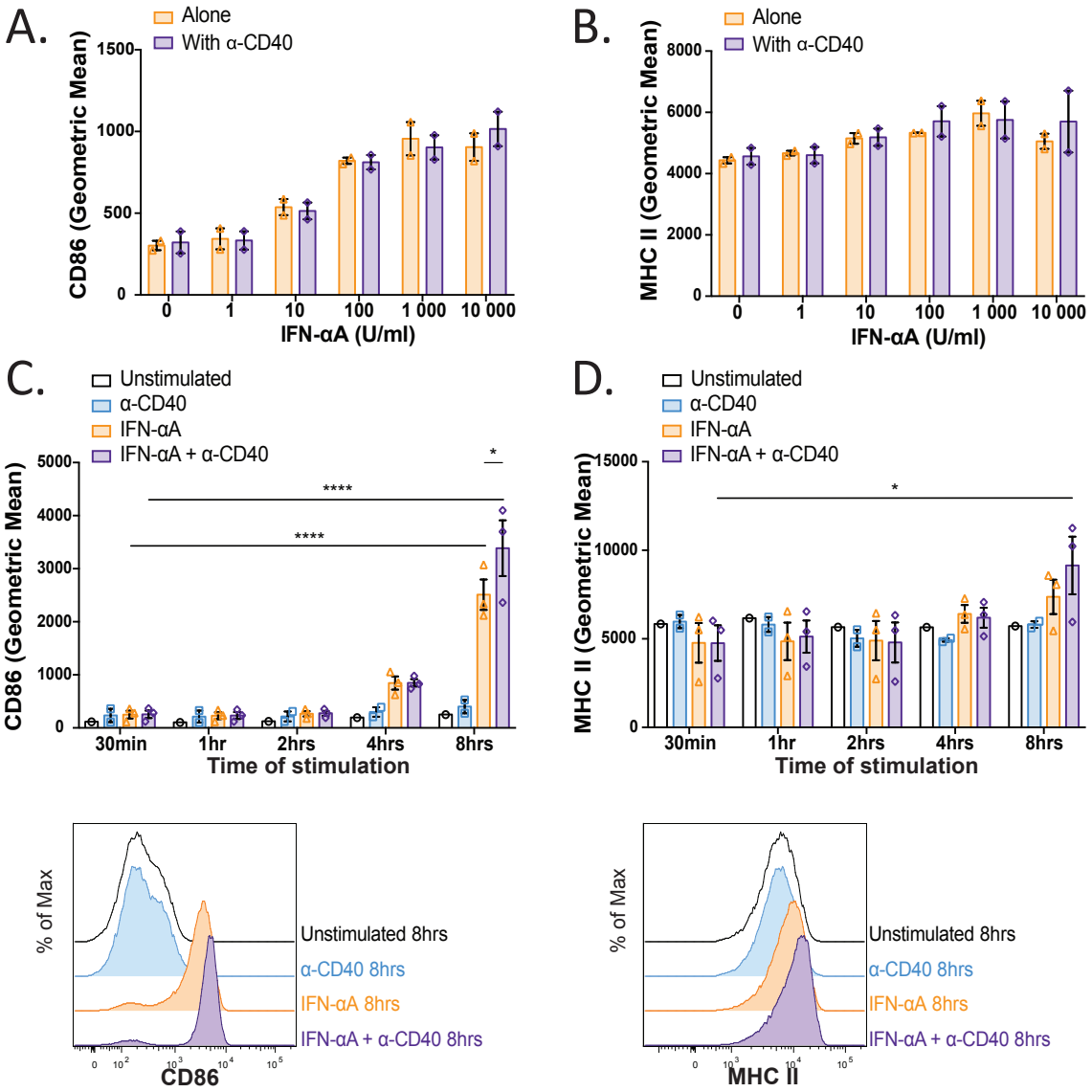



**Figure 3.3: Up-regulation of surface and activation markers on IFN-αA stimulated eCD8⁺ DCs.**

Geometric Mean of CD86 **(A.)** or of MHC II **(B.)** expression measured on eCD8⁺ DCs stimulated with escalating concentrations of IFN-αA, in the presence (purple) or absence (orange) of α-CD40 mAb (Pool from 2 independent experiments).

Geometric Mean (upper part) and representative histogram (lower part) of CD86 **(C.)** or of MHC II **(D.)** expression measured on eCD8⁺ DCs stimulated from 30 minutes to 8 hours with media only (Unstimulated, white), α-CD40 mAb (10 µg/ml) (α-CD40, blue), IFN-αA (1000 U/ml) (IFN-αA, orange) or IFN-αA (1000 U/ml) with α-CD40 mAb (10 µg/ml) (IFN-αA + α-CD40, purple) (Pool from 2 to 4 independent experiments).

Error bars represent mean +/- SEM. Asterisk indicates statistically significant differences between IFN-αA condition and IFN-αA + α-CD40 condition, or between IFN-αA condition or IFN-αA + α-CD40 condition, each time point being compared to the expression at 30 minutes of the corresponding stimulation as assessed by two-way ANOVA; adjusted p-value: * $p \leq 0.0252$; **** $p < 0.0001$.

no culture were performed for longer than 8 hours. However, the survival rate of unstimulated cells was comparable to cells treated with α-CD40 antibody. Similarly, cell survival following various IFN-αA doses with or without the addition of α-CD40 antibody showed no difference compared to unstimulated cells. Precisely, eCD8$^+$ DCs stimulated with α-CD40 antibody and IFN-αA for only 30 minutes survived at 80% while the ones stimulated for 8 hours reached 40 % of living cells. Therefore, while progressive cell death was observed following the sort of eCD8$^+$ DCs, none of the stimulations applied to those cells increased nor decreased the proportion of cell death induced by the culture conditions.

To assess how the different stimuli and their combination affect the activation of eCD8$^+$ DCs, typical DC activation markers were measured. CD86, CD83, CD40 or MHC class II molecules are expressed at relatively low levels in immature DCs, and increase substantially following activation (Mellman and Steinman 2001). Following 4 hours of stimulation with a low dose of IFN-αA (1 U/ml), eCD8$^+$ DCs expressed the same level of maturation marker CD86 than unstimulated cells (**Figure 3.3.A**). However, escalating doses of type I IFN increased CD86 expression on their surface in a dose dependent manner. Reaching a peak at the IFN-αA concentration of 1000 U/ml, this expression was twofold higher than unstimulated cells. Stimulated cells showed a slight up-regulation of MHC II expression, which similarly reached a maximum of surface expression with 1000 U/ml of IFN-αA (**Figure 3.3.B**). The addition of α-CD40 antibody treatment to the IFN-αA stimulation had no influence on the expression of these two maturation markers (**Figure 3.3.A** and **B**). To ensure this activated state would last over longer stimulation, sorted eCD8$^+$ DCs were cultured from 30 minutes to 8 hours under the four different conditions: no stimulation, stimulation with α-CD40 antibody only or IFN-αA only and stimulation with the combination of both. Following 8 hours of culture with media only CD86 (**Figure 3.3.C**) and MHC II (**Figure 3.3.D**) expressions were not increased on the cell surface. Although CD40 ligation has been shown to induce phenotypic changes on DCs including the up-regulation of CD86 (van Kooten and Banchereau

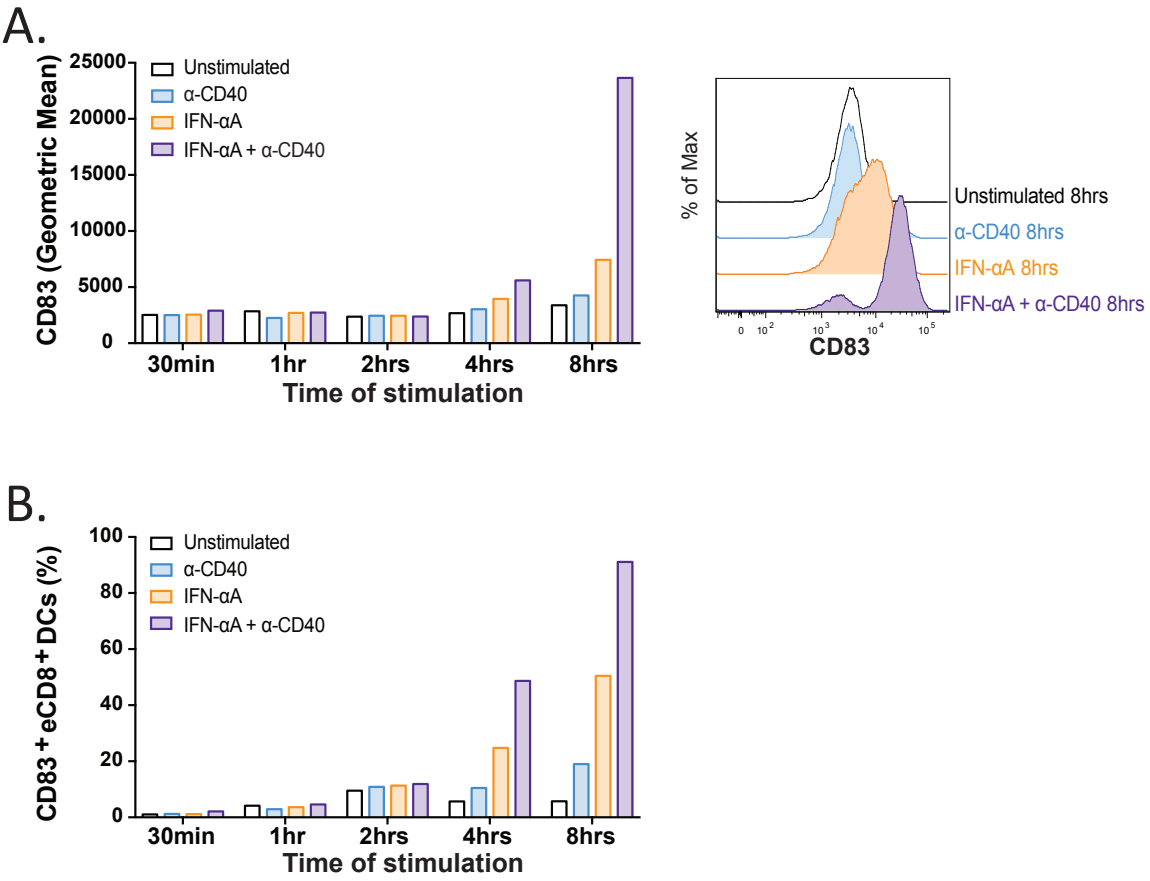
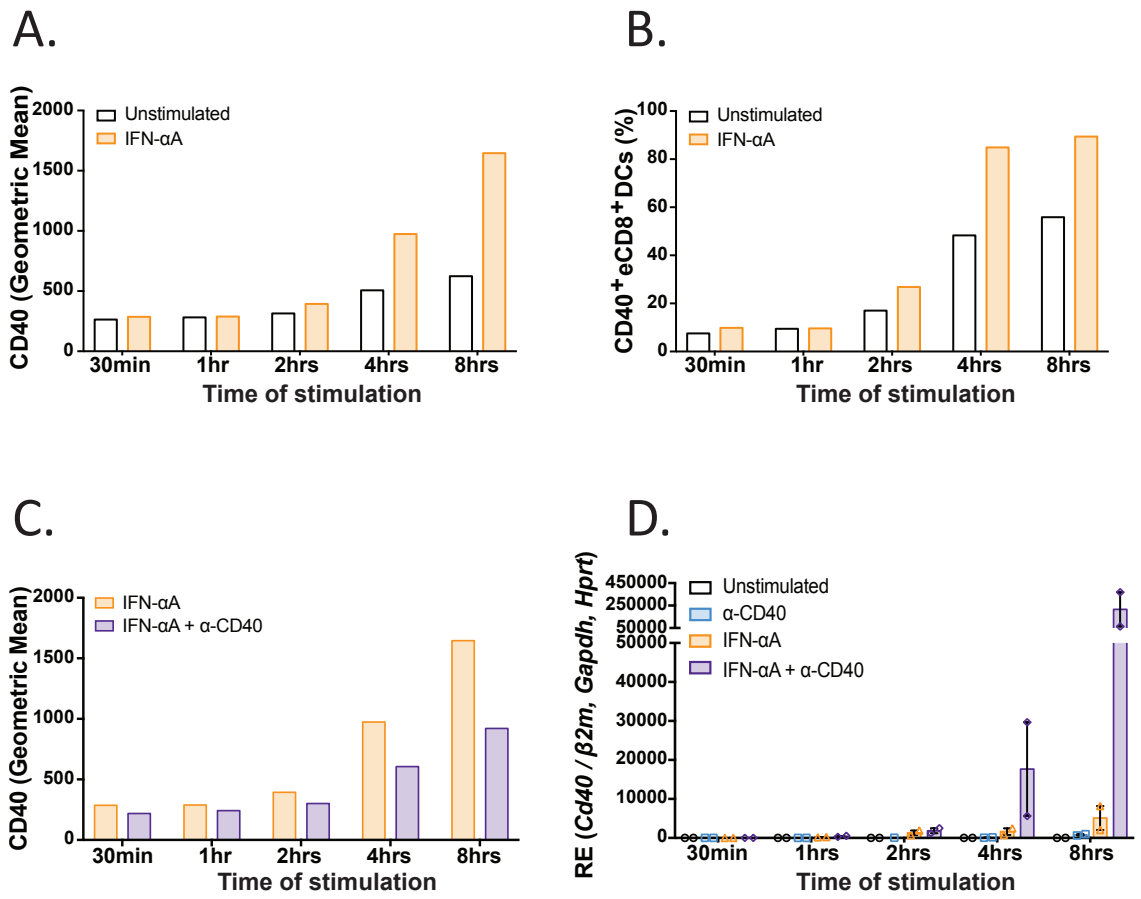



**Figure 3.4: Up-regulation of CD83 on IFN-αA stimulated eCD8⁺ DCs.**
Geometric Mean (left part) and histogram (right part) of eCD8⁺ DCs CD83 expression **(A.)** or percentage of CD83⁺ eCD8⁺ DCs **(B.)** measured following time course from 30 minutes to 8 hours of stimulation with media only (Unstimulated, white), α-CD40 mAb (10 µg/ml) (α-CD40, blue), IFN-αA (1000 U/ml) (IFN-αA, orange) or IFN-αA (1000 U/ml) with α-CD40 mAb (10 µg/ml) (IFN-αA + α-CD40, purple) (n=1).

2000), α-CD40 stimulated cells did not up-regulate CD86 or MHC II (**Figure 3.3.C** and **D**). IFN-αA stimulation strongly increased CD86 expression by 10-fold (**Figure 3.3.C**). Finally, a slightly higher expression of CD86 and MHC II markers was observed from 8 hours of stimulation with both IFN-αA and α-CD40 antibody (**Figure 3.3.C** and **D**). CD83 is another marker up-regulated by DCs in response to activation and has been correlated with functional T cell activation (Aerts Toegaert et al. 2007). While 8 hours of IFN-αA stimulation alone induced a moderate up-regulation of CD83 surface expression, the addition of α-CD40 antibody treatment led to a clear increase of the Geometric Mean Fluorescence of this marker, reaching more than 20,000 with the combination of these two stimuli, 5-times higher than under the unstimulated condition (**Figure 3.4.A**). Likewise, the percentage of $eCD8^+$ DCs positive for this maturation marker progressively increased from 4 hours of IFN-αA stimulation (**Figure 3.4.B**). The α-CD40 antibody treatment in addition enhanced the proportion of $CD83^+$ cells to 90 % compared to 50 % with 8 hours of IFN-αA stimulation alone.

CD40, the receptor for CD40L (or CD154), involved in the transmission of the help signal (Ridge et al. 1998; Schoenberger et al. 1998) can be up-regulated following DC maturation (O'Sullivan and Thomas 2003). While without any stimulation CD40 expression was not increased, $eCD8^+$ DCs treated with IFN-αA up-regulated CD40 from 4 hours of stimulation (**Figure 3.5.A**). Moreover, the percentage of cells expressing CD40 also increased over time: only 10 % of $eCD8^+$ $CD40^+$ DCs at 30 minutes of IFN-αA stimulation while more than 80 % expressed this marker after 4 hours of stimulation. Interestingly, we also observed an increase of the CD40 positive cells percentage under no stimulation, from 10 % at the start of the culture to 50 % of $eCD8^+$ DCs positive for CD40 from 4 hours post-sort (**Figure 3.5.B**). To assess if α-CD40 antibody treatment could, as for IFN-αA stimulation, lead to an increase of CD40 surface expression, a competition assay was performed. The α-CD40 antibody used for stimulation may mask the CD40 receptor from a fluorochrome conjugated antibody used for its staining. Thus, $eCD8^+$ DCs were stimulated over a time




**<u>Figure 3.5</u>: CD40 up-regulation on IFN-αA stimulated eCD8⁺ DCs.**
Geometric Mean of eCD8⁺ DCs CD40 expression **(A.)** or percentage of CD40⁺ eCD8⁺ DCs **(B.)** measured following time course from 30 minutes to 8 hours of stimulation with media only (Unstimulated, white) or IFN-αA (1000 U/ml) (IFN-αA, orange) (n=1).
**(C.)** Competition assay. Geometric Mean of eCD8⁺ DCs CD40 expression measured following time course from 30 minutes to 8 hours of 1000 U/ml IFN-αA stimulation with (purple) or without (orange) pre-staining with α-CD40 mAb (n=1).
**(D.)** eCD8⁺ DCs regulation of *Cd40* assessed following time course from 30 minutes to 8 hours of stimulation with media only (Unstimulated, white), α-CD40 mAb (10 µg/ml) (α-CD40, blue), IFN-αA (1000 U/ml) (IFN-αA, orange) or IFN-αA (1000 U/ml) with α-CD40 mAb (10 µg/ml) (IFN-αA + α-CD40, purple) (Pool from 2 independent experiments).

course with IFN-αA, at 1000 U/ml, dose previously shown to induce CD40 up-regulation (**Figure 3.5.A**). Following stimulation, each sample was split in two with some cells directly stained using the fluorescently conjugated α-CD40 antibody and the other cells first incubated with α-CD40 antibody mimicking the help for 30 minutes, and then stained with the fluorescently conjugated α-CD40 antibody (**Figure 3.5.C**). While the two sets of samples were subject to the same IFN-αA stimulation, cells that had been pre-incubated with the α-CD40 antibody used for the treatment were lower in CD40 surface expression, suggesting that the binding of fluorescently conjugated α-CD40 antibody is occluded. The efficiency of the α-CD40 antibody binding to the CD40 receptor therefore prevented the precise assessment of a potential α-CD40-driven up-regulation of CD40 receptor on the surface of DCs. Nonetheless, α-CD40 antibody treatment on its own did not induce *Cd40* expression at the mRNA level. However, α-CD40 antibody treatment in combination with IFN-αA triggered a 250,000-fold increase in *Cd40* mRNA expression compared to unstimulated control (**Figure 3.5.D**).

With MHC II, CD86, CD83 and CD40 all up-regulated from 4 hours of culture with IFN-αA, sorted eCD8$^+$ DCs phenotypically responded to stimulation. Moreover, eCD8$^+$ DCs reached maturation when stimulated with IFN-αA from a concentration of 1000 U/ml. This concentration was therefore used for the subsequent functional experiments. In order to ensure that the experimental conditions did not trigger activation of the cells, an unstimulated condition for each time point performed, was included as a control of the inactivated state of the cells. In summary, these results support the use of BM-derived eCD8$^+$ DCs as an *in vitro* system to study DCs functional response to stimuli.

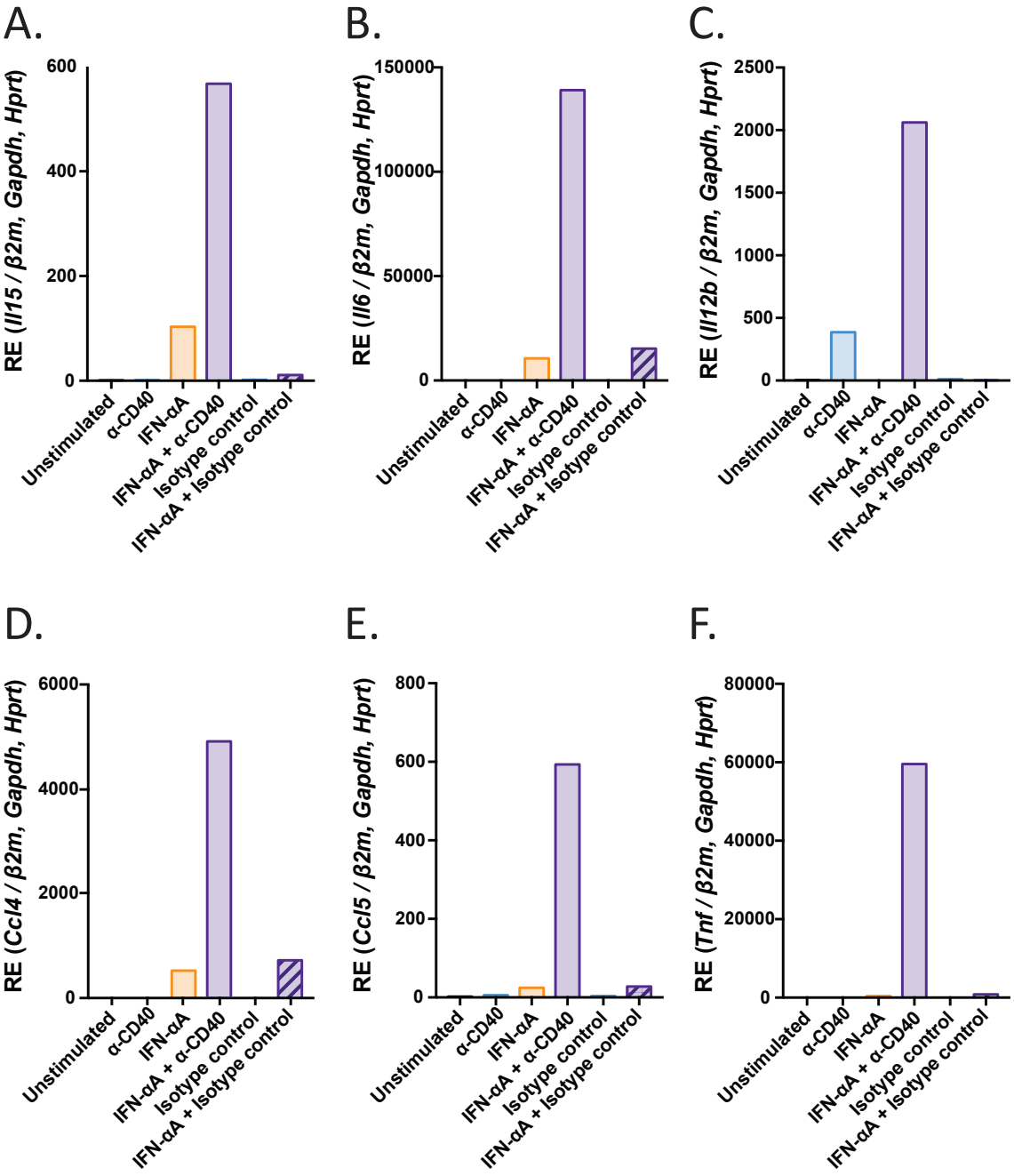
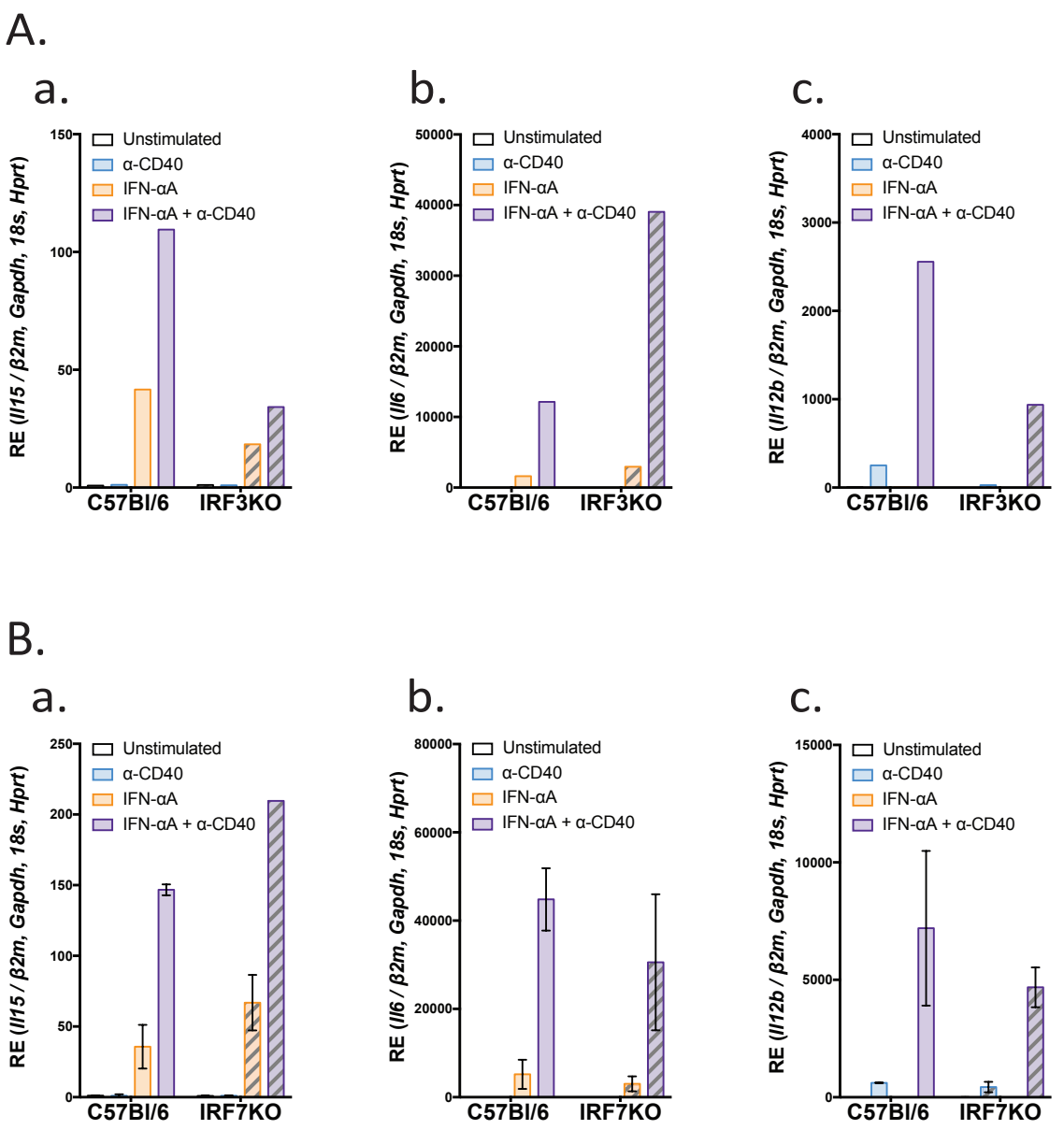



**Figure 3.6: Amplification of cytokine and chemokine gene expression triggered by the stimulation of CD40 on eCD8⁺ DCs.**
eCD8⁺ DCs cultured for 4 hours with media only (Unstimulated, white), α-CD40 mAb (10 µg/ml) (α-CD40, blue), IFN-αA (1000 U/ml) (IFN-αA, orange), IFN-αA (1000 U/ml) with α-CD40 mAb (10 µg/ml) (IFN-αA + α-CD40, purple), rat IgG2a (10 µg/ml) (Isotype control, hatched blue) or IFN-αA (1000 U/ml) with rat IgG2a (10 µg/ml) (IFN-αA + Isotype control, hatched purple). Following stimulation, the regulation of *Il15* **(A.)**, *Il6* **(B.)**, *Il12b* **(C.)**, *Ccl4* **(D.)**, *Ccl5* **(E.)**, *Tnf* **(F.)** was assessed (n=1).

### 3.2.2 CD40-driven amplification of IFN-αA-initiated cytokine and chemokine expression

In order to characterize the CD40-driven amplification of the responses induced by this IFN-αA stimulation, eCD8$^+$ DCs gene regulation was assessed following 4 hours under various conditions (**Figure 3.6**). Without any stimulation, none of the various cytokines and chemokines measured was expressed at the mRNA level. Stimulation with IFN-αA alone induced the expression of different cytokines, such as *Il15* (**Figure 3.6.A**) or *Il6* (**Figure 3.6.B**) but not of the cytokine *Il12b* (**Figure 3.6.C**). The three chemokines measured, *Ccl3* (data not shown), *Ccl4* (**Figure 3.6.D**) and *Ccl5* (**Figure 3.6.E**) were also all induced by IFN-αA stimulation. Interestingly, mRNA expression of *Il12b,* a sub-unit of IL-12 cytokine (Henry et al. 2008), was promoted by α-CD40 antibody treatment on its own (**Figure 3.6.C**), in accordance with previous observations (Gately et al. 1998; Schulz et al. 2000). Importantly, the mRNA expression of these cytokines and chemokines was highly induced when α-CD40 antibody treatment was added to the IFN-αA stimulation reaching an expression level at least 4-times higher than the one triggered by IFN-αA alone. This illustrates an amplification of IFN-αA-mediated expression triggered by the stimulation of CD40 receptor. Remarkably, while IFN-αA stimulation alone induced an expression of *Tnf* that was almost undetectable, the addition of α-CD40 antibody enhanced its expression to a level 60,000-times higher than the unstimulated control (**Figure 3.6.F**). *Il12b* mRNA expression could be measured as well following IFN-αA and α-CD40 antibody combined stimulation and was visibly higher than the expression induced under the stimulation of CD40 receptor alone (**Figure 3.5.C**). In order to confirm that these results illustrated the outcome of a specific CD40 stimulation, eCD8$^+$ DCs were stimulated with the isotype control of the α-CD40 monoclonal antibody at the same concentration and under the same experimental conditions. None of the cytokines and chemokines measured could be detected in the presence of the isotype control alone. When the cells were stimulated with the combination of IFN-αA and the isotype control, the detected mRNA expression reached a level




**<u>Figure 3.7</u>: α-CD40-driven amplification of IFN-I-induced cytokine expression independent of IRF3 and IRF7 transcription factors.**
C57Bl/6 (control), IRF3KO **(A.)** or IRF7KO **(B.)** eCD8$^{+}$ DCs cultured for 4 hours with media only (Unstimulated, white), α-CD40 mAb (10 µg/ml) (α-CD40, blue), IFN-αA 4 hours (1000 U/ml) (IFN-αA, orange) or IFN-αA 4 hours (1000 U/ml) with α-CD40 mAb 4 hours (10 µg/ml) (IFN-αA + α-CD40, purple). Following stimulation, the regulation of *Il15* **(a.)**, *Il6* **(b.)** and *Il12b* **(c.)** was assessed (n=1 or pool from 2 independent experiments). Error bars represent mean +/- SEM.

comparable to the one induced by IFN-αA stimulation alone showing that the specific stimulation of CD40 is required to trigger the amplification of the IFN-αA-initiated responses (**Figure 3.6**). In summary, IFN-αA stimulation promotes eCD8$^+$ DCs cytokine and chemokine responses. The *in vitro* amplification of these responses requires the specific stimulation of CD40 receptor.

One possible explanation of this enhancement of the expression of IFN-αA-triggered genes is that the stimulation of CD40 receptor could lead to the production of IFN-α/β, which would then generate a positive feedback loop, essentially increasing the level of IFN-αA stimulation in an autocrine manner. Type I IFN responses can be induced via the activation of the transcription factors IRF3 or IRF7 (Marié et al. 1998). Accordingly, these two transcription factors could potentially be involved in the amplification of IFN-αA-induced responses. Stimuli responses from eCD8$^+$ DCs derived from C57Bl/6 mice and eCD8$^+$ DCs deficient for Irf3 expression were compared (**Figure 3.7.A**). While the level of *Il15* (**Figure 3.7.A.a**), *Il6* (**Figure 3.7.A.b**) and *Il12b* (**Figure 3.7.A.c**) mRNA expression were different from the control C57Bl/6 cells, Irf3 deficiency did not alter the α-CD40-triggered amplification of IFN-αA-induced responses. Similarly, the stimulation of Irf7$^{-/-}$ eCD8$^+$ DCs with the combination of IFN-αA and α-CD40 antibody for 4 hours induced a clear amplification of IFN-αA-triggered *Il15* (**Figure 3.7.B.a**) and *Il6* (**Figure 3.7.B.b**) mRNA production as well as *Il12b* expression (**Figure 3.7.B.c**). In order to inhibit the potential feedback loop of IFN-α/β signalling, a blocking antibody against the IFN receptor 1 (IFNAR1), MAR1-5A3 was used. IFNAR1 blockade instantly inhibited type I IFN pathway as the simultaneous addition of αIFNAR1 and IFN-αA to eCD8$^+$ DCs for 4 hours entirely prevented the IFN-αA-driven mRNA expression of various cytokines and chemokines such as *Il15* (**Figure 3.8.A.a**), *Il6* (**Figure 3.8.A.b**), and *Tnf* (**Figure 3.8.A.c**). By decreasing the duration of αIFNAR1 treatment, the initiation of IFN-αA responses became slightly detectable. While eCD8$^+$ DCs were stimulated for 4 hours with IFN-αA stimulation, type I IFN recognition was blocked for the last 3 hours, the last 2 hours, or for the last hour only. In this last case, when the cells were stimulated with IFN-αA for 3 hours

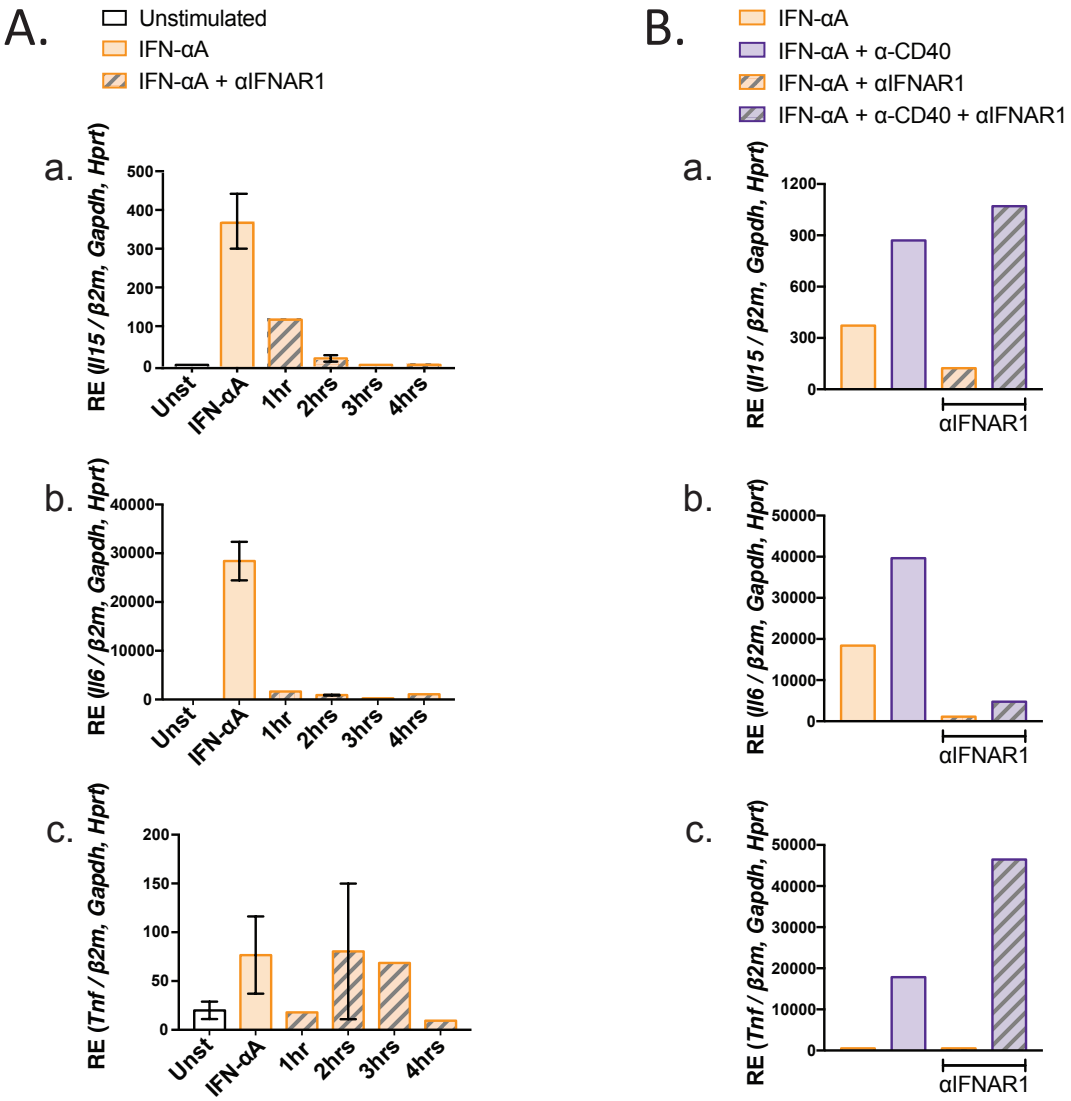



**Figure 3.8: IFN-I feedback stimulation not required for α-CD40-driven amplification of IFN-αA-induced responses.**

**(A.)** Efficiency of a blocking monoclonal antibody specific for IFN-α/β receptor subunit 1 MAR1-5A3 (αIFNAR1) to block IFN-αA-induced gene regulation. eCD8⁺ DCs were cultured with media only (Unst or Unstimulated, white), IFN-αA 4 hours (1000 U/ml) (IFN-αA, orange) or IFN-αA 4 hours (1000 U/ml) with anti-IFNAR1 (10 μg/ml) (IFN-αA + αIFNAR1, grey striped orange) either for the entire time of the experiment, either for the last 3, 2 or 1 hour(s) (1 to 2 independent experiments pooled for each time point). Error bars represent mean +/- SEM.

**(B.)** IFN-αA-independent amplification of IFN-αA-induced responses. eCD8⁺ DCs were cultured with IFN-αA 4 hours (1000 U/ml) (IFN-αA, orange), IFN-αA 4 hours (1000 U/ml) with α-CD40 mAb 1 hour (10 μg/ml) (IFN-αA + α-CD40, purple), IFN-αA 4 hours (1000 U/ml) with anti-IFNAR1 1 hour (10 μg/ml) (IFN-αA + αIFNAR1, grey striped orange), or IFN-αA 4 hours (1000 U/ml) with α-CD40 mAb 1 hour (10 μg/ml) and anti-IFNAR1 1 hour (10 μg/ml) (IFN-αA + α-CD40 + αIFNAR1, grey striped purple) (n=1, representative of 2 independent experiments).

Following stimulation, the regulation of *Il15* **(a.)**, *Il6* **(b.)**, *Tnf* **(c.)** was assessed.

and its recognition blocked for the last hour of culture, a slight expression of the three cytokines could be observed (**Figure 3.8.A**). Therefore, the αIFNAR1 treatment was able to efficiently block the IFN-αA-induced pathway and the cells required at least 2 to 3 hours of stimulation to induce their cytokine gene expressions. The mRNA expression induced after 4 hours of IFN-αA with IFNAR1 blocking for the last hour, was lower than 4 hours of IFN-αA stimulation only (**Figure 3.8.A** and **B**). eCD8$^+$ DCs were again stimulated for 4 hours with IFN-αA and received αIFNAR1 treatment for the last hour of culture. Knowing that the blocking effect of this antibody was instantaneous, the α-CD40 antibody was quickly added to the cells following the start of the IFNAR1 blocking. Despite this blocking, the α-CD40 antibody treatment induced the amplification of the expression of all three cytokines (**Figure 3.8.B**). In some cases such as for *Il15* (**Figure 3.8.B.a**) and *Tnf* (**Figure 3.8.B.c**), the expression induced when IFN-αA recognition was blocked was comparable and even higher than the α-CD40-mediated amplification of IFN-αA-induced expression without any IFNAR1 blocking. The amplification triggered via CD40 stimulation while the receptor for type I IFN was blocked showed that the gene expression enhancement is not due to an autocrine effect of IFN-α/β produced by DCs. Therefore, CD40 stimulation in the context of a danger signal such as IFN-αA, induces an enhancement of the DCs responses that relies on another mechanism than the simple intensification of IFN-α/β production itself.

The results so far depict the *in vitro* induction of α-CD40-driven amplification of IFN-αA-induced mRNA expression. This amplification depends on the specific stimulation of CD40 receptor on DCs and leads to the enhancement of various cytokines and chemokines expression without requiring an autocrine effect of IFN-α/β.

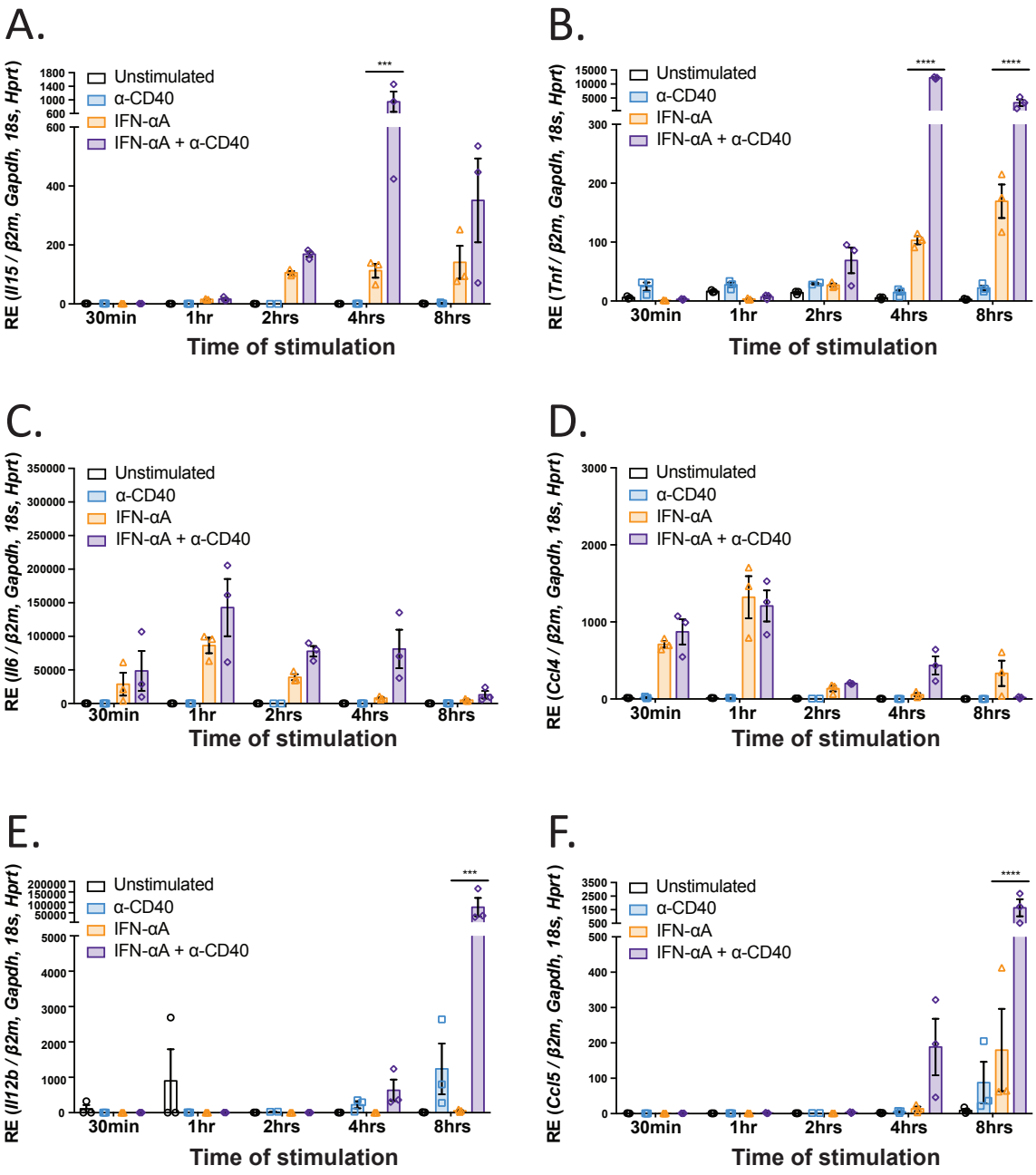


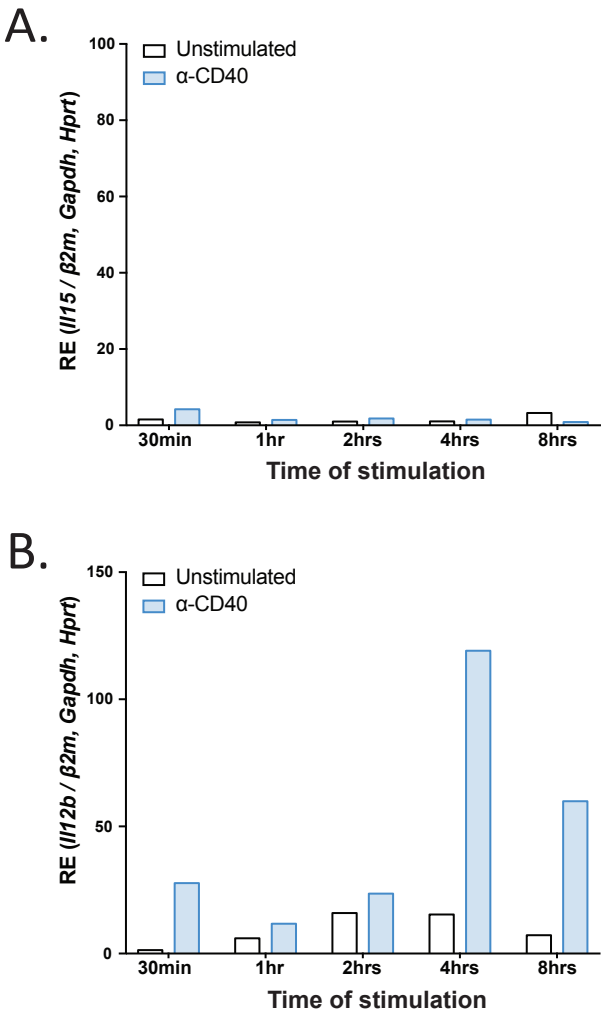

**Figure 3.9: Time course of α-CD40-driven amplification of IFN-αA-induced cytokine and chemokine gene expression.**

eCD8⁺ DCs cultured for time course from 30 minutes to 8 hours with media only (Unstimulated, white), α-CD40 mAb (10 μg/ml) (α-CD40, blue), IFN-αA (1000 U/ml) (IFN-αA, orange) or IFN-αA (1000 U/ml) with α-CD40 mAb (10 μg/ml) (IFN-α A + α-CD40, purple).

Following stimulation, the regulation of *Il15* **(A.)**, *Tnf* **(B.)**, *Il6* **(C.)**, *Ccl4* **(D.)**, *Il12b* **(E.)**, *Ccl5* **(F.)** was assessed (Pool from 2 to 3 independent experiments). Error bars represent mean +/- SEM. Asterisks indicate statistically significant differences between IFN-αA condition and IFN-αA + α-CD40 condition as assessed by two-way ANOVA; adjusted p-value: *** p≤0.0002; **** p<0.0001.

### 3.2.3 Kinetics of CD40 signal requirement in the context of an IFN-αA-driven activation

BM-derived eCD8$^+$ DCs can be used effectively *in vitro* to investigate the CD40-driven amplification of IFN-αA-induced signals. So far, mRNA assessment was performed following 4 hours of stimulation and discrepancies between the expression of the different cytokines could be observed. For instance, *Tnf* was almost not detectable under IFN-αA stimulation (**Figure 3.6.F.**) while *Il12b* was not induced at all under this condition and was measured under α-CD40 antibody treatment alone (**Figure 3.6.C.**). Such differences could be due to the precise time point of 4 hours chosen with all cytokines and chemokines having the potential to respond the same way to stimulation but following different kinetics in response to a danger signal. To investigate this possibility, time course experiments were performed by stimulating eCD8$^+$ DCs from 30 minutes to 8 hours with IFN-αA alone, with α-CD40 antibody alone or with the combination of both of these stimuli. As previously, the gene expression of cytokines and chemokines was assessed. Interestingly, each cytokine and chemokine showed specific dynamic patterns of help-driven amplification (**Figure 3.9**). Expression of *Il15* was induced after 2 hours of IFN-αA stimulation while the addition of α-CD40 antibody showed a peak of significant amplification occurring at 4 hours of stimulation (**Figure 3.9.A**). The same trend was observed for *Tnf* with a noticeable high mRNA expression under IFN-αA and α-CD40 antibody combined stimulation (**Figure 3.9.B**). Other genes where induced earlier, such as *Il6*, with a peak of α-CD40 antibody-mediated amplification occurring after 1 hour of stimulation and a tempered amplification maintained throughout the time course (**Figure 3.9.C**). A similar pattern was observed for *Ccl3* (data not shown) and *Ccl4* mRNA measurement (**Figure 3.9.D**). Both of these chemokines showed an early peak of gene expression occurring at 1 hour after IFN-αA stimulation. The early expression of these genes was unaffected by the addition of α-CD40 antibody. However, a second later peak occurred after 4 hours of stimulation indicating a late CD40-mediated amplification profile. Finally, other genes such as *Il12b*




**<u>Figure 3.10</u>: α-CD40-driven amplification of *Il12b* independently of IFN-I signal.**
IFNARKO eCD8⁺ DCs cultured for time course from 30 minutes to 8 hours with media only (Unstimulated, white) or α-CD40 mAb (10 μg/ml) (α-CD40, blue). Following stimulation, the regulation of *Il15* **(A.)** and *Il12b* **(B.)** was assessed (n=1).

(**Figure 3.9.E**) and *Ccl5* (**Figure 3.9.F**) were induced later during the time course, with a peak of significant CD40-induced amplification occurring at 8 hours compared to the mRNA expression induced by IFN-αA only. Again, anti-CD40 stimulation on its own led to a slight *Il12b* expression from 4 hours but this was clearly enhanced when stimulation was combined with IFN-αA for 8 hours. To confirm that this *Il12b* expression was independent of type I IFN signalling pathway, eCD8$^{+}$ DCs derived from the BM of IFNAR KO mice that lack the expression of IFN-I receptor were stimulated over a similar time course. As expected, without type I IFN signalling, the expression of most of the genes, such as *Il15* didn't show any expression pattern (**Figure 3.10.A**). However, *Il12b* was expressed in a type I IFN-independent manner following α-CD40 antibody treatment (**Figure 3.10.B**).

At a protein level, the cytokines and chemokines measured followed two main patterns of secretion. Cytokines and chemokines such as Il-12p40 (**Figure 3.11.A**), Ccl5 (**Figure 3.11.B**) and Tnf-α (**Figure 3.11.C**) were not detectable in the supernatant of the cells stimulated with IFN-αA stimulation alone. Upon combined treatment these levels were highly and significantly secreted, reaching concentration of about 1500 pg/ml, 350 pg/ml and 130 pg/ml respectively. For those three proteins, the secretion occurred at 8 hours of stimulation. By contrast, Il-6 (**Figure 3.11.D**) and Ccl4 (**Figure 3.11.E**) were detectable in the supernatant following IFN-αA stimulation alone, with both proteins secretion amplified from 4 hours with the addition of α-CD40 antibody. Ccl4 reached a concentration of 150 pg/ml in the supernatant of the cells stimulated with IFN-αA and α-CD40 antibody compared to 100 pg/ml from the cells stimulated with IFN-αA only.

The cytokine IL-15 is a transpresented cytokine, meaning that the DCs secrete IL-15 and express at their surface the α-chain of the receptor (IL-15Rα) as well. When secreted, IL-15 interacts with IL-15Rα and is presented to the two other chains of the receptor, β and γ at the surface of the target cells (**Figure 3.12.A**) (Jabri and Abadie 2015). Therefore, its measurement as a

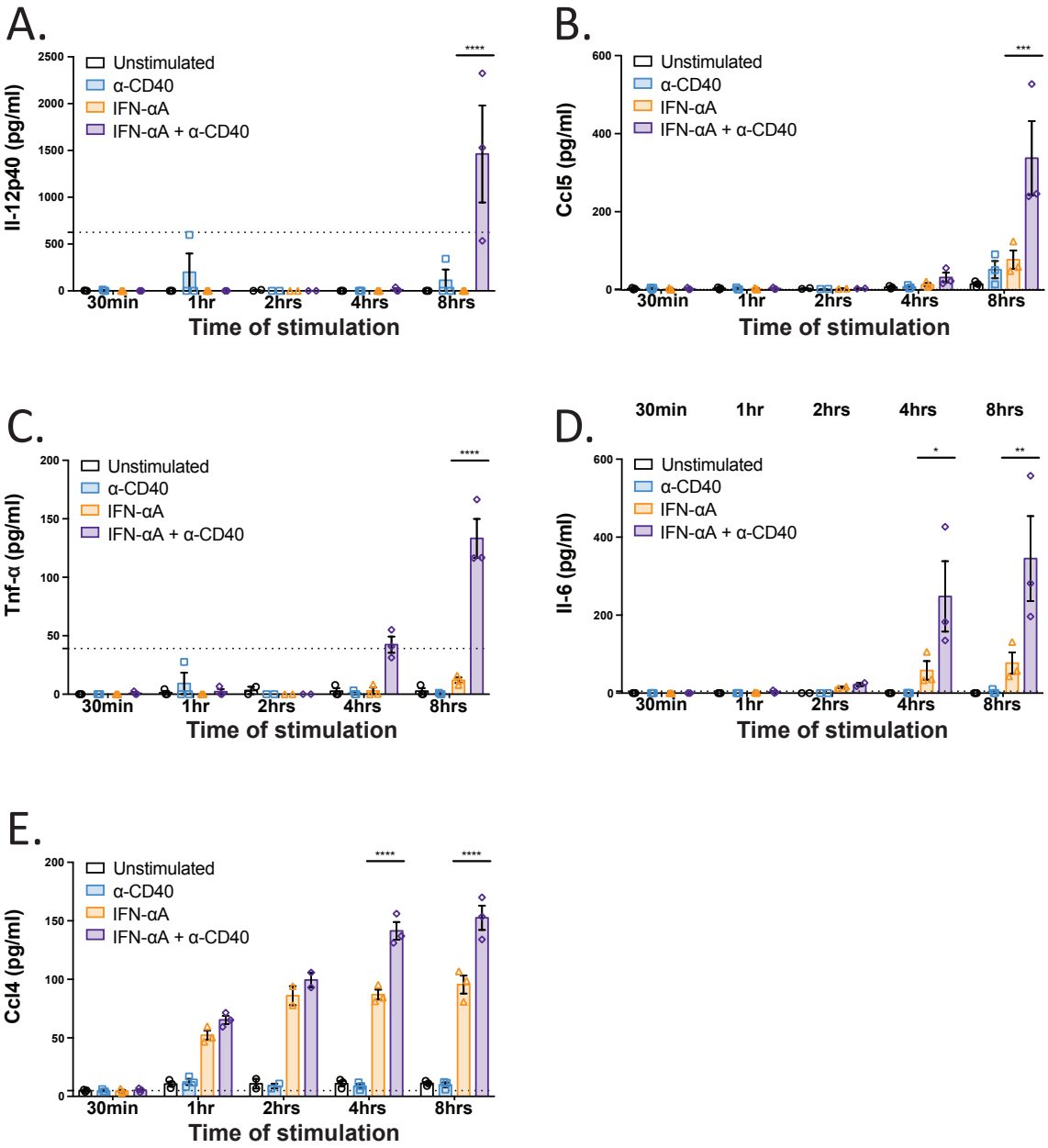



**Figure 3.11: Time course α-CD40-driven amplification of IFN-αA-induced cytokine and chemokine secretion.**
eCD8⁺ DCs cultured for time course from 30 minutes to 8 hours with media only (Unstimulated, white), α-CD40 mAb (10 µg/ml) (α-CD40, blue), IFN-αA (1000 U/ml) (IFN-αA, orange) or IFN-αA (1000 U/ml) with α-CD40 mAb (10 µg/ml) (IFN-α A + α-CD40, purple). Following stimulation, the secretion of Il-12p40 **(A.)**, Ccl5 **(B.)**, Tnf-α **(C.)**, Il-6 **(D.)**, Ccl4 **(E.)** was assessed (Pool from 2 to 3 independent experiments for each time point). Dotted line indicates the limit of detection of each cytokine/chemokine for the assay performed. Error bars represent mean +/- SEM. Asterisks indicate statistically significant differences between IFN-αA condition and IFN-αA + α-CD40 condition as assessed by two-way ANOVA; adjusted p-value: * p≤0.0332; ** p≤0.0021; *** p≤0.0002; **** p<0.0001.

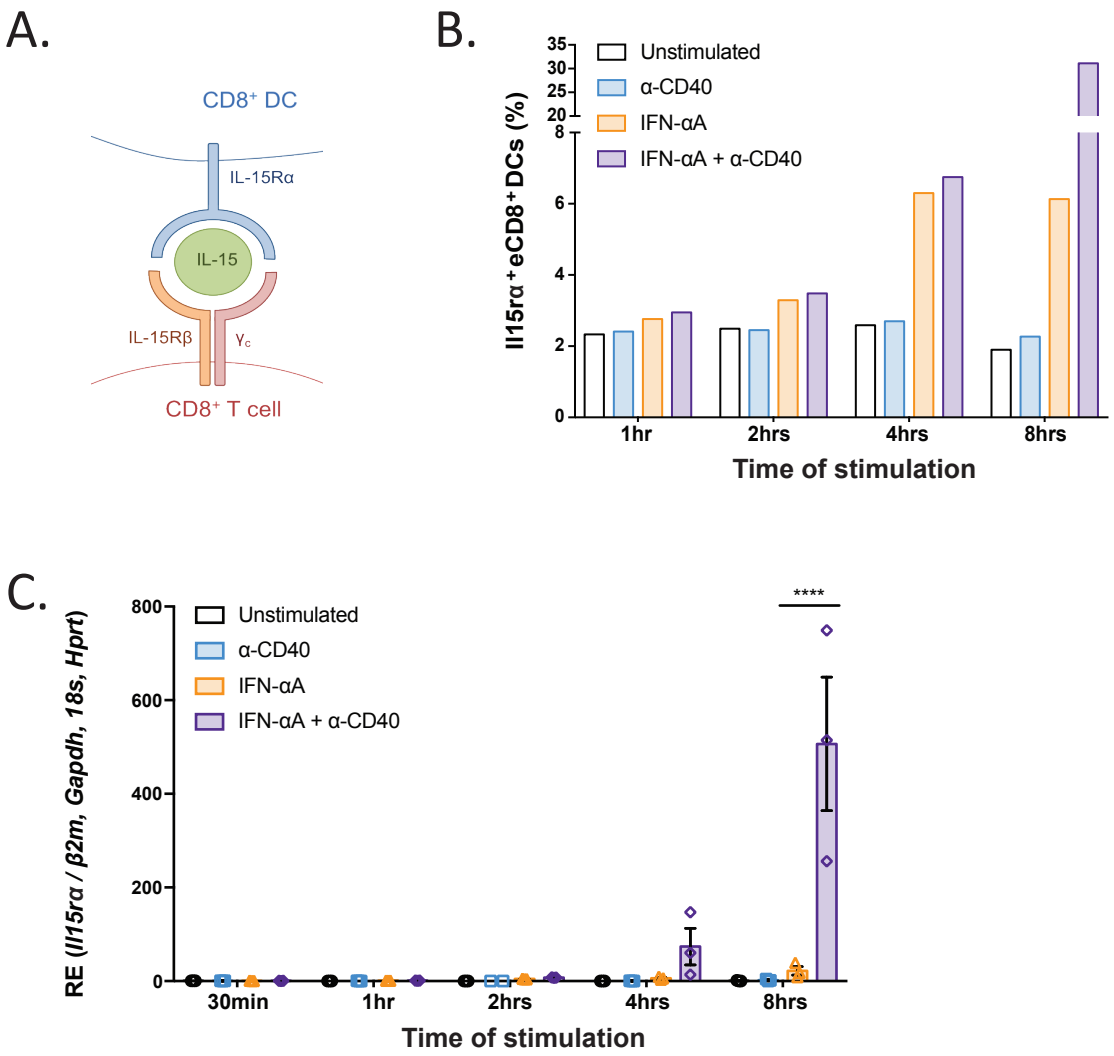
secreted cytokine during DC stimulations is challenging and can be unrepresentative of its actual level. The intracellular staining of Il-15rα in combination with its surface staining allows the investigation of its dynamic expression (**Figure 3.12.B**). Interestingly, Il-15rα expression was induced following 4 hours of IFN-αA stimulation and was amplified via α-CD40 treatment after 8 hours of stimulation. These results are in accordance with the *Il15ra* gene expression measurement showing that *Il15ra* is significantly induced under IFN-αA and α-CD40 stimulation for 8 hours reaching a relative expression about 500-times higher than the unstimulated control condition (**Figure 3.12.C**).

Finally, mRNA expression and protein formation and secretion are processes that potentially consume cell energy and resources (Kafri et al. 2016). To investigate if the entirety of the IFN-α/β-induced gene sets was amplified following treatment with α-CD40 antibody, the gene regulation of other Interferon-Stimulated Genes (ISG) such as *Irf7* (**Figure 3.13.A**) and *Isg20* (**Figure 3.13.B**) were measured. These genes, were, as expected, induced at the mRNA level following IFN-αA stimulation. However, the addition of α-CD40 antibody treatment did not result in any significant amplification of this IFN-αA-driven expression. These results reveal that CD40-driven amplification targets a precise set of genes only.

These experimental results provide key insights into the kinetics of the CD40-mediated amplification of the innate pathways in eCD8$^+$ DCs with distinct but specific patterns of gene and protein expressions of various molecules involved in inflammatory responses.

### 3.2.4 CD40-CD40L interaction requirement for amplification of DC responses

In order to investigate if the use of an antibody targeting CD40 receptor recreates the effect of the CD40L expressed on the surface of the T cell, an

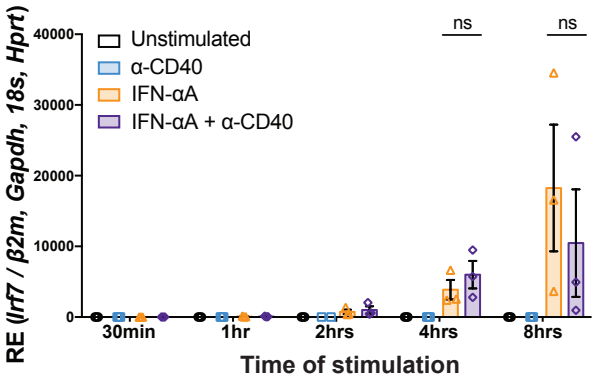



**<u>Figure 3.12</u>: α-CD40-driven amplification of *Il-15rα* expression.**
**(A.)** Trans-presentation of the cytokine IL-15. DCs secrete IL-15 and express the α-chain of the receptor (IL-15Rα) allowing the cytokine to bind and its presentation to the two other chains of the receptor, β and γ at the surface of the T cells (Jabri & Abadie 2015).
Percentage of Il-15rα⁺ eCD8⁺ DCs **(B.)** or eCD8⁺ DCs regulation of *Il15rα* **(C.)** measured following time course from 30 minutes or 1 hour to 8 hours of stimulation with media only (Unstimulated, white), α-CD40 mAb (10 µg/ml) (α-CD40, blue), IFN-αA (1000 U/ml) (IFN-αA, orange) and IFN-αA (1000 U/ml) with α-CD40 mAb (10 µg/ml) (IFN-αA + α-CD40, purple) (1 to 3 independent experiments pooled). Error bars represent mean +/- SEM. Asterisks indicate statistically significant differences between IFN-αA condition and IFN-αA + α-CD40 condition as assessed by two-way ANOVA; adjusted p-value: **** $p<0.0001$.

experiment that would allow the interaction between CD40L expressed by the $CD4^+$ T cell and CD40 on the DCs was set up. First, the up-regulation of CD40L on $CD4^+$ T cells was assessed. For this, naïve $CD4^+$ T cells were isolated from C57Bl/6 LNs and stimulated *in vitro* using anti-CD3/CD28 coated plates (**Figure 3.14***)*. CD40L expression was measured by flow cytometry over time and interestingly the surface CD40L expression increased at 6 hours after stimulation and decreased progressively thereafter, reaching a level close to the resting $CD4^+$ T cells at 48 hours after *in vitro* stimulation (**Figure 3.14.A** and **B**).

The 6 hours time point was chosen to perform a co-culture experiment of $CD4^+$ T cells with $eCD8^+$ DCs (**Figure 3.15**). $CD4^+$ T cells were enriched from OT-II mice that have been engineered to produce $CD4^+$ T cells expressing transgenic TCR specific for the $OVA_{323-339}$ peptide from ovalbumine (OVA). *In vivo*, CD40 stimulation triggered by CD40L requires a cognate $CD4^+$ T cell to increase the strength of the interaction. Therefore, OT-II $CD4^+$ T cells were *in vitro* stimulated and co-cultured in the presence of their specific OVA peptide with IFN-αA-stimulated DCs. The co-culture system is not amenable to mRNA unless the cell types were sorted again which would cause confounding factors to the analysis. Therefore, the cytokines and chemokines secreted in the supernatant of the co-culture were measured. Control $eCD8^+$ DCs were stimulated with IFN-αA alone, α-CD40 antibody alone or combined IFN-αA and α-CD40 antibody. As observed previously, IFN-αA stimulation induced Il-6 secretion that was amplified with the addition of α-CD40 antibody (**Figure 3.15.A**). There was no Il-6 secreted from unstimulated $eCD8^+$ DCs co-cultured with activated OT-II $CD4^+$ T cells, with or without their specific OVA peptide. However, when $eCD8^+$ DCs were stimulated with IFN-αA and co-cultured with activated OT-II $CD4^+$ T cells, the levels of Il-6 secreted trended to be higher than IFN-αA-stimulated DCs alone, although not to a significantly different level (**Figure 3.15.A**). This amplified secretion induced by the presence of activated OT-II $CD4^+$ T cells with IFN-αA-stimulated $eCD8^+$ DCs was also observed for the chemokines Ccl4 (**Figure 3.15.B**) and Ccl5 (**Figure 3.15.C**), compared to the IFN-αA stimulation alone. Moreover, the addition of OT-II $CD4^+$ T cell

A.




B.

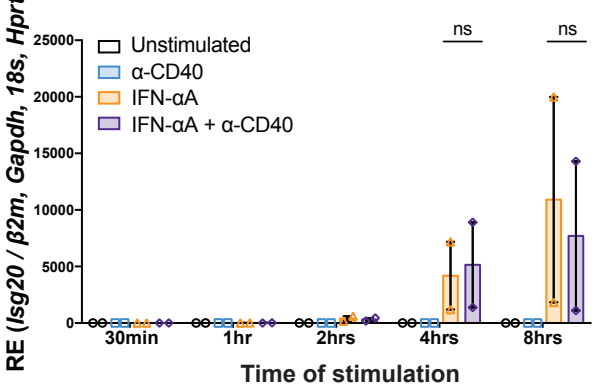



**Figure 3.13: No α-CD40-driven amplification for some IFN-αA-induced genes.**

eCD8⁺ DCs cultured for time course from 30 minutes to 8 hours with media only (Unstimulated, white), α-CD40 mAb (10 μg/ml) (α-CD40, blue), IFN-αA (1000 U/ml) (IFN-αA, orange) or IFN-αA (1000 U/ml) with α-CD40 mAb (10 μg/ml) (IFN-α A + α-CD40, purple).

Following stimulation, the regulation of *Irf7* **(A.)** and *Isg20* **(B.)** was assessed (Pool from 2 to 3 independent experiments for each time point). Error bars represent mean +/- SEM. ns = non significant difference between IFN-αA condition and IFN-αA + α-CD40 condition as assessed by two-way ANOVA.

specific OVA peptide seemed to slightly increase the secretion of each cytokine and chemokine, showing a significant higher secretion of 45 pg/ml of Ccl5 when compared to only 15 pg/ml secreted by eCD8$^+$ DCs stimulated with IFN-αA only (**Figure 3.15.C**). The finding that cytokine and chemokine secretion under IFN-αA-stimulated eCD8$^+$ DCs co-cultured with activated OT-II CD4$^+$ T cells without cognate ligand was comparable to the secretion by eCD8$^+$ DCs stimulated with IFN-αA and α-CD40 antibody together indicates that the CD40L up-regulation alone could allow CD40L-CD40 interaction and help signal deliverance *in vitro*. The addition of the OVA-specific peptide potentially induced a stronger and longer interaction leading to an even greater secretion (**Figure 3.15**). Interestingly, it was observed that OT-II CD4$^+$ T cells co-cultured with unstimulated eCD8$^+$ DCs could secrete Ccl4 and Ccl5, this secretion being comparable to eCD8$^+$ DCs stimulated with IFN-αA alone. However, a similar level of secretion was detectable when the OT-II CD4$^+$ T cells were cultured on their own or in the presence of IFN-αA (data not shown). The secretion of Ccl4 and Ccl5 in the supernatant of the cultured cells was thus linked to OT-II CD4$^+$ T cells anti-CD3/CD28 stimulation. The amplification of chemokines secretion observed when eCD8$^+$ DCs were stimulated with IFN-αA and co-cultured with activated OT-II CD4$^+$ T cells did not originate from the OT-II CD4$^+$ T cells themselves.

These results utilising *in vitro* activated CD4$^+$ T cells correlated with the initial findings using the surrogate α-CD40 antibody stimulations. This depicts the ability of CD40L-CD40 interaction to induce a signal able to amplify the IFN-I-induced pathways within eCD8$^+$ DCs.

### 3.2.5 CD40-driven amplification of various innate signals

The results so far demonstrate an enhancement of IFN-αA-triggered responses via CD40L-CD40 interaction. DCs can respond to many external stimuli other than type I IFN. For instance TLRs stimulation enhances the ability

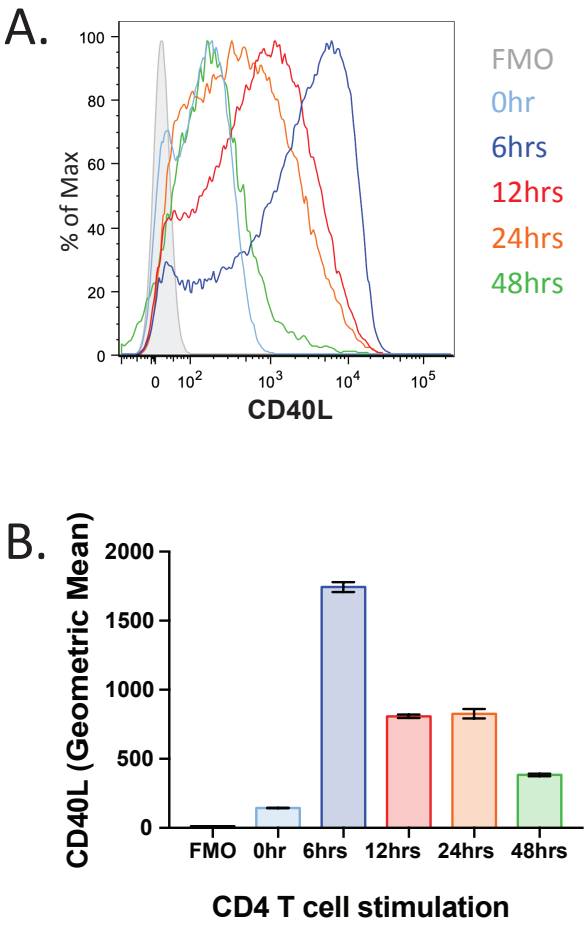
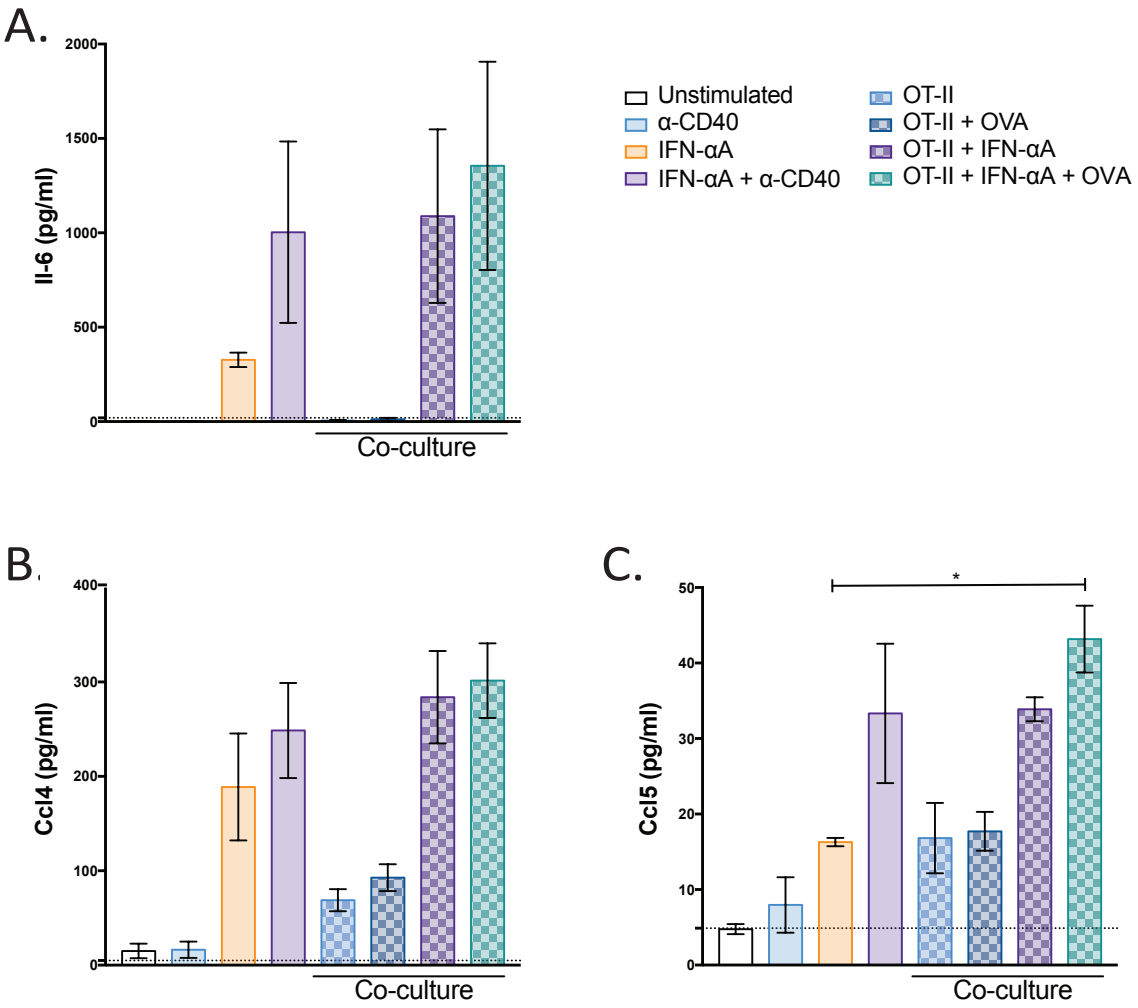



**Figure 3.14: Time course of CD40L up-regulation following CD4 T cell stimulation.**
Histogram **(A.)** or Geometric Mean of CD40L **(B.)** measurement on anti-CD3/CD28 antibody-stimulated $CD4^+$ T cells (Pool from 2 independent experiments). Measurement compared to the cells stained with the same fluorochrome-conjugated antibodies except the fluorochrome-conjugated CD40L (Fluorescence minus one, "FMO"). Error bars represent mean +/- SEM.

of DC to provide cytokines and chemokines (Reis e Sousa 2004). To investigate if interaction of CD40L-CD40 could also act to amplify responses to other innate stimuli eCD8$^+$ DCs were stimulated with different TLR agonists: CpG (TLR9 agonist), LPS (TLR4 agonist) and poly(I:C) (TLR3 agonist), in the presence or the absence of α-CD40 antibody for 4 hours (**Figure 3.16**). In order to compare the TLR-induced responses to IFN-αA-triggered responses previously observed (**Figure 3.6**), eCD8$^+$ DCs were stimulated with TLR agonists for 4 hours. The three TLR agonists triggered *Il15* mRNA production on their own, at various levels of expression (**Figure 3.16.A**). Importantly, these TLR-induced expressions were slightly higher following the addition of α-CD40 antibody (**Figure 3.16.A**). Interestingly, the level of expression reached by the combination of innate stimuli and CD40 triggering depended on the type of innate stimulation received. LPS triggered an expression of *Il15* that was 30-times higher than the unstimulated condition, with α-CD40 antibody addition enhancing this expression. p(I:C) stimulation on its own led to an expression 500-times higher than the unstimulated condition and a slight increase was observed with α-CD40 antibody addition (**Figure 3.16.A**). In the same way, *Il6* (**Figure 3.16.B**) and *Il12b* (**Figure 3.16.C**) expressions were clearly induced following TLR9 stimulation and slightly induced following TLR3 and 4 stimulation. In many cases, these expressions could be enhanced thanks to CD40 signalling (**Figure 3.16.B** and **C**). For instance, the addition of α-CD40 antibody treatment to the CpG stimulation triggered a significant amplification of these two cytokines, reaching an expression 4-times higher than the expression induced by CpG stimulation on its own. As TLRs ligation can induce IFN-α/β production as well, it is not possible with these results to confirm that the higher mRNA expression we observed was due to the specific amplification of the TLR9, TLR4 or TLR3 pathways rather than the amplification of the autocrine IFN-I-induced response. To investigate this question, eCD8$^+$ DCs were stimulated with LPS in the presence of the αIFNAR1, blocking the type I IFN signalling, therefore blocking its potential feedback stimulation. Under αIFNAR1 treatment, LPS stimulation induced *Il15* (**Figure 3.17.A**), *Il-6* (**Figure 3.17.B**) and *Tnf* (**Figure 3.17.C**) mRNA expression, presumably via LPS-specific




**<u>Figure 3.15</u>: *In vitro* assay of CD4⁺ T cell-CD40L-induced amplification of DC response to IFN-αA stimulation.**

On the left, eCD8⁺ DCs cultured for 4 hours with media only (Unstimulated, white), α-CD40 mAb 3 hours (10 µg/ml) (α-CD40, blue), IFN-αA 4 hours (1000 U/ml) (IFN-αA, orange) or IFN-αA 4 hours (1000 U/ml) with α-CD40 mAb 3 hours (10 µg/ml) (IFN-αA + α-CD40, purple).

On the right, eCD8⁺ DCs were co-cultured for 3 hours with stimulated OT-II CD4⁺ T cells only (OT-II, tiled light blue), with stimulated OT-II CD4⁺ T cells and their specific ovalbumin peptide (OT-II + OVA, tiled dark blue), with stimulated OT-II CD4⁺ T cells and IFN-αA (1000 U/ml) (OT-II + IFN-αA, tiled purple), with stimulated OT-II CD4⁺ T cells, their specific ovalbumin peptide and IFN-αA (1000 U/ml) (OT-II + IFN-αA + OVA, tiled green). Following stimulation, the secretion of Il-6 **(A.)**, Ccl4 **(B.)**, Ccl5 **(C.)** was assessed (Pool from 2 independent experiments). Dotted line indicates the limit of detection of each cytokine/chemokine for the assay performed. Error bars represent mean +/- SEM. Asterisk indicates statistically significant differences between IFN-αA condition and OT-II + IFN-αA + OVA condition as assessed by one-way ANOVA; adjusted p-value: * p≤0.0480.

pathways. These same pathways were amplified, when α-CD40 treatment was added leading to a greater mRNA expression of the three cytokines, even in the presence of the IFNAR1 blocking antibody (**Figure 3.17**).

In conclusion, the α-CD40-driven amplification of TLR-induced responses demonstrates the possibility of enhancing the responses of DCs to various innate signals via CD40 engagement.

## 3.3 Discussion

This chapter characterized an *in vitro* system of CD40-driven amplification of innate pathways in eCD8$^+$ DCs. This amplification could be achieved by using either an activating α-CD40 antibody or through the addition of antigen-activated CD4$^+$ T cells, thus representing an *in vitro* model of T cell help. IFN-αA stimulation of eCD8$^+$ DCs induced their activation and maturation illustrated by the up-regulation of co-stimulatory molecules (Hensley et al. 2005; Shortman and Heath 2010; Simmons et al. 2012) and the production of cytokines and chemokines. Importantly, the specific stimulation of CD40 on the DCs, while not triggering any responses on its own, intensified these innate responses in a manner that followed distinct patterns of mRNA expression and protein secretion.

The priming of naïve T cells requires three different signals from the DCs: antigen presentation (signal 1), co-stimulatory molecules expression (signal 2) and cytokines secretion (signal 3) (Mescher et al. 2006). CD4$^+$ T cell help has been suggested to be involved in the provision of signal 3 by DCs (Rajasagi et al. 2009; Wiesel et al. 2011), with reports implicating the secretion of IL-12 (Filatenkov et al. 2005), IL-15 (Oh et al. 2008) or CCL3 and CCL4 (Castellino et al. 2006). eCD8$^+$ DCs responded to simultaneous stimulation with IFN-αA and the α-CD40 antibody by transcribing and secreting cytokines and chemokines with distinct kinetics. For example, while Il-12p40, Ccl5 or Tnf-α

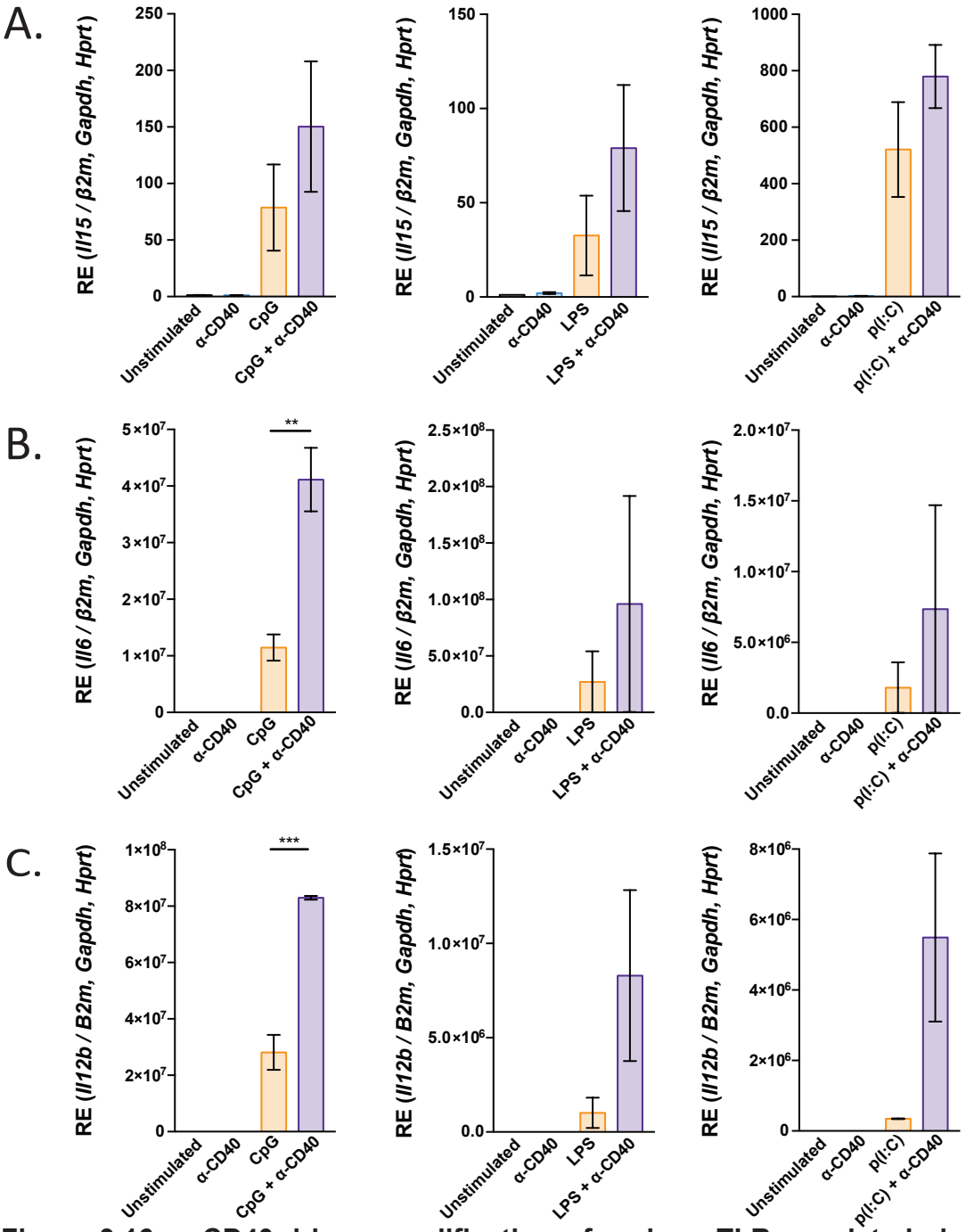



**Figure 3.16: α-CD40-driven amplification of various TLR-agonists-induced responses.**

eCD8$^+$ DCs cultured for 4 hours with media only (Unstimulated, white), α-CD40 mAb (10 µg/ml) (α-CD40, blue), CpG (1 µg/ml), LPS (10 µg/ml) or poly(I:C) (10 µg/ml) alone (CpG/LPS/p(I:C), orange) or with α-CD40 mAb (10 µg/ml) (CpG/LPS/p(I:C) + α-CD40, purple). Following stimulation, the regulation of *Il15* **(A.)**, *Il6* **(B.)**, *Il12b* **(C.)** was assessed (Pool from 2 independent experiments). Error bars represent mean +/- SEM. Asterisk indicates statistically significant differences between TLR-agonist condition and TLR-agonist + α-CD40 condition as assessed by one-way ANOVA; adjusted p-value: ** p≤0.0053; *** p≤0.0006.

secretion displayed a late enhancement, the provision of Il-6, Ccl3 and Ccl4 was amplified from 4 hours of stimulation. This unique pattern aligns with the described kinetics of *in vivo* supply of those molecules. The chemokines CCL3 and CCL4 are secreted by licensed DCs interacting with $CD4^+$ T cells and the injection of blocking antibodies against CCL3 and CCL4 leads to a diminution of naïve $CD8^+$ T cells accumulation in the draining LN (Castellino et al. 2006; Castellino and Germain 2007). On the other hand, the up-regulation of Il-15rα after 8 hours of stimulation with the combination of IFN-αA and α-CD40 antibody, and the amplification of *Il15* from 4 hours of stimulation suggested that Il-15 as a protein is likely to be secreted and trans-presented from 8 hours of stimulation. This cytokine has been shown to be required for optimal clonal expansion of specific $CD8^+$ T cells in the context of VSV and HSV infections (Schluns and Lefrançois 2003; Greyer et al. 2016). It is therefore possible that the enhanced secretion of Ccl3 and Ccl4 we observed *in vitro* aims to increase the $CD8^+$ T cell chemoattraction towards the antigen-bearing DCs potentially increasing the chance of meeting and engaging the antigen specific $CD8^+$ T cells. Il-15 could subsequently play a role in the expansion of specific $CD8^+$ T cells. While many of the IFN-αA-induced genes and proteins assessed were amplified by the addition of α-CD40 antibody, not all ISGs expression was enhanced, revealing that the effect of CD40 treatment is specific to some genes. Therefore the α-CD40-driven amplification of innate DC responses is precise in terms of kinetics and targets.

Our culture conditions were associated with expected rates of cell death with a half-life of 1.5 days *in vivo,* splenic $CD8^+$ DCs are subject to a rapid turnover (Kamath et al. 2000). Similarly, Flt3-DCs that are isolated from the spleen and cultured in nutritive medium display significant cell death (Vremec et al. 2015). While apoptotic cells release signals and molecules to attract phagocytes to the site of cell death (Peter et al. 2010), no up-regulation of maturation markers such as CD86 or CD83 was observed and therefore no spontaneous maturation occurred over the culture time of $eCD8^+$ DCs. Interestingly, the addition of CD40 stimulation left the cells in an immature state,

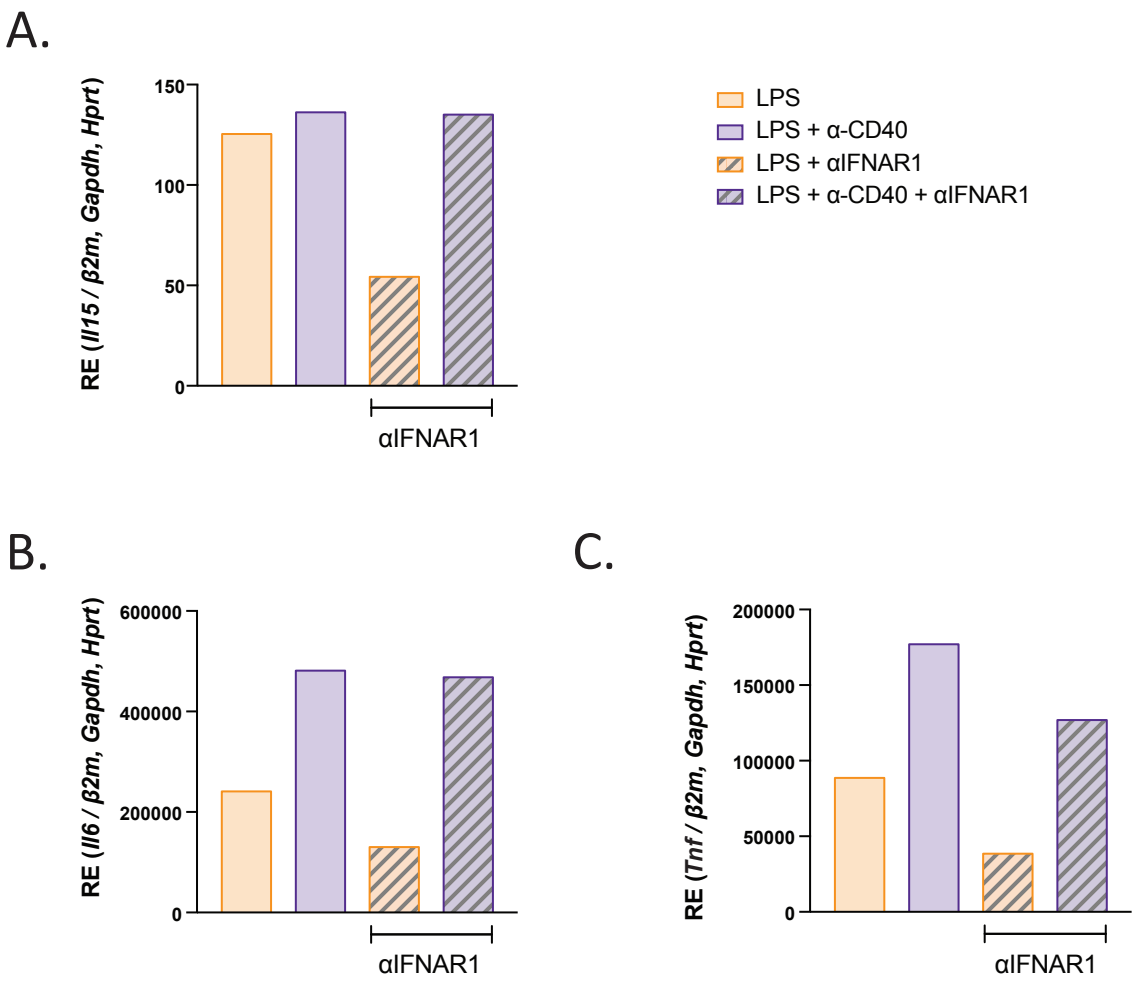



**Figure 3.17: CD40-driven amplification of LPS-induced responses independently of IFN-α/β recognition.**
eCD8⁺ DCs cultured over 4 hours under various conditions. Cells were cultured with LPS 4 hours (10 µg/ml) (LPS, orange), LPS 4 hours (10 µg/ml) with α-CD40 mAb for 1 hour (10 µg/ml) (LPS + α-CD40, purple), LPS for 4 hours (10 µg/ml) with anti-IFNAR1 for 1 hour (10 µg/ml) (LPS + αIFNAR1, grey striped orange), or LPS for 4 hours (10 µg/ml) with α-CD40 mAb for 1 hour (10 µg/ml) and anti-IFNAR1 1 hour (10 µg/ml) (LPS + α-CD40 + αIFNAR1, grey striped purple). Following stimulation, the regulation of *Il15* **(A.)**, *Il6* **(B.)**, *Tnf* **(C.)** was assessed (n=1).

suggesting that whatever danger signals released during the culture-associated cell death, these were not significantly amplified by CD40. In 2016, Greyer et al. observed that the outcome of DCs cytokine response is dictated by the stimulus they received. Exposing mice to cell-associated OVA and challenging them with either LPS or poly(I:C) drove different cytokine responses and requirements for the generation of specific CTL priming. However, in both cases these $CD8^+$ T cell responses were helper dependent (Greyer et al. 2016). We observed the CD40-driven enhancement of various TLR-induced pathways, such as those triggered by LPS or CpG, indicating that T cell help is not tied to the amplification of a single innate pathway. Corroborating these results, CD40L-transfected fibroblasts and CpG stimulation act together synergistically on $CD8^+$ DCs to produce an higher amount of IL-12 than both stimuli on their own (Schulz et al. 2000). While the potential danger signals released due to spontaneous cell death would not activate $eCD8^+$ DCs nor be amplified following CD40 stimulation, DCs sense and precisely respond to the innate signals from the microenvironment in which they encounter antigen. In turn, CD4 T cell help enhances those responses. These results allow us to consider the CD40-mediated amplification as a general principle applicable to a broad range of microbial or danger signals sensed by the DCs.

The cytokine IL-12 has been shown to increase the proliferation and cytotoxic abilities of $CD8^+$ T cells (Henry et al. 2008). Consistent with previous work, *Il12b* was expressed *in vitro* following CD40 stimulation alone (Gately et al. 1998; Schulz et al. 2000) while CD40 stimulation on its own did not trigger the expression of most of the other cytokines and chemokines assessed. Although *Il12b* was not induced by IFN-αA stimulation alone, the synergy of this stimulation with CD40 signalling triggered its up-regulation (Schulz et al. 2000; Reis e Sousa 2001). Without stimulation, DCs constitutively express low levels of CD40, which was sufficient to induce a low IL-12 expression when cells receive the CD40L stimulus alone. On the other hand, activating signals such as microbial challenge (Schulz et al. 2000) or IFN-α/β stimulation (**Figure 3.5**), induce CD40 surface expression on DCs and increase the number of cells

expressing it along with other maturation markers. This receptor up-regulation could be enough to increase the CD40L-induced IL-12 expression. Therefore, in the case of genes adopting the same pattern of expression, the enhancement might be caused by the innate signal-mediated increase of the DCs ability to respond to CD40 signalling. Moreover, this phenomenon might depend on the innate signal received by the DCs as their stimulation with TLR-agonists on their own led to significant *Il12b* mRNA production and to an IFN-α/β-independent enhancement following the addition of α-CD40 antibody treatment. Therefore, the ability of DCs to produce IL-12 in response to CD40 stimulation *in vivo* is dependent on the exposure to microbial stimuli, a conclusion that aligns with a report by Schulz et al. (Schulz et al. 2000).

Although some experiments would gain from experimental replicates, the findings presented herein characterised eCD8$^+$ DCs responses to α-CD40 antibody treatment and innate stimulation. Knowing that CD40L-CD40 interaction is required for providing the help signal (Bennett et al. 1998; Ridge et al. 1998; Schoenberger et al. 1998), these results support the use of the *in vitro* system of BM-derived DCs to resolve the molecular mechanisms that enable the “help”-mediated amplification of innate pathways.

# Chapter 4

## Molecular mechanisms involved in CD40 signalling

## 4.1 Introduction

The priming of antigen-specific $CD8^+$ T cells involves carefully orchestrated interactions between naïve $CD8^+$ T cells and DCs. In many situations the effectiveness of this interaction also depends on $CD4^+$ T cells triggering CD40 via CD40L in a process referred to as T cell help. Various studies have highlighted the relative kinetics of the $CD8^+$ T cell (Mempel et al. 2004; Garcia et al. 2007; Henrickson et al. 2008) and $CD4^+$ T cell (Miller et al. 2004; Celli et al. 2007) response, but little is known about the dynamics through which activated $CD4^+$ T cells provide such help to the antigen-bearing DC. Cross-presenting $XCR1^+$ DCs serve as important platforms allowing dynamic interactions between $CD4^+$ and $CD8^+$ T cells (Eickhoff et al. 2015; Hor et al. 2015) and *in vivo* imaging indicates that $CD4^+$ T cells can have surprisingly short, transient interactions with $CD8^+$ T cell-DCs clusters (Hor et al. 2015). This suggests that activated cognate $CD4^+$ T cells are able to rapidly move between the DCs, triggering dynamic and short-lived interactions required for CTL priming. Furthermore, CD40L expression occurs in a transient manner following activation and its interaction with CD40 is tightly controlled (van Kooten and Banchereau 2000). These findings raise the possibility that the provision of T cell help to DC is a surprisingly rapid event.

The binding of CD40L to CD40 leads to the recruitment of TRAF proteins (Bishop et al. 2007). In contrast to TRAF6 that binds to a unique domain of the cytoplasmic tail of CD40, TRAF2 and TRAF3 associate with an overlapping region of the receptor. Moreover, TRAF5 only indirectly interacts with CD40 via TRAF2 or TRAF3 (van Kooten and Banchereau 2000; Ajibade et al. 2013). Accordingly, the combined deficiency for CD40-TRAF2/3/5 signalling causes a different phenotype compared to the single deficiency for CD40-TRAF6 signalling (Chatzigeorgiou et al. 2014). Upon recruitment, TRAF2 and TRAF3 are degraded leading to the activation of NIK and therefore to the processing of p100 into p52 and stimulation of the non canonical NF-κB pathway (Zarnegar et al. 2008; Elgueta et al. 2009). TRAF6 recruitment leads to TAK1 activation.

TAK1 is a kinase that can phosphorylate and activate MAPKs (p38, JNK and ERK) and IKK-NF-κB pathways (Ajibade et al. 2013) (**Figure 1.4**).

The components of these different signalling pathways, their crosstalk and outcomes have been extensively studied and described. The integration of a signal can occur overtime, triggering gradual transcriptional changes. For instance, histone modifications accumulate in a temporal manner, following prolonged LPS stimulation and these epigenetic modifications are linked to and potentially depend on the activation of transcription factors (TFs) (Vandenbon et al. 2018). A TF can translocate to the nucleus with different rates depending on the stimulus received by the cells and these nuclear translocation dynamics influence the gene expression. For instance, NF-κB can move in and out of the nucleus, adopting an oscillatory behaviour and controlling the gene expression into waves (Zambrano et al. 2016; Lane et al. 2017). Moreover, these distinct expression patterns are linked to specific molecular functions: in response to TNF stimulation, an early cluster of NF-κB-dependent genes encodes cytokines while a late cluster of genes encodes cell surface receptors and adhesion molecules (Tian et al. 2005). These studies highlight an integration of signal overtime leading to dynamic changes in transcription, but it is unclear if such signalling integration also occurs in DCs that have received T cell help.

Our previous results revealed α-CD40-driven amplification of IFN-αA-induced gene expression and protein secretion after 4 to 8 hours of stimulation. However, all stimulations were applied on the cells simultaneously. To better understand the underlying kinetics and molecular regulation, we set up an *in vitro* system allowing us to study the timing of CD40-driven amplification of innate response in DCs and investigate the underlying molecular mechanisms.

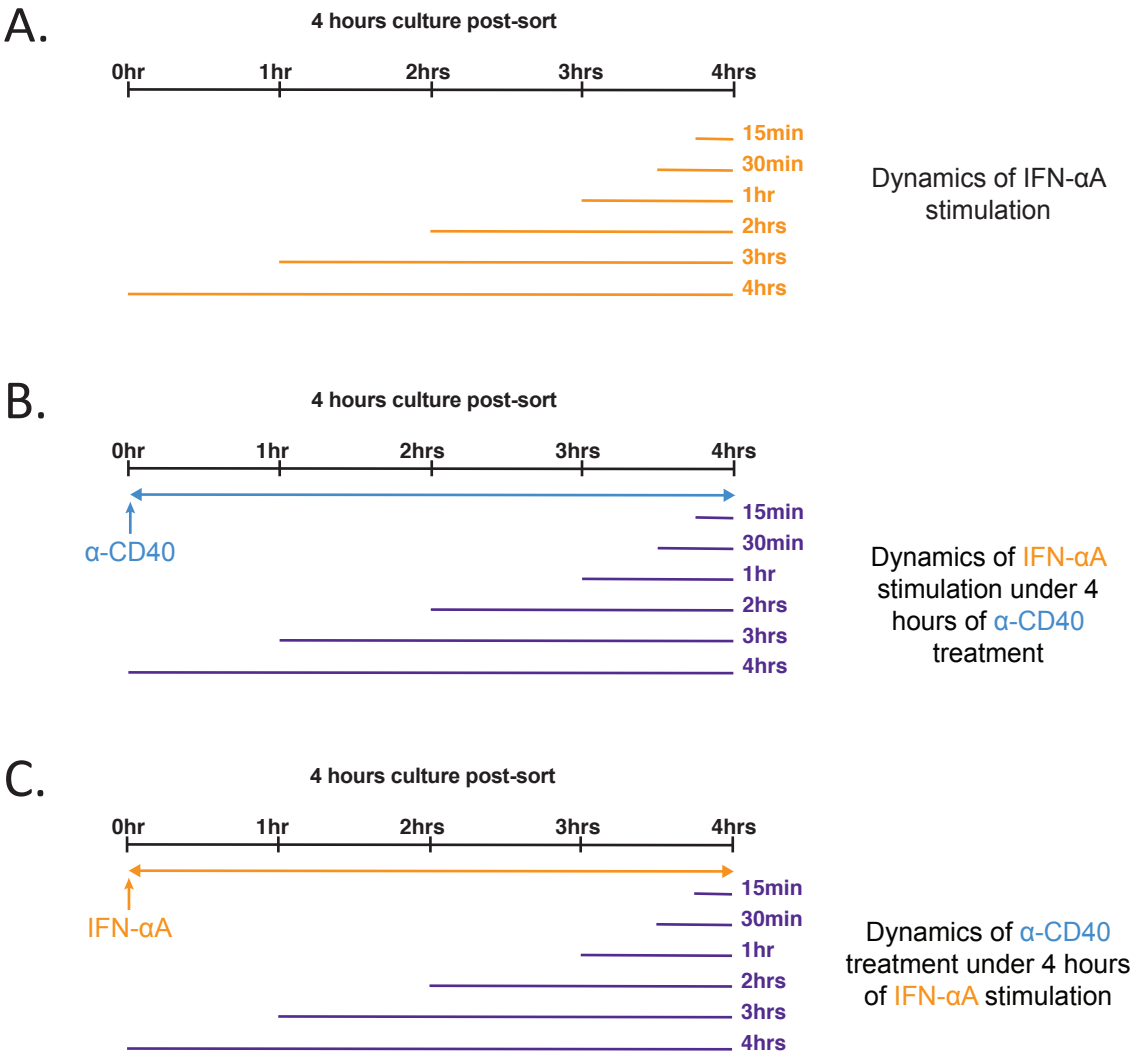



**Figure 4.1: Investigation of the dynamics of CD40-driven amplification of IFN-αA-initiated pathway.**
eCD8$^+$ DCs cultured for 4 hours under various conditions:
**(A.)** Dynamics of IFN-αA-induced responses. Cells were stimulated with IFN-αA only (1000 U/ml) either for 4 hours, either for the last 3 hours, 2 hours, 1 hour, 30 minutes or 15 minutes.
**(B.)** Dynamics of IFN-αA responses under 4 hours of α-CD40 treatment. Cells were stimulated with α-CD40 mAb (10 μg/ml) (α-CD40) for 4 hours while being also stimulated with IFN-αA (1000 U/ml) either for 4 hours, either for the last 3 hours, 2 hours, 1 hour, 30 minutes or 15 minutes.
**(C.)** Dynamics of responses to α-CD40 treatment under 4 hours of IFN-αA stimulation. Cells were stimulated with IFN-αA (1000 U/ml) for 4 hours while being stimulated with α-CD40 mAb (10 μg/ml) (α-CD40) either for 4 hours, either for the last 3 hours, 2 hours, 1 hour, 30 minutes or 15 minutes.

## 4.2 Results

### 4.2.1 Dynamics of α-CD40-mediated amplification

In order to investigate molecular pathways triggered by CD40 stimulation, the precise dynamics of eCD8$^+$ DCs responses first required to be clarified. When eCD8$^+$ DCs were stimulated with IFN-αA and treated with α-CD40 antibody, the expression of many cytokines and chemokines, such as *Il-15*, *Tnf* or *Ccl5*, were highly induced from 4 hours of IFN-αA stimulation (Chapter 3). This timing was therefore chosen as a constant duration of stimulation. Knowing that α-CD40 antibody treatment on its own does not trigger many cytokine and chemokine responses (Chapter 3), the first point that required clarification was the dynamics of the responses to IFN-αA stimulation on its own. Therefore, eCD8$^+$ DCs were subjected to 4 hours of culture and received IFN-αA stimulus either from the beginning, either for the last 3 hours, the last 2 hours, the last hour, the last 30 minutes or the last 15 minutes (**Figure 4.1.A**). This way, the minimum amount of time required for inducing IFN-αA-mediated responses would be assessed. In order to precisely investigate the effect of one signal on the outcome of another one, the treatment with α-CD40 antibody was first administered ahead of IFN-αA stimulation, instead of applying these two signals simultaneously as performed previously (Chapter 3). This experimental set up interrogated whether stimulating CD40 receptor would "prepare" the DCs to, for instance, respond faster to the subsequent challenge with an innate signal. Thus, eCD8$^+$ DCs were treated with α-CD40 antibody for 4 hours and received IFN-αA for various amounts of time within this α-CD40 treatment: for 4 hours, for the last 3 hours, 2 hours, 1 hour, 30 minutes or 15 minutes of culture post-sort (**Figure 4.1.B**). Comparing these results with the dynamics of IFN-αA stimulation alone as explained in Figure 4.1.A would therefore highlight the effect of α-CD40 antibody pre-treatment on DCs. Another hypothesis on the dynamics of the two different signals is that once the DCs have been licensed through the recognition of an innate signal, they might be

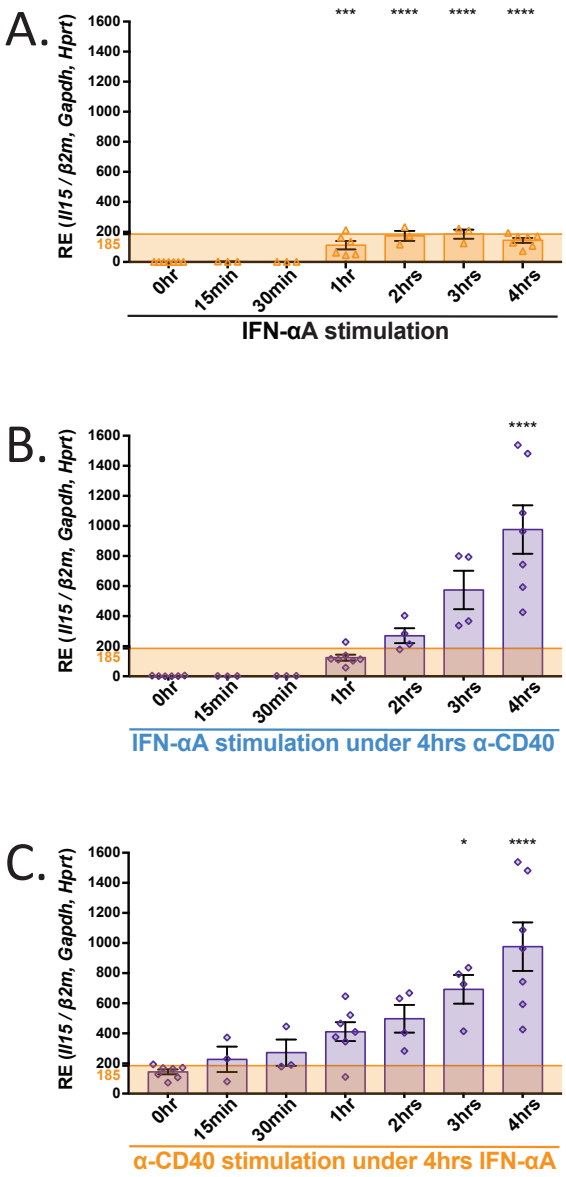



**Figure 4.2: One hour of α-CD40 signaling efficient for the initiation of *Il15* mRNA amplification.**

**(A.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.A**. **(B.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.B**. **(C.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.C**.

Following stimulation, the regulation of *Il15* was assessed. The shaded area indicates the highest mRNA expression induced by IFNαA stimulation, value indicated in orange (Pool from at least 3 independent experiments for each time point). Error bars represent mean +/- SEM. Asterisks indicate statistically significant differences between the IFNαA condition itself versus the unstimulated condition **(A.)** or between the condition itself versus the highest mRNA expression induced by IFN αA stimulation alone **(B.)** and **(C.)** as assessed by one-way ANOVA; adjusted p-value: * p≤0.0454; *** p≤0.0006; **** p<0.0001.

able to respond more quickly to α-CD40 signal. Therefore, through 4 hours of IFN-αA stimulation, eCD8$^+$ DCs received α-CD40 antibody treatment for the entirety of the culture post-sort, or for the last 3 hours, 2 hours, 1 hour, 30 minutes or 15 minutes (**Figure 4.1.C**). Knowing that 4 hours of IFN-αA stimulation led to the activation of eCD8$^+$ DCs (Chapter 3), the addition of α-CD40 stimulus for various amounts of time will highlight the shortest duration required for this signal to induce the enhancement of the response of licensed DC.

#### 4.2.1.1 Rapid amplification of IFN-αA-induced Il-15 and Il-6 gene expression followed by enhancement of protein secretion

As observed previously, IFNα/β stimulation activates DCs and can trigger IL-15 production (Verbist and Klonowski 2012; Greyer et al. 2016) (Chapter 3). While eCD8$^+$ DCs were cultured for 4 hours following sort, the IFN-αA stimulation for 15 minutes or for 30 minutes was not sufficient to trigger *Il15* mRNA expression (**Figure 4.2.A**). However, this mRNA expression was significantly induced from 1 hour of stimulation and reached a plateau of expression with a maximum of expression about 185-times higher than the unstimulated control. When the cells were treated for 4 hours with α-CD40 antibody, 1 hour of IFN-αA stimulation was still required to start inducing *Il15* mRNA expression (**Figure 4.2.B**). Once *Il15* expression was initiated, it further increased upon longer IFN-αA stimulation. When compared to the highest expression induced by IFN-αA alone (185 of relative expression compared to unstimulated condition, **Figure 4.2.A**), this amplified expression became significant when cells were treated with both stimulations for 4 hours. Interestingly, when eCD8$^+$ DCs were stimulated with IFN-αA, the treatment with α-CD40 antibody for only the last 30 minutes of the experiment was enough to show signs of amplification of the *Il15* expression (**Figure 4.2.C**). This amplification gradually increased the longer the cells were treated with α-CD40 antibody, reaching an expression significantly higher than the expression

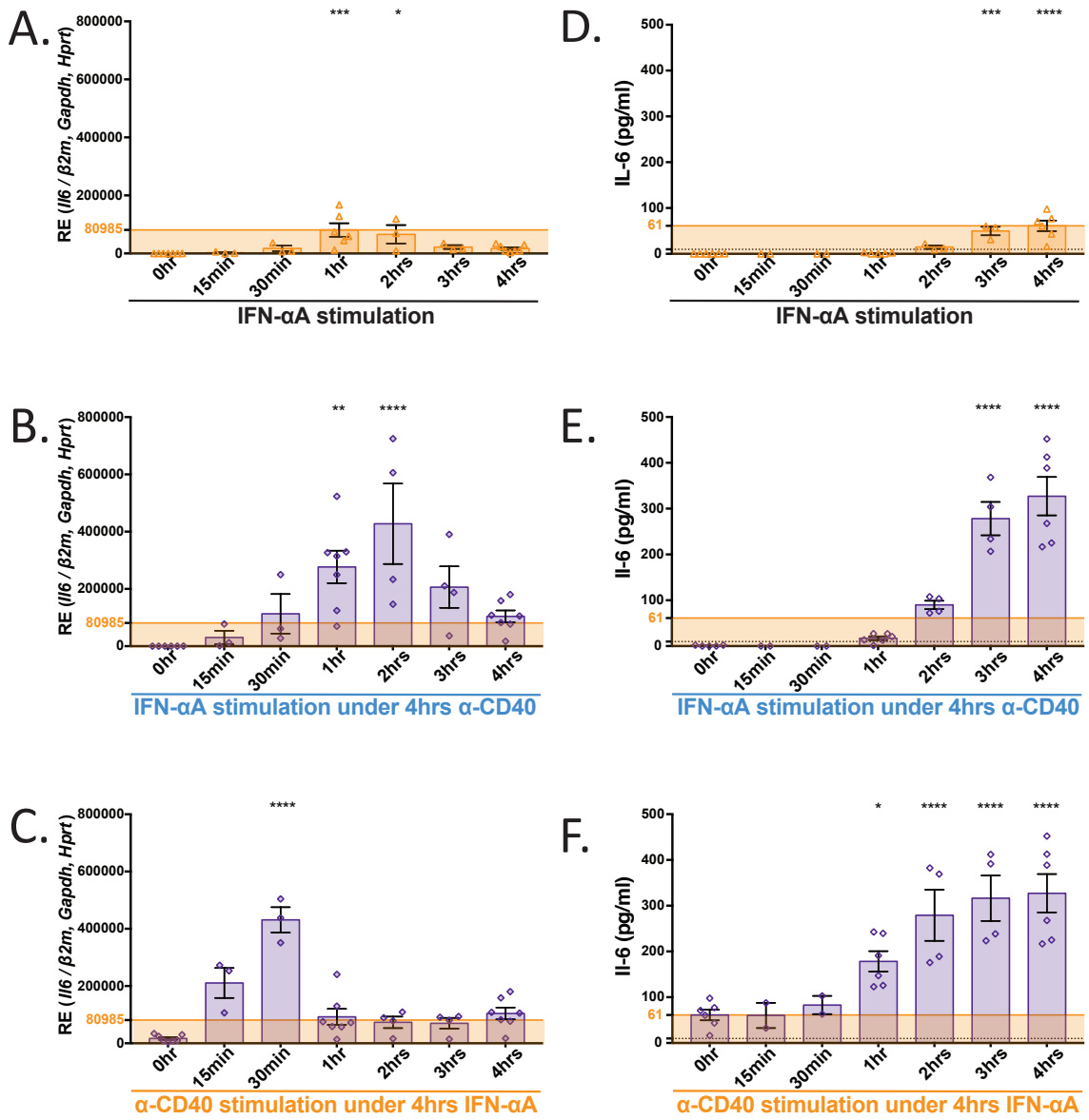



**Figure 4.3: Rapid initiation of *Il6* mRNA amplification followed by Il-6 secretion via α-CD40 signalling.**

**(A.**, **D.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.A**. **(B.**, **E.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.B**. **(C.**, **F.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.C**.

Following stimulation, the regulation of *Il6* **(A.**, **B.**, **C.)** and Il-6 **(D.**, **E.**, **F.)** was assessed. The shaded area indicates the highest mRNA expression induced by IFN-αA stimulation, value indicated in orange, while the dotted line indicates the limit of detection of the cytokine for the assay performed (Pool from at least 2 independent experiments for each time point). Error bars represent mean +/- SEM. Asterisks indicate statistically significant differences between the IFN-αA condition itself versus the unstimulated condition **(A.**, **D.)** or between the condition itself versus the highest expression/secretion induced by IFN-αA stimulation alone **(B.**, **C.**, **E.**, **F.)** as assessed by one-way ANOVA; adjusted p-value: * p≤0.0325; ** p≤ 0.0084; *** p≤0.0009; **** p<0.0001.

induced by IFN-αA alone from 3 hours of α-CD40 antibody treatment. Therefore, in the case of *Il15* expression, at least about 1 hour of IFN-αA stimulation is necessary for DCs to start responding and inducing its mRNA production. Pre-treating the cells with α-CD40 antibody for 3 hours before the addition of IFN-αA did not prepare the eCD8$^+$ DCs to respond faster to this innate stimulus. Conversely, if the cells were pre-treated with IFN-αA, only a short duration, about 30 minutes, of α-CD40 antibody treatment efficiently induces the amplification of these IFN-αA-triggered responses (**Figure 4.2**). Moreover, this short CD40 stimulation in the context of IFN-αA-stimulated cells was also sufficient to trigger *Tnf* and *Ccl5* mRNA amplification (data not shown). This shows that IFN-αA stimulation endows the DC with a remarkably quick responsiveness to CD40 stimulation.

As for *Il15*, *Il6* mRNA expression required about 1 hour of IFN-αA stimulation to be induced, this IFN-αA-triggered expression was about 81 000-times higher than the basal expression under unstimulated condition (**Figure 4.3.A**). When eCD8$^+$ DCs were pre-treated with α-CD40 antibody, the amplification of the *Il6* expression did not occur following only 15 or 30 minutes of IFN-αA stimulation (**Figure 4.3.B**). However, 1 hour of IFN-αA stimulation under α-CD40 constant presence, triggered *Il6* mRNA expression and this expression was significantly amplified, when compared to its highest IFN-αA-induced expression as observed in **Figure 4.3.A**. Interestingly, when eCD8$^+$ DCs were stimulated over 4 hours with IFN-αA, treatment with α-CD40 antibody only for the last 15 minutes was sufficient to induce the enhancement of *Il6* mRNA expression, significantly triggered with 30 minutes of this treatment (**Figure 4.3.C**). This amplification then quickly stopped even with longer α-CD40 antibody treatment, returning to the IFN-αA-triggered level of mRNA expression. Following *Il6* mRNA expression occurring after 1 hour of IFN-αA, the Il-6 cytokine could be measured in the supernatant of the eCD8$^+$ DCs stimulated for 3 hours with IFN-αA (**Figure 4.3.D**). This secretion reached a maximum of 61 pg/ml in the cell stimulated for 4 hours. Correlating with the amplification of *Il6* observed after 1 hour of IFN-αA in the presence of α-CD40 antibody, the

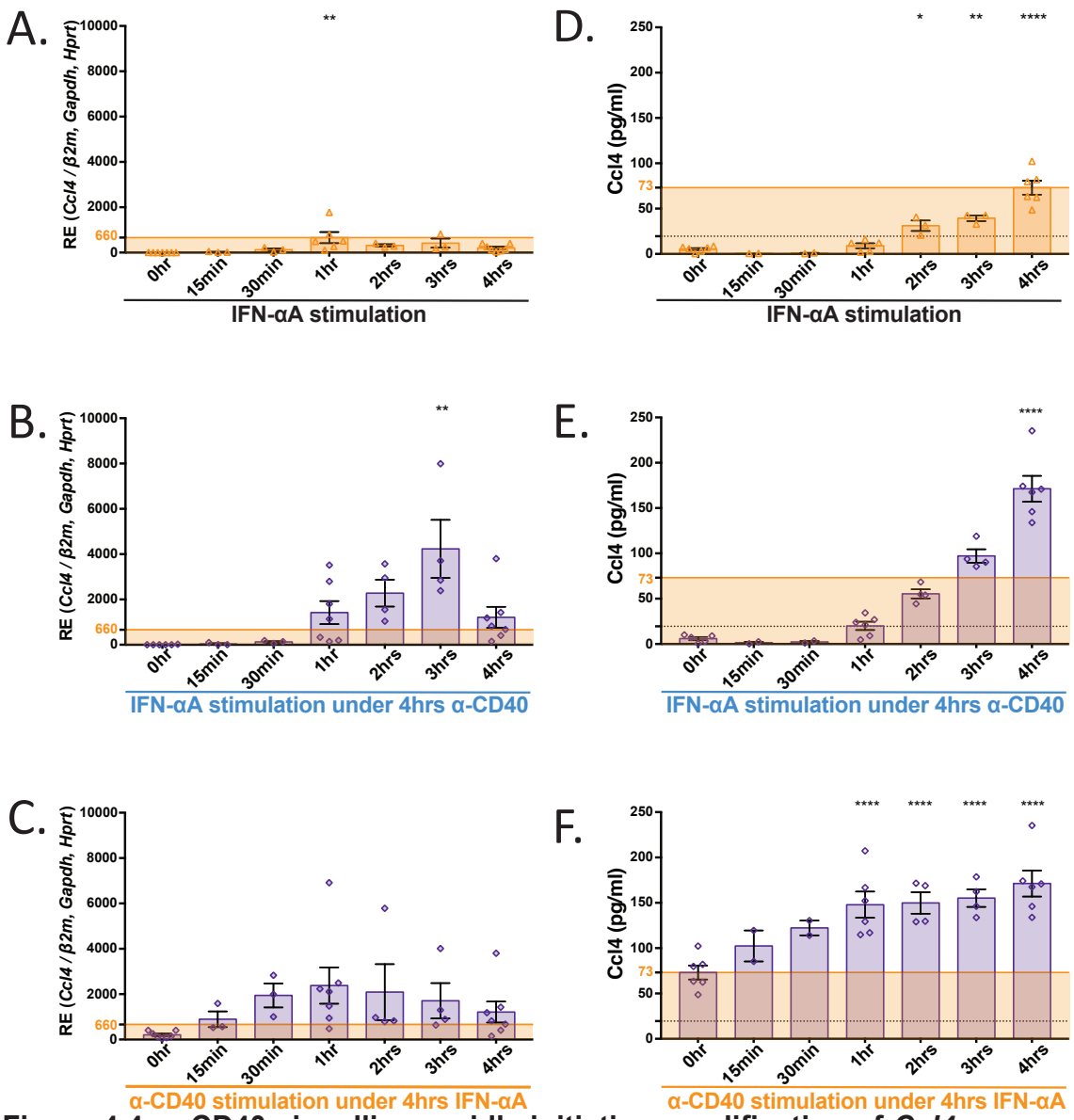



**Figure 4.4: α-CD40 signalling rapidly initiating amplification of *Ccl4* expression and Ccl4 secretion by IFN-αA-stimulated cells.**

**(A.**, **D.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.A**. **(B.**, **E.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.B**. **(C.**, **F.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.C**.

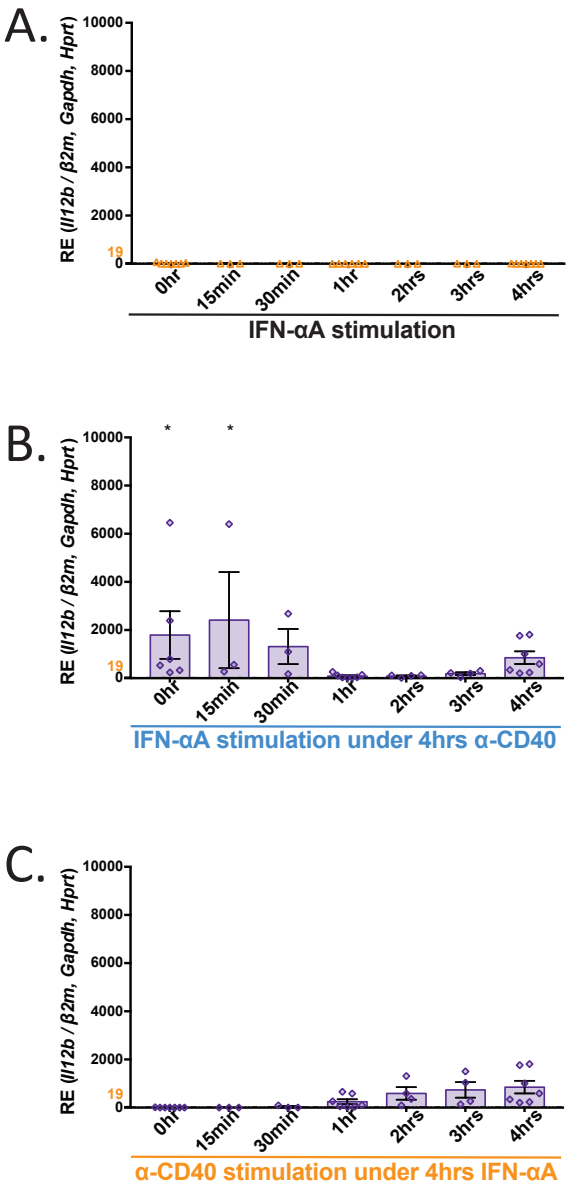
Following stimulation, the regulation of *Ccl4* **(A.**, **B.**, **C.)** and Ccl4 **(D.**, **E.**, **F.)** was assessed. The shaded area indicates the highest mRNA expression induced by IFN-αA stimulation, value indicated in orange, while the dotted line indicates the limit of detection of the cytokine for the assay performed (Pool from at least 2 independent experiments for each time point). Error bars represent mean +/- SEM. Asterisks indicate statistically significant differences between the IFN-αA condition itself versus the unstimulated condition **(A.**, **D.)** or between the condition itself versus the highest expression/secretion induced by IFN-αA stimulation alone **(B.**, **C.**, **E.**, **F.)** as assessed by one-way ANOVA; adjusted p-value: * p≤0.0130; ** p≤ 0.0027; *** p≤0.0006; **** p<0.0001.

secretion of Il-6 was significantly amplified after 3 hours of IFN-αA stimulation under CD40 engagement (**Figure 4.3.E**). When eCD8$^+$ DCs were treated with IFN-αA for 4 hours, the secretion of Il-6 was significantly amplified from only 1 hour of α-CD40 antibody stimulation, secreted at a concentration more than twice higher than the IFN-αA-mediated secretion (**Figure 4.3.F**). Interestingly, the amplification of the secretion gradually increased with longer stimulation of CD40 receptor until reaching a plateau at about 300 pg/ml of protein secreted in the supernatant.

In conclusion, *Il15*, and *Il6* expressions display similar dynamics with IFN-αA-induced expression occurring after at least 1 hour of stimulation. This stimulation duration requirement was unchanged with α-CD40 antibody pre-treatment. However, when eCD8$^+$ DCs were stimulated for the overall 4 hours of the experiment with IFN-αA, the addition of α-CD40 antibody for a short amount of time induced a quick and clear amplification of the IFN-αA-mediated mRNA expression. More importantly, the concentration of secreted Il-6 cytokine was as well amplified by the treatment with α-CD40 antibody under constant IFN-αA stimulation. This amplified secretion occurred subsequently to the observed mRNA amplification.

#### 4.2.1.2 Rapid enhancement of both IFN-αA-induced Ccl4 gene expression and secretion

As for many cytokines, when eCD8$^+$ DCs were stimulated for increasing amounts of time with IFN-αA, at least 1 hour of stimulus was required for inducing *Ccl4* mRNA expression (**Figure 4.4.A**). This timing of stimulus also induced the maximum of *Ccl4* expression, at about 660-times higher than unstimulated cells. When eCD8$^+$ DCs were treated over 4 hours with α-CD40 antibody, 1 hour of IFN-αA stimulation was again required to trigger *Ccl4* expression higher than the expression induced by IFN-αA on its own (**Figure 4.4.B**). However, when the cells received IFN-αA stimulus for the entire time of




**<u>Figure 4.5</u>: Early inhibition of α-CD40-driven *Il12b* mRNA expression by IFN-αA stimulation.**

**(A.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.A**. **(B.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.B**. **(C.)** eCD8⁺ DCs stimulated as described in **Figure 4.1.C**.

Following stimulation, the regulation of *Il12b* was assessed. The highest mRNA expression induced by IFN-αA stimulation value is indicated in orange (Pool from at least 3 independent experiments for each time point). Error bars represent mean +/- SEM. Asterisks indicate statistically significant differences between the IFN-αA condition itself versus the unstimulated condition **(A.)** or between the condition itself versus the highest mRNA expression induced by IFN-αA stimulation alone **(B.)** and **(C.)** as assessed by one-way ANOVA; adjusted p-value: * p≤0.0307.

the experiment, only 30 minutes of α-CD40 antibody was enough to increase the IFN-αA-mediated *Ccl4* expression (**Figure 4.4.C**). Ccl4 chemokine was detectable in the supernatant of eCD8$^+$ DCs from 2 hours of IFN-αA stimulation (**Figure 4.4.D**). This secretion reached a maximum of 73 pg/ml from cells stimulated for 4 hours with IFN-αA. Even in the presence of α-CD40 antibody treatment, the 2 hours of IFN-αA stimulation were again required for detectable Ccl4 secretion (**Figure 4.4.E**). This secretion was clearly higher under the effect of CD40 stimulation reaching a significant amplification of IFN-αA-mediated after 4 hours of α-CD40 antibody treatment. When the cells received IFN-αA stimulation for 4 hours, 15 minutes of α-CD40 antibody was sufficient to observe a slight increase to Ccl4 secretion (**Figure 4.4.F**). Only 1 hour of CD40 engagement was sufficient to trigger a significant amplification of this secretion. As for Il-6, this secretion mediated by the combination of α-CD40 antibody and IFN-αA stimulation reached a plateau at 150 pg/ml that would not be exceeded even with longer duration of CD40 stimulation. Interestingly, while *Ccl4* amplification occurred from 30 minutes to 1 hour of α-CD40 antibody treatment, the amplification of Ccl4 secretion was already triggered by the activated DCs with only 15 to 30 minutes of CD40 engagement.

Therefore, as for *Il6* mRNA expression, *Ccl4* required 1 hour at least of IFN-αA stimulation irrespective of pre-treatment with α-CD40 antibody. IFN-αA-stimulated eCD8$^+$ DCs could quickly respond to CD40 stimulation and triggered *Ccl4* amplification. More importantly, a rapid amplification of the IFN-αA-mediated Ccl4 secretion in the supernatant of the cells was also observed. Thus the effect of α-CD40 antibody in the amplification of Ccl4 IFN-αA-mediated secretion and of *Ccl4* mRNA expression occurred with similar dynamics.

#### 4.2.1.3 Early inhibition of α-CD40-driven *Il12b* expression

As observed previously, *Il12b* mRNA expression was hardly triggered by IFN-αA stimulation (**Figure 4.5.A**). In our previous results, the cytokine was not

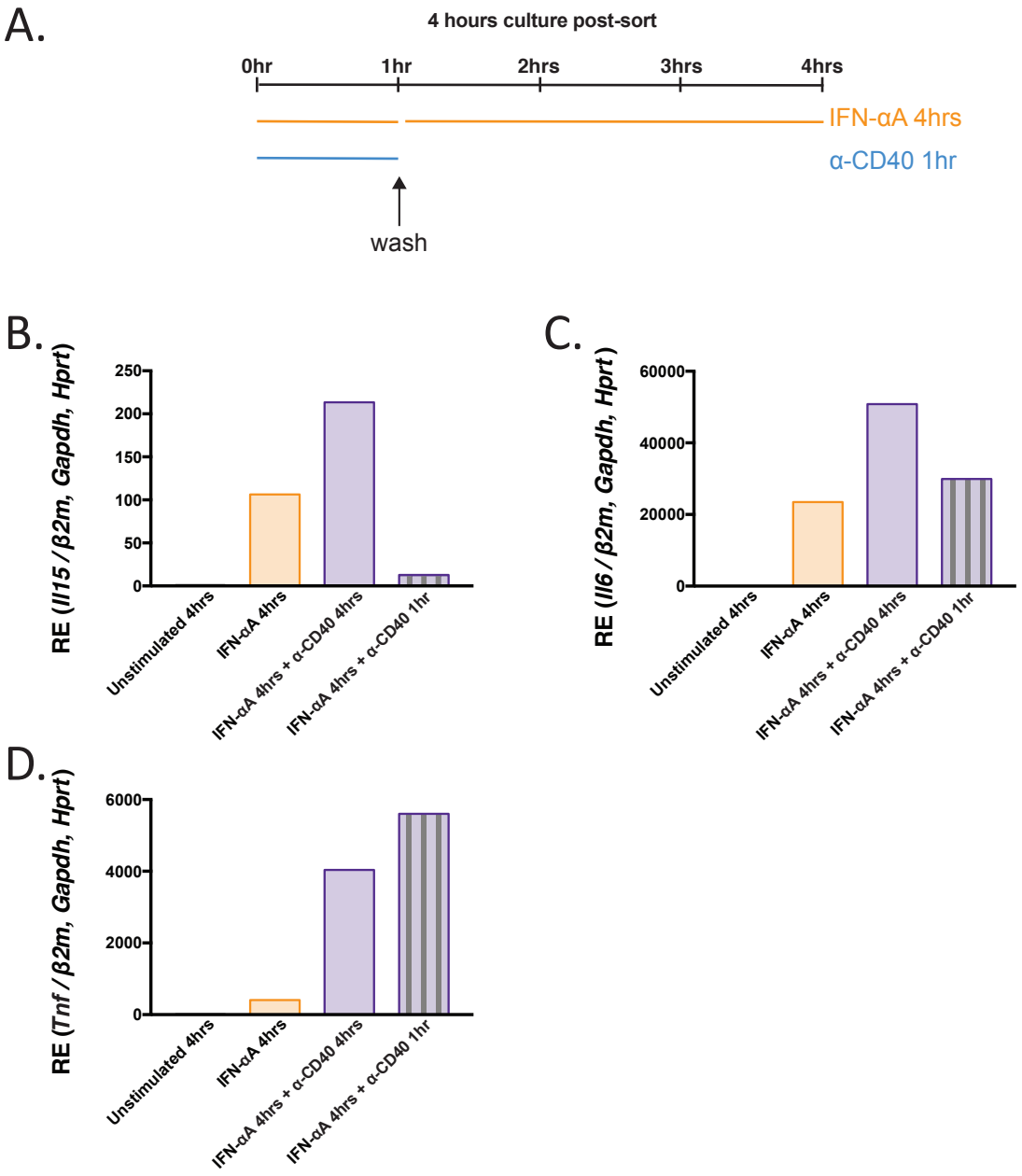



**Figure 4.6: Transient effect of α-CD40 through IFN-αA stimulation.**
eCD8⁺ DCs cultured with media only (Unstimulated 4hrs, white), IFN-αA 4 hours (1000 U/ml) (IFN-αA 4hrs, orange), IFN-αA 4 hours (1000 U/ml) with α-CD40 mAb 4 hours (10 µg/ml) (IFN-αA 4hrs + α-CD40 4hrs, purple) or IFN-αA 4 hours (1000 U/ml) with α-CD40 mAb 1 hour (10 µg/ml) (IFN-αA 4hrs + α-CD40 1hr, grey striped, purple). For this last sample, cells were cultured with IFN-αA and α-CD40 mAb for 1 hour, then cells were collected, washed and resuspended with IFN-αA in fresh media for the remaining time of the experiment **(A.)**. Following stimulation, the regulation of *Il15* **(B.)**, *Il6* **(C.)**, *Tnf* **(D.)** was assessed (n=1).

detectable in the supernatant of stimulated cells for 4 hours or less. Despite the really low induction of *Il12b* at this time point, α-CD40 antibody treatment alone triggered a slight mRNA expression when compared to the unstimulated condition (Chapter 3). Interestingly, while eCD8$^+$ DCs stimulated with both signals IFN-αA and α-CD40 antibody for 4 hours expressed *Il12b* mRNA at a level about 1000-times higher than the IFN-αA-induced expression, *Il12b* was also detected when CD40 was stimulated for 4 hours alone (**Figure 4.5.B**). Compared to *Il12b* expression induced by IFN-αA stimulation, its levels was significantly higher by α-CD40 antibody for 4 hours alone or with 15 minutes of IFN-αA stimulation. Interestingly, this expression seemed to gradually decrease when the duration of IFN-αA stimulation increased and became almost undetectable after 1 hour of IFN-αA stimulation. When constantly stimulated with IFN-αA, eCD8$^+$ DCs initiated *Il12b* mRNA expression from 1 hour of α-CD40 antibody treatment and gradually increased this expression (**Figure 4.5.C**).

The results displayed in Chapter 3 highlighted the requirement of the stimulation with the combination of IFN-αA and α-CD40 antibody to induce a detectable *Il12b* expression. However α-CD40 antibody pre-treatment revealed a significant expression of *Il12b* via CD40 stimulation alone and more importantly its active inhibition triggered by increasing IFN-αA stimulation. This inhibition was subsequently abolished and allowed *Il12b* expression under the combination of IFN-αA and CD40 stimulations.

#### 4.2.1.4 Transient effect of α-CD40 engagement

Once eCD8$^+$ DCs have been stimulated with IFN-αA, they are ready to quickly respond to α-CD40 antibody treatment displaying signs of amplification from as early as 15 minutes of CD40 stimulation (**Figures 4.2.C**, **4.3.C**, **4.4.C**). However, this experimental set up did not demonstrate whether this short hit with α-CD40 antibody is sufficient to subsequently lead to amplification

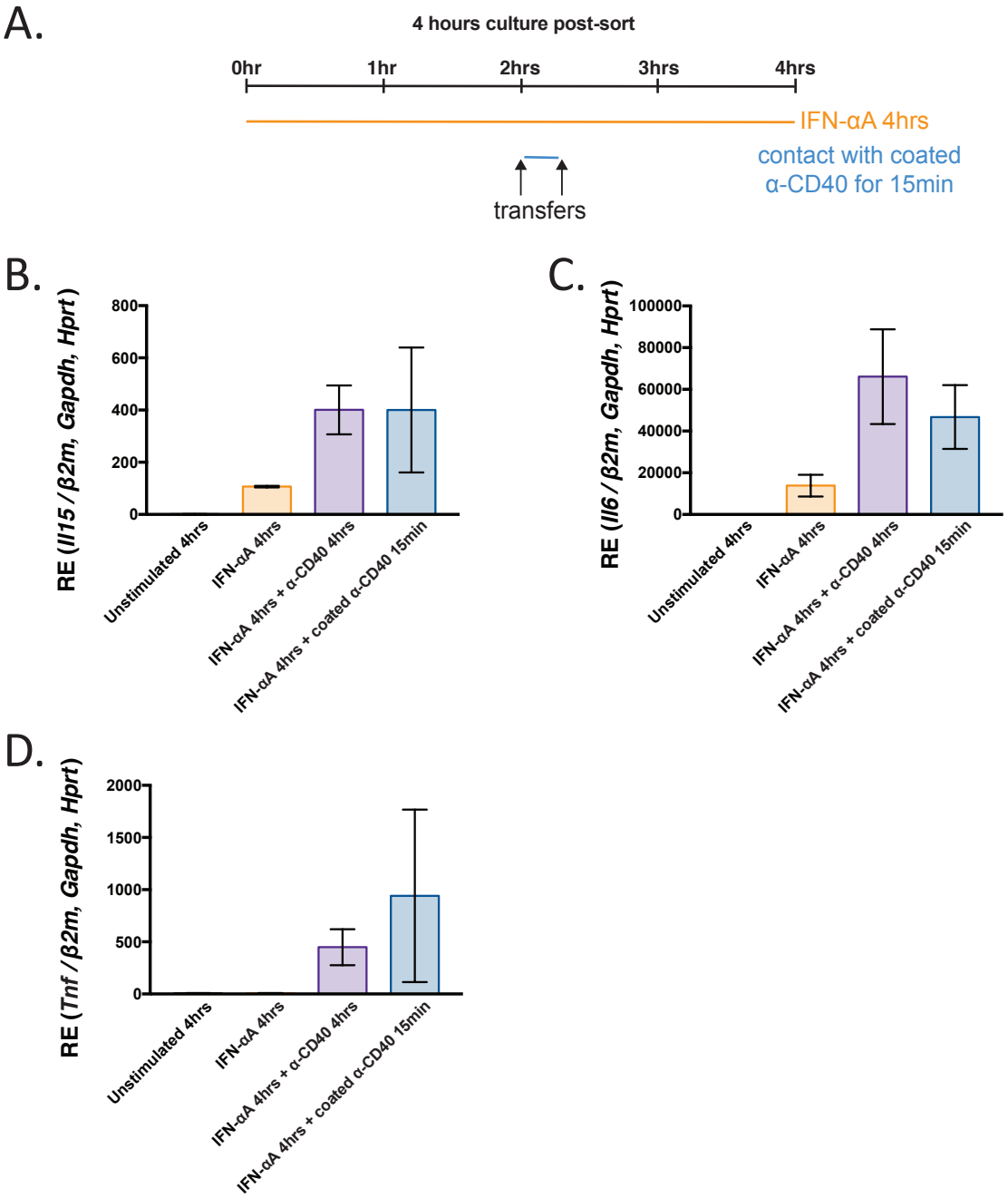



**Figure 4.7: α-CD40 coated plate assay for transient effect of help-driven amplification.**
eCD8⁺ DCs cultured with media only (Unstimulated, white), IFN-αA 4 hours (1000 U/ml) (IFN-αA, orange), IFN-αA 4 hours (1000 U/ml) with α-CD40 mAb 4 hours (10 µg/ml) (IFN-αA 4hrs + α-CD40 4hrs, purple) or IFN-αA 4 hours (1000 U/ml) added to α-CD40 mAb-coated wells for 15 minutes (50 µg/ml) (IFN-αA + α-CD40 coated, dark blue). For this last condition, from 2 hours of IFN-αA stimulation, cells were transferred in α-CD40 coated plate wells for 15 minutes **(A.)** (Pool from 3 independent experiments). Error bars represent mean +/- SEM. Following stimulation, the regulation of *Il15* **(B.)**, *Il6* **(C.)**, *Tnf* **(D.)** was assessed.

comparable with longer CD40 stimulation or whether 4 hours of CD40 engagement is required for an optimal α-CD40-driven amplification of IFN-αA-triggered expression. In order to investigate these two possibilities, CD40 was stimulated in a transient manner in the context of IFN-αA stimulus. eCD8$^+$ DCs were stimulated with both IFN-αA and α-CD40 antibody for an hour. The cells were then collected, washed and cultured again in fresh media for 3 additional hours with IFN-αA only (**Figure 4.6.A**). eCD8$^+$ DCs stimulated with the combination of IFN-αA and α-CD40 antibody for 4 hours displayed the expected amplification of IFN-αA-triggered *Il15* mRNA expression (**Figure 4.6.B**). However, this expression was impaired when the cells were subjected to the transient α-CD40 antibody treatment. On the other hand, those cells displayed level of *Il6* mRNA expression comparable to the level induced by IFN-αA stimulation (**Figure 4.6.C**). Moreover, *Tnf* expression was amplified by the transient effect of α-CD40 antibody at a level comparable to the CD40 stimulation for 4 hours in the presence of IFN-αA (**Figure 4.6.D**). In the context of IFN-αA stimulation, the measurement of *Tnf* regulation seemed to highlight a short CD40 stimulation sufficient to induce its amplification. However, the other cytokines did not respond the same way to the stimulations.

Optimally, eCD8$^+$ DCs would be stimulated with IFN-αA and subsequently treated with α-CD40 antibody for a short amount of time without wash that would lead to cell loss and/or additional stimulation. For a transient and controlled CD40 stimulation, plates were coated with α-CD40 antibody overnight at a concentration of 50 µg/ml, a concentration that induced an α-CD40-driven amplification of IFN-αA-triggered cytokines expression comparable to the one measured when the antibody was diluted in the media (data not shown). eCD8$^+$ DCs were stimulated for 2 hours with IFN-αA, as this duration was enough to induce the mRNA expression of cytokines (**Figures 4.2.A**, **4.3.A**, **4.4.A**) (**Figure 4.7.A**). Activated DCs were then transferred to α-CD40 antibody coated wells for only 15 minutes before pursuing the culture in their original well. *Il15* mRNA expression induced under this condition displayed and amplification about 2-times higher than the IFN-αA-induced *Il15* (**Figure 4.7.B**).

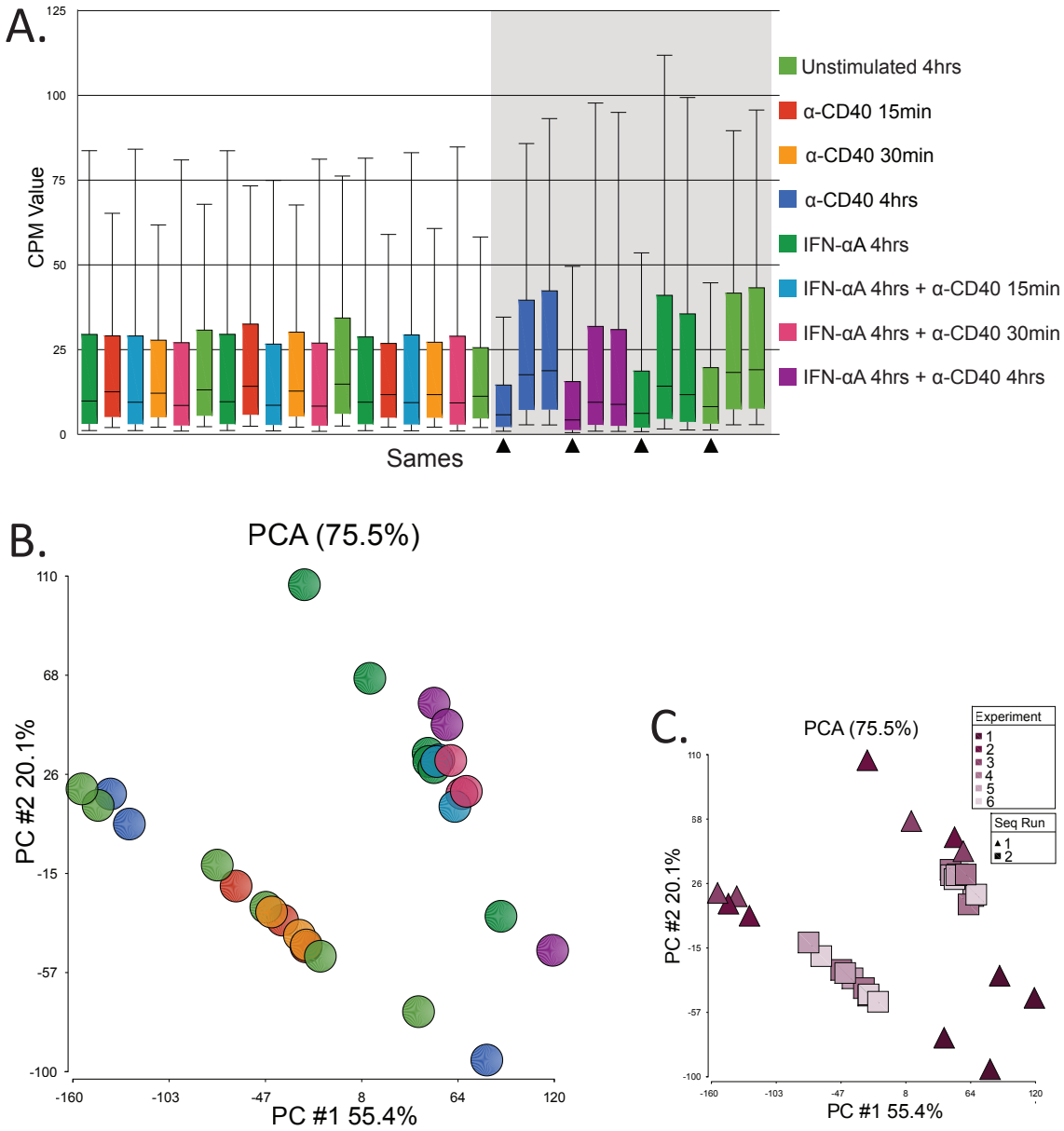



**Figure 4.8: Quality control for samples of 2 independent sequencing runs in a combined analysis.**

RNA sequencing analysis of eCD8⁺ DCs stimulated with media only (Unstimulated), α-CD40 mAb for various timings (10 µg/ml) (α-CD40 4hrs, 30min or 15min), IFN-αA 4 hours (1000 U/ml) (IFN-αA 4hrs) or IFN-αA 4 hours (1000 U/ml) with α-CD40 mAb for various timings (10 µg/ml) (IFN-αA 4hrs + α-CD40 4hrs, 30min or 15min). Samples are from 2 independent sequencing runs, 3 independent experiments for each sequencing runs.

**(A.)** Box Plot of normalised Counts per Million (CPM) values for each sample. Grey background identifies the samples of the sequencing run number 1. White background identifies the samples of the sequencing run number 2. Marked with an arrow are the 4 samples behaving slightly differently than the others. **(B.**, **C.)** Principal Component Analysis (PCA) of 10,222 present genes. Stimulatory conditions, α-CD40 treatment timing and sequencing runs are colored as indicated.

Moreover, this expression was comparable to the one induced in cells stimulated with both IFN-αA and diluted α-CD40 antibody for 4 hours. Similarly, *Il6* (**Figure 4.7.C**) and *Tnf* (**Figure 4.7.D**) IFN-αA-induced respective expression was amplified with this short contact of 15 minutes with α-CD40 antibody coated well. However, an important variability was observed for instance for *Il15* and *Tnf* expression and the effect of transferring DCs during the experiment was difficult to assess. Moreover, a consequent amount of cells remained at the bottom of the coated wells, observed via microscope following the experiments. Therefore, this method was not utilized to pursue the investigation of α-CD40-mediated amplification kinetics.

These results support the hypothesis of a short hit of CD40 stimulation being sufficient to induce the amplification of IFN-αA-triggered cytokine expression. While 1 to 2 hours of IFN-αA stimulation is required to promote DC activation, a brief encounter with the α-CD40 antibody is sufficient to generate the amplification of cytokine and chemokine expressions. Interestingly, while the α-CD40-mediated amplification of Il-6 secretion occurred subsequently of the amplification of its IFNαA-induced mRNA expression, Ccl4 chemokine showed a slightly different pattern. Only 30 minutes of CD40 stimulation efficiently induced the amplification of the IFNαA-mediated Ccl4 secretion in the supernatant and *Ccl4* mRNA expression, these two events occurring with similar dynamics.

### 4.2.2 Investigation of the α-CD40-mediated amplification mechanisms

In order to study the potential pathway(s) responsible for the early and late transcriptional regulation in response to CD40 stimulation, the response of $eCD8^+$ DCs to stimulation was assessed at the transcriptomic level. $eCD8^+$ DCs were kept unstimulated or were treated with α-CD40 antibody and IFN-αA in combination or separately. The α-CD40 antibody treatment was performed for

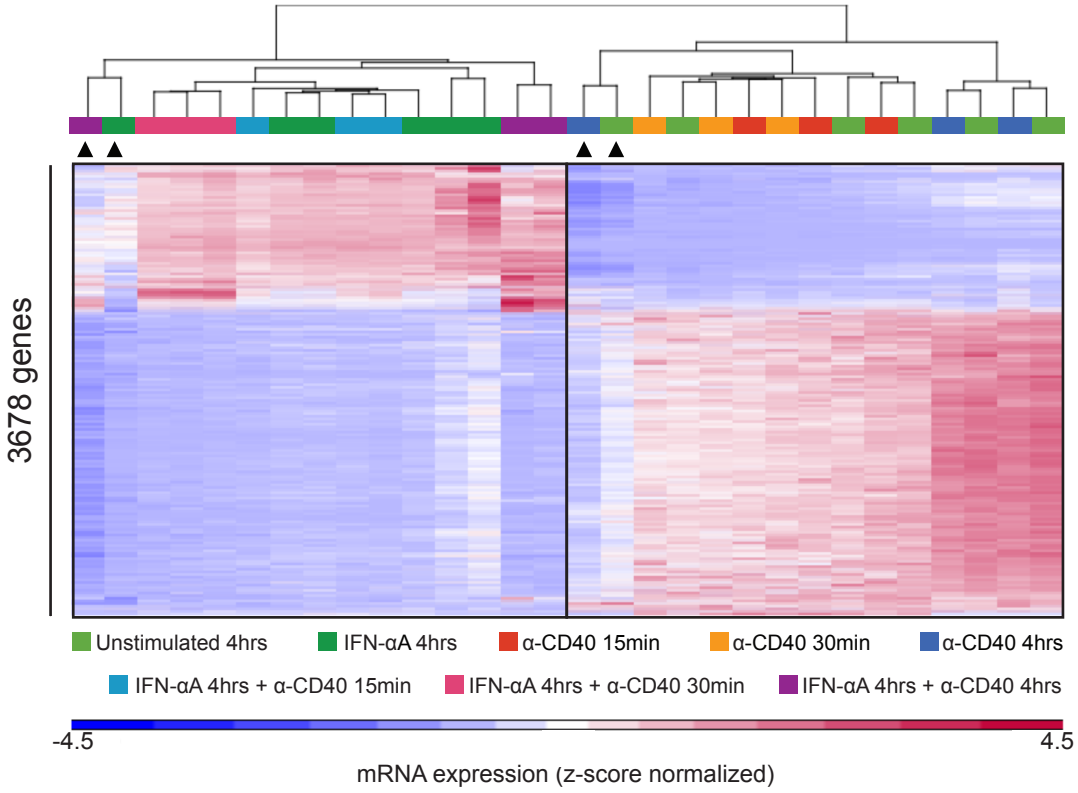



**Figure 4.9: Hierarchical clustering of samples according to the similar expression profiles by the most variable genes through the different conditions.**
Heatmap displaying z-score transformed CPM expression values. FDR adjusted p-value ≤ 0.01. Marked with an arrow are the 4 samples behaving slightly differently than the others.

15 minutes, 30 minutes and 4 hours, to investigate the early and late mRNA regulation as described in **Figure 4.1**. The preparation of the samples was performed into two sets of three experiments, each set sequenced separately. Each experiment contained an unstimulated condition and an IFN-αA stimulated condition: therefore the two independent sequencing runs could be subsequently combined for analysis. 10,349 genes were obtained from normalized and batch corrected data and the distribution of all gene counts, as normalized Counts per Million (CPM) values, per sample was plotted for quality control reasons (**Figure 4.8.A**). The samples from the second sequencing run showed clear homogeneity compared to the samples from the first sequencing run with 4 samples, deriving from one experiment, that behaved differently. However, the distribution of the gene reads for each sample showed a median value consistent through both sequencing runs allowing the comparison of the responses to the respective conditions (**Figure 4.8.A**). About 10,222 present genes were extracted on the basis of the mean CPM values of at least one condition equal or higher than the defined background value. The similarities and differences within the conditions were further assessed by reducing the dimension of the data in form of a Principal Component Analysis (PCA) on the normalized CPM values (**Figure 4.8.B**). The two first principal components, PC1 and PC2, summarized more than 75% of the overall variance of the data set. The samples clearly clustered into two groups according to similar expression patterns. One cluster contained the unstimulated DCs and the samples treated with α-CD40 antibody alone. All the samples stimulated with IFN-αA with or without α-CD40 antibody clustered together separately, demonstrating that IFN-αA triggered important changes in the transcriptional programming of DCs. While the four samples of the first experiment displayed a higher variance than the others, those were still clustering in accordance with their conditions within the two different groups (**Figure 4.8.C**). Therefore, the quality of the data set allowed further analysis.

In addition of providing a first insight in the gene regulation through the various conditions, the hierarchical clustering of the 3678 most variable genes

A.

| Comparisons | Number of DE genes | |
|---|---|---|
| | Up | Down |
| Unstimulated 4hrs vs α-CD40 15min | 0 | |
| Unstimulated 4hrs vs α-CD40 30min | 0 | |
| Unstimulated 4hrs vs α-CD40 4hrs | 0 | |
| Unstimulated 4hrs vs IFN-αA 4hrs | 5186 | |
| | 1237 | 3949 |
| IFN-αA 4hrs vs IFN-αA 4hrs + α-CD40 15min | 21 | |
| | 21 | 0 |
| IFN-αA 4hrs vs IFN-αA 4hrs + α-CD40 30min | 115 | |
| | 112 | 3 |
| IFN-αA 4hrs vs IFN-αA 4hrs + α-CD40 4hrs | 295 | |
| | 232 | 63 |

B.

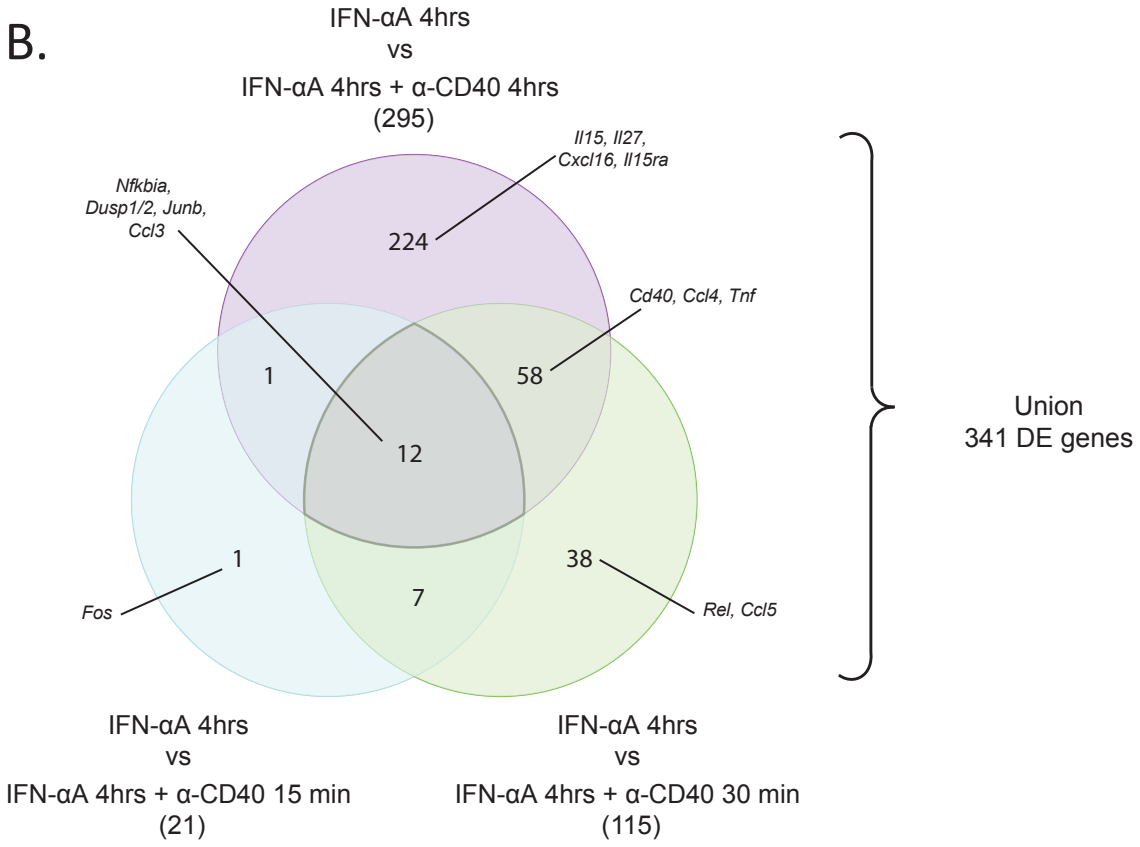
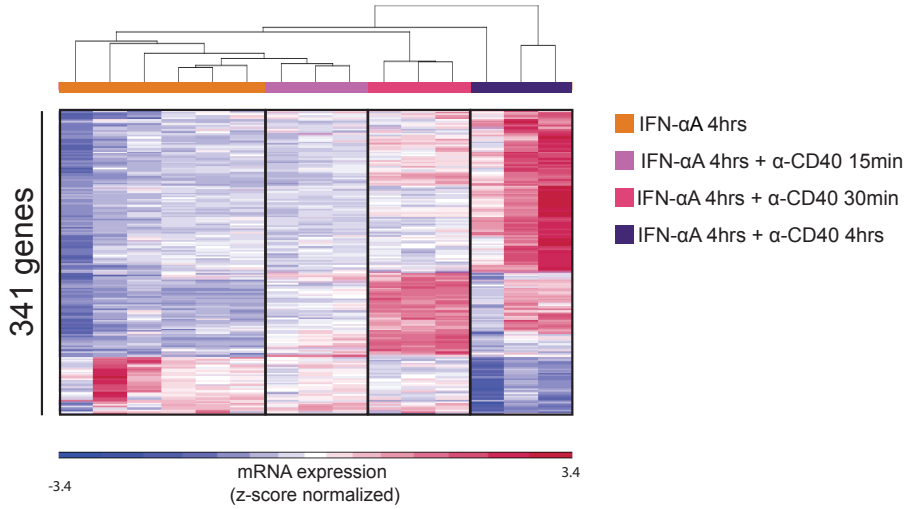



**Figure 4.10: Overviews of proportions of differentially expressed (DE) genes between stimulatory conditions.**
**(A.)** Analysis of 10,222 present genes with changes in expression greater than Fold Change (FC) |1.5| and a FDR-corrected step-up p-value ≤ 0.05 (Benjamini & Hochberg) were defined as differentially expressed (DE). Total number of DE genes are marked in green, increased expression are marked in red (Up) and decreased expression are marked in blue (Down).
**(B.)** Venn diagram of the 341 DE genes between IFN-αA 4 hours and all IFN-αA 4 hours + α-CD40 conditions.

corroborated the PCA results (**Figure 4.9**). Samples from unstimulated DCs clustered again with samples treated with α-CD40 antibody for different durations. This result indicates that the stimulation of CD40 alone does not lead to significant changes in the DC transcriptional regulation. In contrast, IFN-αA-stimulated samples in absence and presence of α-CD40 antibody clustered together (**Figure 4.9**, left part). Again, four samples from the first experiment displayed a slightly different behaviour. However, their overall gene regulation was mainly dictated by IFN-αA stimulation and followed the same trend compared to the samples they clustered with. Moreover, the number of differentially expressed (DE) genes proportionally illustrated the gene regulation and therefore the impact of stimuli on the DCs (**Figure 4.10.A**). The comparison of the gene regulation under the various conditions did not reveal significant differences induced by the α-CD40 antibody treatment alone. As expected from the PCA and heatmap of the most variable genes (**Figures 4.8** and **4.9**), a large number of DE genes were identified when IFN-αA stimulation was compared to the unstimulated control, with about 1237 genes up-regulated and 3949 genes down-regulated. This IFN-αA stimulation were further compared to the responses induced by the combination of IFN-αA with α-CD40 antibody treatment. Interestingly, the number of DE genes relative to the IFN-αA alone group progressively increased the longer the treatment duration was. Indeed, 21 genes were significantly regulated following only 15 minutes of CD40 stimulation while 30 minutes triggered the regulation of 115 genes. Finally, 295 genes were significantly regulated by 4 hours of α-CD40 antibody added to IFN-αA stimulation. In total, 341 genes were significantly regulated when IFN-αA with α-CD40 treatment conditions were compared to IFN-αA stimulation alone (**Figure 4.10.B**). Therefore, while the strongest gene regulation was induced by IFN-αA stimulation, the addition of α-CD40 treatment induced increasing changes in the mRNA expression of various genes.

Focusing only on the 341 DE genes induced when α-CD40 antibody was added to IFN-αA stimulation, the samples clustered together depending on their respective condition, showing that the gene regulation the cells displayed was

A.




B.

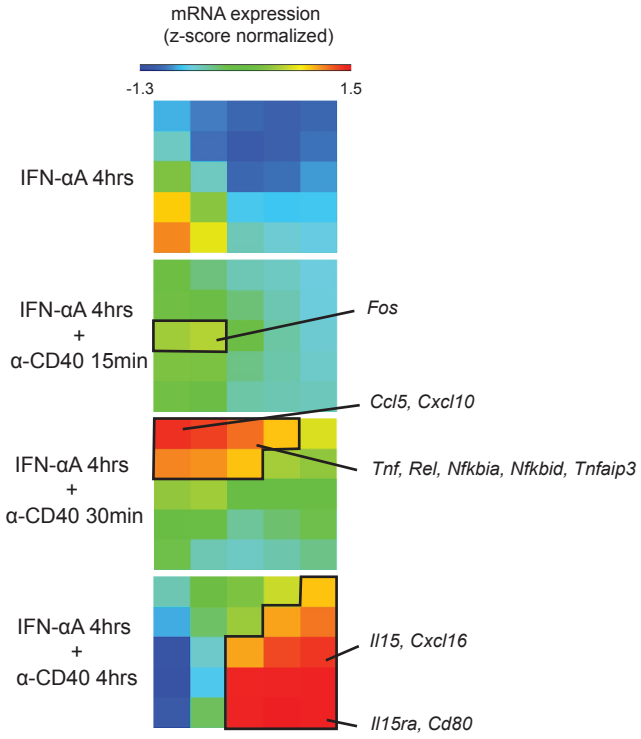



**Figure 4.11: Time kinetics of α-CD40-mediated gene regulation.**
**(A.)** Hierarchical clustering of samples according to similar expression profiles by the union of 341 DE genes as determined in **Figure 4.11.B**. Standardised expression values were shifted to mean zero and scaled to standard deviation of one.
**(B.)** Self Organizing Map (SOM) of genes belonging to the union of 341 DE genes as determined in **Figure 4.11.B** according to their expression into 25 clusters. Clusters following similar patterns of expression are highlighted. Some genes are identified within their respective cluster.

caused by the stimulation they received (**Figure 4.11.A**). The self-organizing map (SOM) clustering is an unsupervised technique of pattern recognition through different conditions. Therefore, SOMs can be used to define groups of genes with similar expression patterns. Performing SOM clustering on the 341 DE genes defined above allowed the separation of the DE genes into 24 different patterns of gene expression throughout the conditions (**Figure 4.11.B**) (see Appendix **Table A.1** for a complete list of the 341 genes divided into the 24 clusters). One square represents one cluster of genes following the same pattern of regulation. For instance, the upper right square, dark blue under IFN-αA stimulation, became light blue/ light green with 15 to 30 minutes of α-CD40 and orange with 4 hours of treatment. Therefore, the group of genes contained in this cluster became gradually up-regulated with longer duration of CD40 stimulation. Taken together, hierarchical and SOM clustering analysis revealed a gradual regulation of the genes, the majority of which is not induced by treatment with IFN-αA alone (**Figure 4.10.A** and **B**). Indeed, the effect of the addition of α-CD40 antibody treatment for the last 15 minutes of IFN-αA stimulation was confined to a small group of genes specifically regulated at that time point, noticeably containing *Fos*. With 30 minutes of treatment added to IFN-αA stimulation, up-regulated genes were grouped into 7 clusters. Chemokines such as *Ccl5*, *Cxcl10* or NF-κB-related genes belonged to this clusters and followed the same pattern of expression. Interestingly, a large part of them were not up-regulated anymore when α-CD40 antibody treatment was applied for 4 hours. Instead, the expression of another group of genes increased highlighting a specific group of gene clusters regulated with 4 hours of CD40 ligation, genes that were not rapidly regulated with only 15 or 30 minutes of CD40 stimulation. In accordance with the results presented in Chapter 3, this group of gene clusters contained pivotal genes for the DC functions such as *Il15* or *Il15rα*. The Venn diagram performed on the 341 DE genes between IFN-αA compared to IFN-αA with α-CD40 antibody for the three time points also illustrated the differences and similarities between these lists of genes as well (**Figure 4.10.B**). Only 12 genes were differentially expressed in a sustained manner that occurred at all time points examined after α-CD40

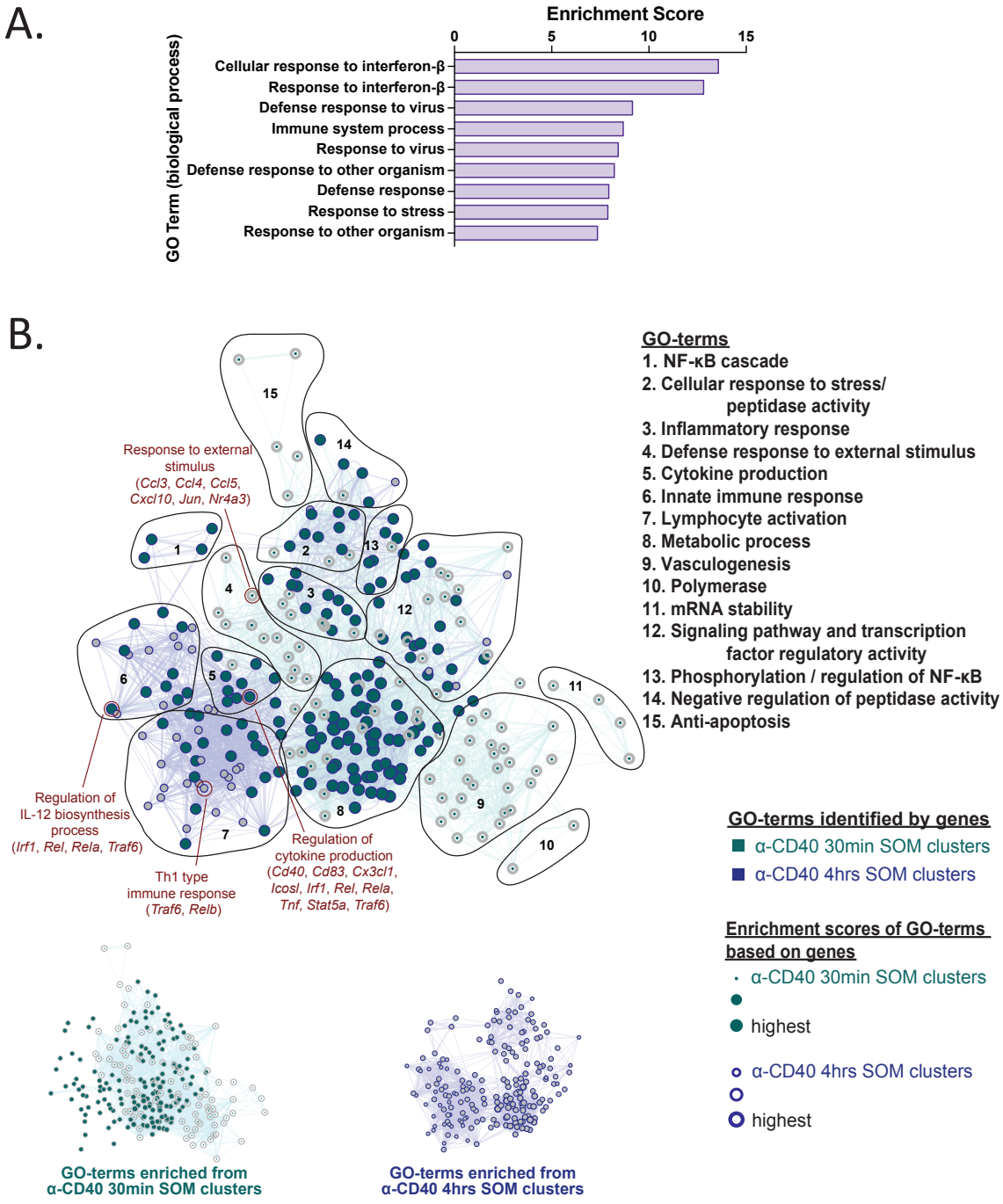


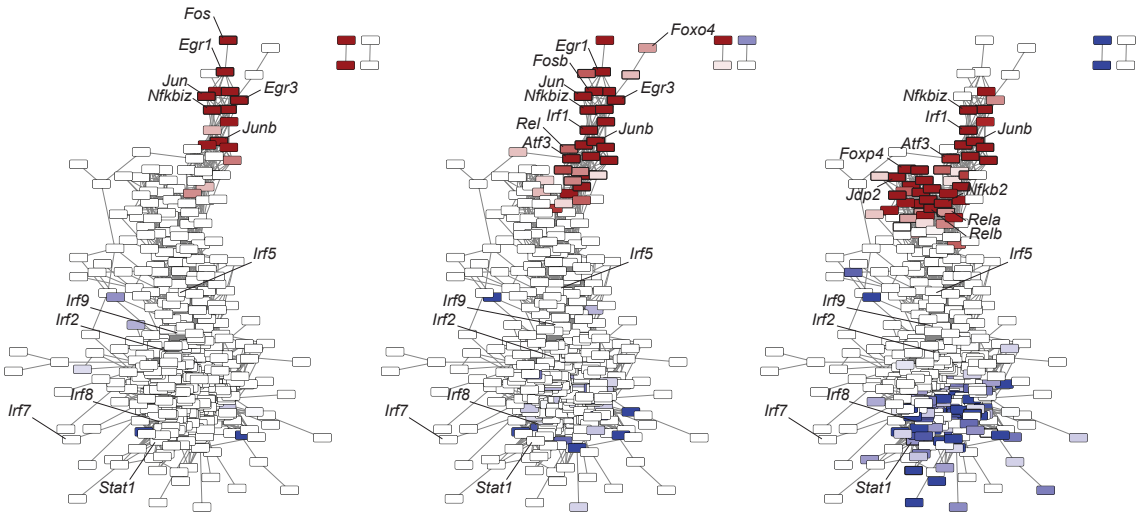

**Figure 4.12: Biological functions of SOM clusters.**
**(A.)** Biological process Gene Ontology (GO)-term enrichment performed on 15 minutes gene clusters (as displayed in **Figure 4.12.B**). GO-terms with a p-value < 0.001 are displayed.
**(B.)** Network of GO-term enrichment performed on 30 minutes gene clusters (as displayed in **Figure 4.12.B**) (turquoise) and 4 hours gene clusters (as displayed in **Figure 4.12.B**) (blue). Nodes represent the GO-terms, edges represent a score inversely proportional of the genes shared between the two GO-terms. GO-terms with a p-value ≤ 0.005 and connected with three or more GO term are displayed.

treatment. Those genes were for instance *Nfkbia* or *Dusp1/2* and *Junb* related to the NF-κB or MAPKinase signalling pathways respectively, or the chemokine *Ccl3*. About 224 genes, including *Il15* and *Il15ra*, were specifically regulated under IFN-αA and α-CD40 antibody 4 hours and 38 genes were following IFN-αA with α-CD40 treatment for 30 minutes. Corroborating the SOM clustering, *Fos* was the only gene specifically and significantly regulated after 15 minutes of CD40.

Overall, the RNA sequencing analysis revealed a major transcriptomic regulation occurring following IFN-αA stimulation. CD40 ligation induced a rapid gene regulation in the context of this IFN-αA stimulation. Interestingly, the duration of CD40 signalling impacts distinct group of genes highlighting time kinetics of α-CD40-mediated gene regulation.

#### 4.2.2.1 α-CD40-mediated functional regulation

To investigate changes in the biological program induced by IFN-αA stimulation over the initial phases of DC activation, 15 minutes, 30 minutes or 4 hours of CD40 stimulation induced the regulation of distinct groups of genes. Gene ontology (GO) analysis was performed to link the DE genes to biological processes of the cells (**Figure 4.12**). Regarding the small group of clusters containing genes that were rapidly up-regulated with the addition of α-CD40 antibody for 15 minutes (see **Figure 4.11.B**), the top enriched GO terms were linked to the response to IFN and to the immune responses to virus or stress (**Figure 4.12.A**). Although only a few genes were part of this group, 14 out of 15 genes belonged to the Interferome (Rusinova et al. 2013) and specifically to the Type I IFN signature genes. The BiNGO plugin in combination with the EnrichmentMap plugin of Cytoscape allows investigating the GO-terms significantly overrepresented within only two sets of genes. The number of genes regulated after only 15 minutes of α-CD40 treatment being low and therefore not included in this analysis. However this tool was used to visualize




IFN-αA 4hrs
vs
IFN-αA 4hrs + α-CD40 15min

IFN-αA 4hrs
vs
IFN-αA 4hrs + α-CD40 30min

IFN-αA 4hrs
vs
IFN-αA + α-CD40 4hrs

**Figure 4.13: Transcription factors network.**
Network of transcription factors genes. Colours represent the expression as standardised to the mean of IFN-αA-induced expression. Red are the up-regulated genes under the indicated comparison; blue are the down-regulated genes under the indicated comparison. Framed are the significant expression with a p-value ≤ 0.05.

the changes in biological functions induced between 30 minutes and 4 hours of α-CD40 antibody treatment added to IFN-αA stimulation (**Figure 4.12.B**). Each node represents a GO-term, enriched either after 30 minutes or 4 hours of α-CD40 treatment. The inner and outer parts of each node represent the enrichment score of the GO-term at the respective time point and are therefore proportional to the contribution of the α-CD40 30 minutes and 4 hours data set respectively. For instance, a large dark blue outer part of the node represents an important contribution of the α-CD40 4 hours SOM clusters for the concerned GO-term. The edges (links between the nodes), thickness and length represent the number of common genes that belong to both GO-terms. Thus, the closer two GO-terms are, the higher is the number of genes they share. GO-terms cluster together, sharing genes and similar functions. Finally, WordCloud was used to determine the most common words in a cluster of GO-terms. As expected, the α-CD40 treatment for 4 hours induced genes that allowed the enrichment of GO-terms related to immune responses (blue borders, **Figure 4.12.B**), with for instance the cytokine production, containing *Cd40*, *Irf1*, *Tnf* and *Traf6* (GO-terms cluster 5), or the lymphocyte activation, with *Traf6* and *Relb* potentially involved in Th1 responses (GO-terms cluster 7). With only 30 minutes of α-CD40 treatment, genes are linked to anti-apoptotic processes (GO-terms cluster 15) and response to stimulus (GO-terms clusters 4 and 9) (turquoise borders, **Figure 4.12.B**). In addition, transcriptional activity was rapidly initiated after 30 minutes of CD40 ligation (GO-terms clusters 10, 11 and 12). Importantly, the NF-κB pathway was highlighted as involved from 30 minutes up to 4 hours, in a sustained manner (GO-terms clusters 1 and 13).

The rapid enrichment of genes associated to the transcriptional activity encouraged the investigation of the regulation of the TF. Filtering the entire data set with the murine TFs and chromatin remodelers, about 580 TFs were expressed by eCD8$^+$ DCs in at least one of the different experimental conditions assessed. Focusing on the IFN-αA 4 hours and all IFN-αA 4 hours with α-CD40 conditions, the expression patterns of 505 co-regulated TFs were chosen to generate a network structure based on Pearson correlation ($\rho$=0.83). Each TF

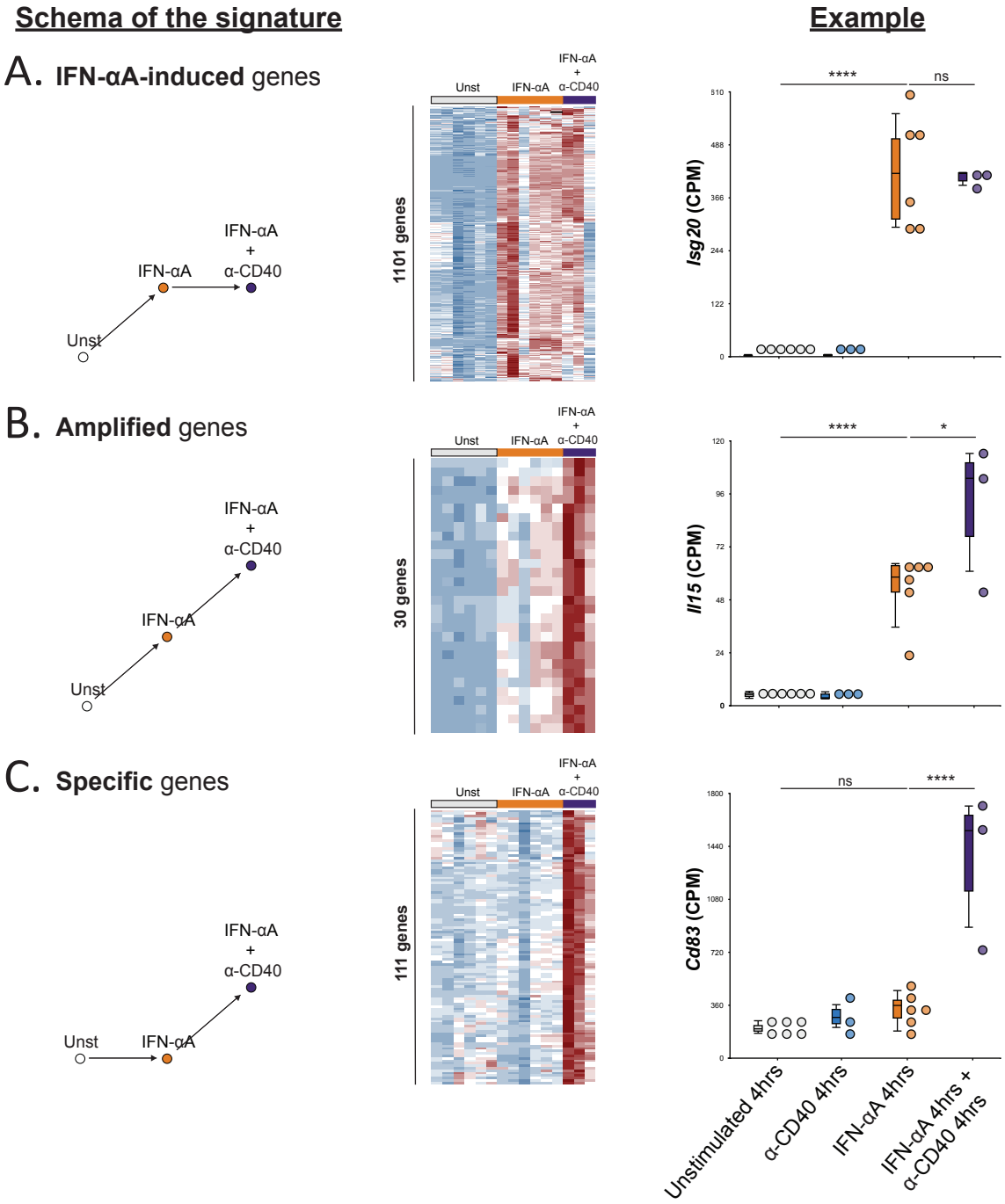



**Figure 4.14: Differential gene expression patterns.**
IFN-αA-induced only genes **(A.)**, amplified genes **(B.)** and specific genes **(C.)** expression patterns illustrated with schema (left), heatmap (middle) and box plot of one example mRNA expression (right). For the box plot, each dot is a sample, the line inside the box is the median, the whiskers represent the variability. Asterisks indicate statistically significant differences between Unstimulated condition and IFN-αA condition or between IFN-αA condition and IFN-αA + α-CD40 condition as assessed by one-way ANOVA; adjusted p-value: ns = non significant; * p≤0.032; **** p<0.0001.

is represented by a node and is linked to other TFs according to similar expression patterns in the data set (**Figure 4.13**). Only 15 minutes of α-CD40 treatment added to IFN-αA stimulation was enough to induce a rapid up-regulation of a group of TFs including key regulators such as *Fos*, *Jun* and *Junb*. 30 minutes of α-CD40 treatment triggered a distinct pattern, allowing the significant up-regulation of *Irf1*, *Rel* and *Atf3*. Furthermore, many of those rapidly regulated TFs were not anymore significantly induced when α-CD40 antibody was applied for 4 hours. Instead, the expression of *Nf-κB2*, *Rela* and *Relb* was newly enhanced with longer CD40 stimulation. Interestingly, while the enhancement of expression of TFs was regulated in a dynamic manner through α-CD40 treatment, a group of IFN-α/β-related TFs such as *Irf2*, *Irf5*, *Irf7* or *Stat1* were unchanged or slightly but not significantly down-regulated under CD40 stimulation (**Figure 4.13**, see Appendix **Figure A.1** for gene regulation of *Irfs* and *Stat1/2*).

The gene regulation established by α-CD40 treatment clearly follows a precise kinetic. This dynamic regulation affects distinct biological functions of the DCs. Genes involved in cell survival and immune defences were rapidly amplified, setting up the responses of cells to their stimulus. These immune responses were further enhanced in a sustained manner with longer CD40 signalling. Importantly, a clear program controlling the transcriptional activity was rapidly induced within only 15 minutes to 30 minutes of CD40 stimulation. This might indicate the *de novo* synthesis of cellular proteins required for the DCs responses to help signal.

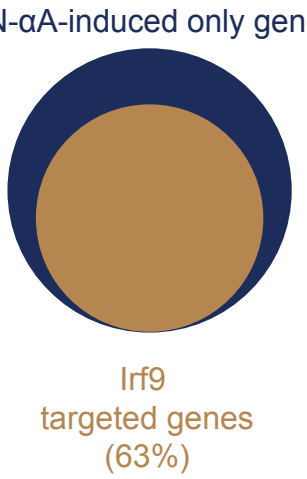
#### 4.2.2.2 Various gene regulation patterns in response to IFN-αA and α-CD40 stimuli

Different processes influence gene expression such as transcriptional regulatory elements, TFs and chromatin rearrangements (Maston et al. 2006). As observed previously, α-CD40 treatment triggered a dynamic program of

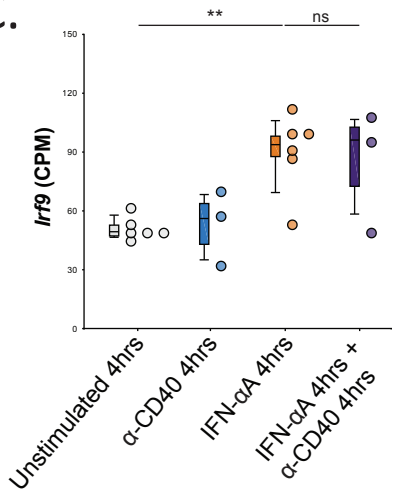
A.

| Cluster | Transcription Factors | NES |
|---|---|---|
| M1 | Irf4, Irf8, Irf5, Irf3, Irf9, Zeb1 | 10.562 |
| M2 | Stat1, Stat6, Stat2, Irf1, Irf2, Irf3, Irf4, Irf5,... | 10.137 |
| M3 | Irf1, Irf2, Irf3, Irf4, Irf5, Irf6, Stat1, Stat2,... | 8.320 |

B.




C.




D.

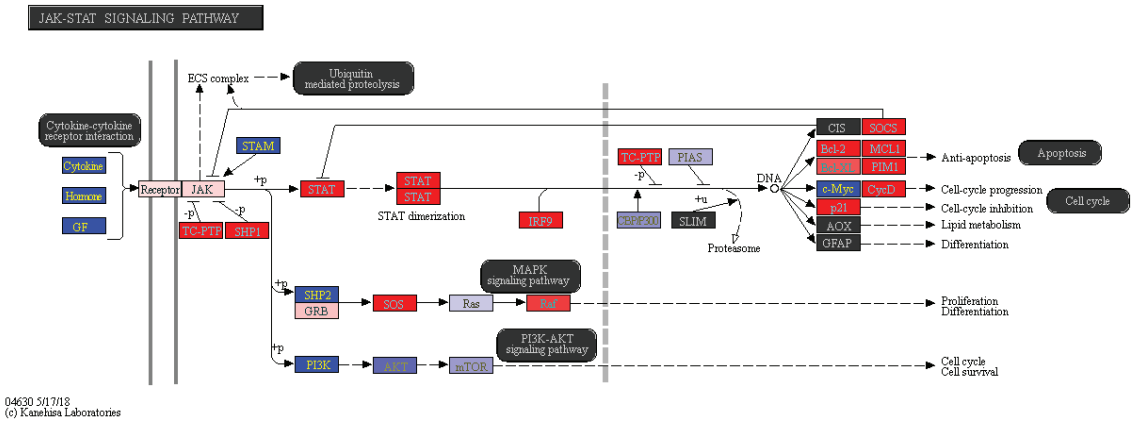



**Figure 4.15: Transcription factors binding prediction IFN-αA-induced genes.**
**(A.)** Top three of the transcription factors (TFs) motifs clusters enriched for IFN-α A-induced genes signature as presented in **Figure 4.15.A**.
**(B.)** Venn diagram of IFN-αA-induced genes targeted by Irf9.
**(C.)** Box plot of *Irf9* expression: each dot is a sample, the line inside the box is the median, the whiskers represent the variability. Asterisks indicate statistically significant differences between Unstimulated condition and IFN-αA condition or between IFN-αA condition and IFN-αA + α-CD40 condition as assessed by one-way ANOVA; adjusted p-value: ns = non significant; ** p≤0.002.
**(D.)** JAK-STAT pathway enrichment, colours represent -1.5 ≤ FC (blue) and FC ≤ 1.5 (red) under the comparison Unstimulated vs IFN-αA 4 hours.

transcriptional activity and processes. Aiming to investigate the possible gene regulation induced by the CD40 stimulation, precise expression patterns were defined (**Figure 4.14**). The expression of the majority of IFN-αA-triggered genes was not affected by α-CD40 antibody treatment (**Figure 4.14.A**). The expression of this group of 1101 IFN-αA responsive genes, including for instance *Isg20*, was induced by IFN-αA stimulation and displayed no change with the addition of α-CD40 treatment. Those genes are called “IFN-αA-induced only” genes. As described previously *Il15* and 29 other genes expression was triggered by IFN-αA stimulation and significantly enhanced when CD40 was stimulated in addition (**Figure 4.14.B**). Those genes are the “amplified” genes. Finally, the RNA sequencing analysis highlighted a third pattern of expression. A group of genes neither triggered by IFN-αA stimulation on its own nor by CD40 stimulation required the combination of both, IFN-αA and α-CD40 signals to be induced (**Figure 4.14.C**). This was the case for 111 genes, including *Cd83*.

The stimulation of DCs with IFN-αA in combination with α-CD40 treatment induces precise and gene specific regulation patterns. Interestingly, within the large amount of IFN-αA-induced genes, some of these genes respond to α-CD40 mAb addition, being amplified, while other genes seem immune to its effect. Under our statistical analysis characteristics, a third pattern of gene expression requiring the combination of IFN-αA and α-CD40 treatment was highlighted. This might indicate various molecular mechanisms responsible for α-CD40-driven amplification and IFN-αA and α-CD40 signalling synergy.

#### 4.2.2.3 Mechanism of α-CD40-mediated amplification of IFN-αA-induced gene expression

To investigate the potential regulatory mechanisms of α-CD40-driven gene amplification, TFs potentially involved in the expression of the DE genes were predicted by iRegulon. In this promoter binding prediction (PBP) analysis,

A. All amplified genes 30 min (46 genes)

| Cluster | Transcription Factors | NES |
|---|---|---|
| M1 | Irf1, Irf2, Irf3, Irf4, Irf5, Irf6, Stat1, Stat2,... | 4.993 |
| M2 | Mef2a, Mef2b, Mef2c, Mef2d,... | 4.754 |
| M3 | Mef2a, Mef2b, Mef2c, Mef2d, Stat1, Stat2,... | 4.632 |

B. All amplified genes 4 hrs (30 genes)

| Cluster | Transcription Factors | NES |
|---|---|---|
| M1 | Irf1, Irf2, Irf3, Irf4, Irf5, Irf6, Stat1, Stat2,... | 6.184 |
| M2 | Nf-κb1, Nf-κb2, Bcl3, E2f1, Relb,... | 5.704 |
| M3 | Myb, Mybl2, Mybl1, Stat6,... | 5.601 |

C.

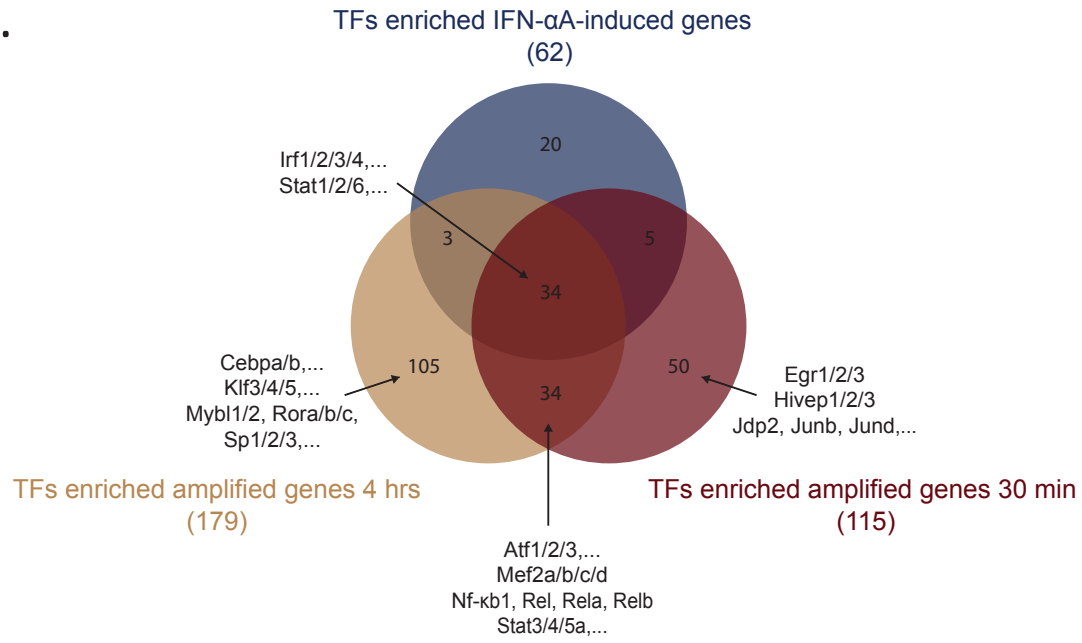



**Figure 4.16: Transcription factors binding prediction amplified genes.**
**(A.)** Top three of the TFs motifs clusters enriched for amplified 30 minutes genes signature as presented in **Figure 4.15.B**.
**(B.)** Top three of the TFs motifs clusters enriched for amplified 4 hours genes signature as presented in **Figure 4.15.B**.
**(C.)** Venn diagram of all TF motifs enriched for IFN-αA -induced genes, amplified genes 30 minutes and amplified genes 4 hours.

similar motifs recognised by TFs are grouped into clusters of enriched motifs. Different TFs can be associated to a motif. Therefore, a cluster of motifs can potentially be recognised by a group of different TFs. Clusters of motifs are classified depending on their Normalized Enrichment Score (NES), ranking the likelihood of a motif to be involved in the regulation of the genes assessed. IFN-α/β stimulation triggers the activation of the JAK-STAT pathway eventually leading to the formation of ISGF3 complex containing IRF9, STAT1 and STAT2 TFs involved in the ISGs expression (Chapter 1, **Figure 1.2**). As expected, the three top scored clusters motifs enriched for the groups of IFN-αA-induced genes included IRFs and STATs TF family members (**Figure 4.15.A**). 63% of the 1101 IFN-αA-induced only genes contained motifs targeted by IRF9 (**Figure 4.15.B**). Like other IRF family members and consistent with the TF footprint (**Figure 4.13**), *Irf9* was significantly induced following IFN-αA stimulation and was not regulated by the addition of α-CD40 antibody treatment (**Figure 4.15.C**). Moreover, when comparing IFN-αA-stimulated eCD8$^{+}$ DCs with unstimulated cells, most of the JAK-STAT pathway actors were up-regulated highlighting a possible positive feedback regulation of IFN-αA (**Figure 4.15.D**). The main characteristic of the amplified genes is that they first undergo an up-regulation triggered by IFN-αA signal alone. Therefore, the underlying mechanism of regulation of these genes could be equivalent to the IFN-αA-induced only genes regulation and involve an additional regulator specific and responsible for the amplification phenomenon. Unfortunately, the number of genes amplified following only 15 minutes of CD40 stimulation was too low to conduct the PBP. The highest enriched cluster of motifs for the amplified genes following 30 minutes (**Figure 4.16.A**), or 4 hours of α-CD40 treatment (**Figure 4.16.B**) was similar, containing Irfs and Stats. When the enrichment results from PBP for the IFN-α-induced only genes and the amplified genes were compared, about 34 TFs were predicted as possibly involved in the expression of all three sets of genes (**Figure 4.16.C**). As expected, the Irf family members as well as the Stats proteins constituted this group of TFs potentially regulating the expression of the three sets of genes. Those TFs are therefore strong candidates for the expression of IFN-αA-induced only genes and for the IFN-

A. Irf9-targeted amplified genes 30 min (23 genes)

| Cluster | Transcription Factors | NES |
|---|---|---|
| M1 | Irf1, Irf2, Irf3, Irf4, Irf5, Irf6, Stat1, Stat2,... | 5.445 |
| M2 | Grhl1 | 4.529 |
| M4 | Spib, Ets1, Elk1, Pura, Myb, Gabpb1,... | 4.397 |
| M5 | Mef2a, Mef2b, Mef2c, Mef2d,... | 4.274 |
| M6 | Nf-κb1, Nf-κb2, Bcl3, Hivep1, Relb,... | 4.106 |

B. Irf9-targeted amplified genes 4 hrs (18 genes)

| Cluster | Transcription Factors | NES |
|---|---|---|
| M1 | Irf1, Irf2, Irf3, Irf4, Irf5, Irf6, Stat1, Stat2,... | 6.446 |
| M2 | Nf-κb1, Nf-κb2, Bcl3, E2f1, Relb,... | 5.951 |
| M3 | Myb, Mybl2, Mybl1, Stat6,... | 5.844 |

**Figure 4.17: Transcription factors binding prediction amplified Irf9-targeted genes.**
**(A.)** Top five of the transcription factors motifs clusters enriched for Irf9-targeted amplified 30 minutes genes signature.
**(B.)** Top three of the transcription factors motifs clusters enriched for Irf9-targeted amplified 4 hours genes signature.

αA-mediated expression of the amplified genes. As highlighted previously, genes rapidly regulated with only 30 minutes of α-CD40 treatment are different than the genes regulated following 4 hours of treatment and can be linked to a slightly different biological program (**Figure 4.12.B**). Therefore, it is not surprising that different TFs could be involved in the expression of the two sets of amplified genes. The genes rapidly amplified display motifs uniquely recognised by Egr, Hivep and Jun families while Klfs and Sp1/2/3 TFs can bind specifically to the genes amplified under 4 hours of α-CD40 treatment. Following the hypothesis that the amplification process could involve the same mechanism at 30 minutes and 4 hours of CD40 stimulation, a group of 34 TFs was commonly enriched for the two sets of genes: the family of NF-κB TFs were found in this group. Importantly, this family of TFs was not enriched for the IFN-αA-induced only genes. Therefore, the presence of motifs recognized by TFs such as NF-κB TFs could govern which of the IFN-αA-induced genes can be amplified.

It has been described earlier that IRF9 is crucial for ISGF3 binding to ISRE and therefore for ISGs expression in response to IFN-α/β (Au-Yeung et al. 2013). Irf9 was further considered as a major candidate regulator of IFN-αA-induced genes. Lists of α-CD40-amplified genes were filtered for the genes possibly targeted by Irf9. When the Irf9-targeted genes were filtered from the 30 minutes amplified genes (**Figure 4.17.A**) and from the 4 hours amplified genes (**Figure 4.17.B**), the NF-κB members appeared in the enriched TF motif clusters with a high NES. Abundantly linked to CD40 stimulation (**Figure 1.4**) (Elgueta et al. 2009; Ma and Clark 2009), this signalling cascade was previously highlighted as regulated from 30 minutes to 4 hours of CD40 stimulation in the SOM clustering analysis (**Figure 4.12.B**). Furthermore, the NF-κB signalling pathway had the highest enrichment score on the DE genes between IFN-αA stimulation and IFN-αA with α-CD40 treatment for 4 hours meaning that many of those DE genes are involved in the signalling pathway (**Figure 4.18**). Focusing on the genes involved in the precise enriched KEGG pathway, most of them, for instance *Traf6*, *Tnf*, *Tnfaip3*, followed a specific gene signature

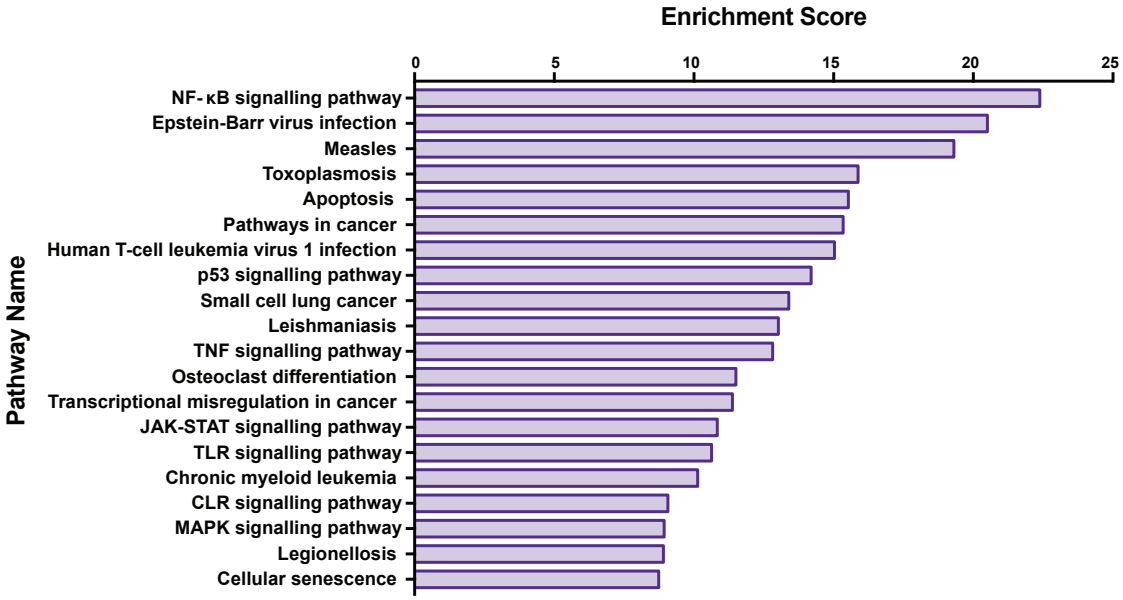



**Figure 4.18: Pathway enrichment analysis of DE genes induced by the addition of α-CD40 treatment to IFN-αA stimulation.**
KEGG pathway enrichment analysis performed on DE genes between IFN-αA 4 hours and IFN-αA 4 hours + α-CD40 4 hours. Pathways enriched with a p-value < 0.0001.
The DE genes were identified with -1.5 ≤ FC ≤ 1.5 and FDR adjusted p-value ≤ 0.05 from 10,222 present genes.

(**Figure 4.19**). The cytokine *Tnf* displayed the highest fold change, with an mRNA expression upon IFN-αA with α-CD40 up to 10-times higher than the expression triggered by IFN-αA alone, while the negative regulator *Nfkbia*, also called IκBα, has the overall highest mean expression under the combination of IFN-αA and α-CD40 stimulation.

While there are various TRAFs proteins that interact with CD40, TRAF6, is the only one that has been clearly shown as involved downstream of CD40 activation in DCs (Ma and Clark 2009). Moreover, TRAF6 mediates the activation of the canonical NF-κB signalling pathway via the recruitment of TAK1 that phosphorylated IKKα and IKKβ leading to the degradation of IκB and therefore to the release of p105/RelA TFs (**Figure 1.4**) (Ghosh and Dass 2016). Interestingly, the expression of *Traf6* was induced following 8 hours of α-CD40 antibody and this expression was intensified when $eCD8^+$ DCs were stimulated with the combination of IFN-αA and α-CD40 antibody for 8 hours (**Figure 4.20.A**). Moreover, the RNA sequencing data set showed a significant amplification of the IFN-αA-initiated *Traf6* expression following the addition of α-CD40 treatment for 4 hours (**Figure 4.20.B**). To investigate a potential function role of TRAF6, we employed the small molecule inhibitor 6877002 that specifically targets CD40-TRAF6 interactions, without interfering with CD40-TRAF2/3/5 (Chatzigeorgiou et al. 2014; Aarts et al. 2017). The dose response treatment with this small molecule inhibitor, from 10 to 100 μM, or with DMSO at a dose equivalent of the highest concentration of inhibitor did not induce spontaneous expression of *Il15* (**Figure 4.21.A**) or *Il12b* (**Figure 4.21.B**). When applied simultaneously, the presence of CD40-TRAF6 inhibitor did not dampen *Il15* expression induced by IFN-αA stimulation. And more importantly, when combined with IFN-αA stimulation and α-CD40 antibody treatment, the compound did not inhibit the amplification of *Il15*. However, a progressive reduction of *Il12b* expression could be observed with increasing dose of CD40-TRAF6 (**Figure 4.21.B**). The minimum dose of 50 μM, inducing an adequate inhibition of *Il12b* expression, was chosen to further interrogate the involvement of CD40-TRAF6 interaction in the α-CD40-driven amplification of IFN-αA

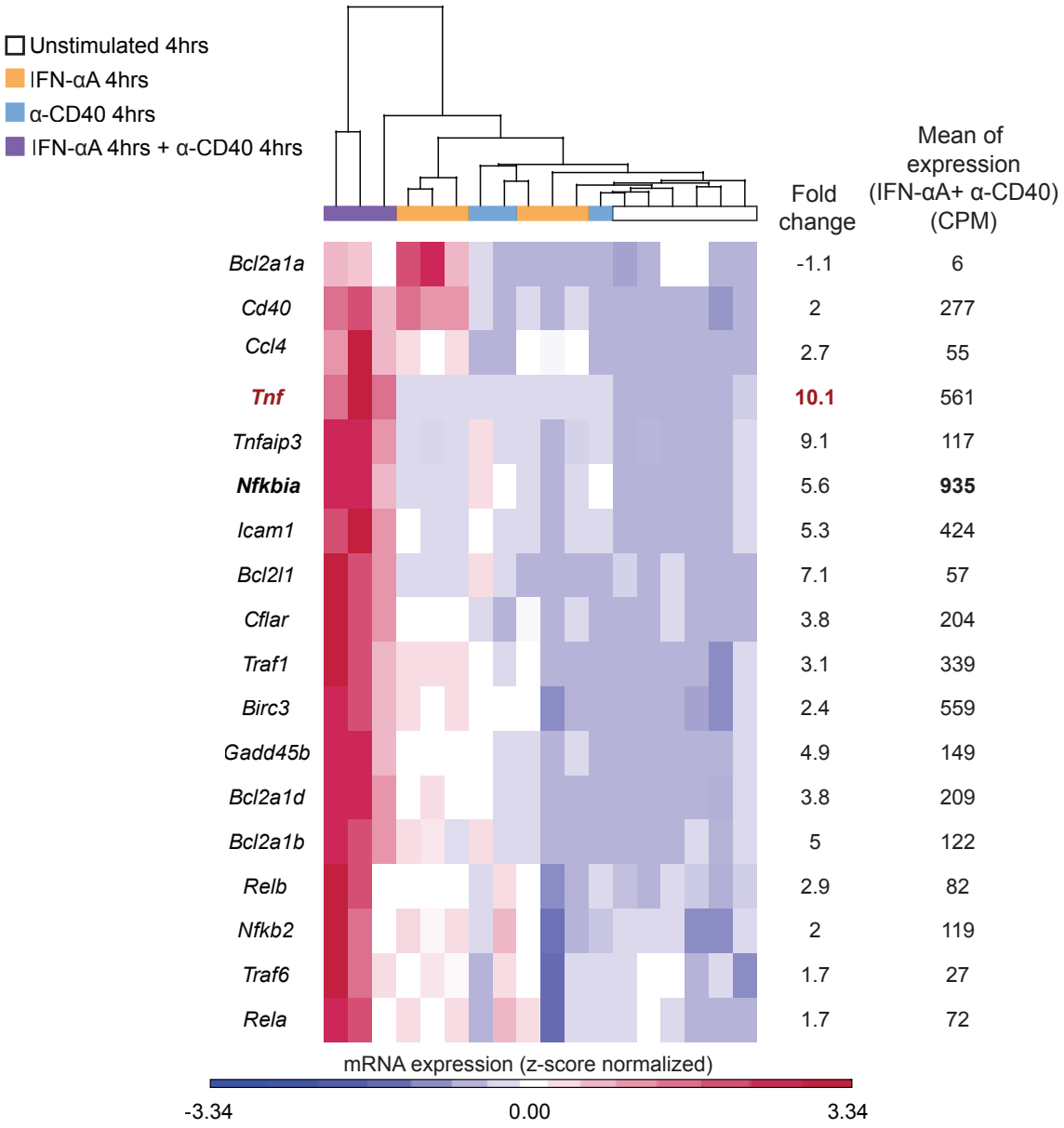



**Figure 4.19: Hierarchical clustering of NF-κB signalling pathway enriched DE genes.**
Hierarchical clustering of the DE genes between IFN-αA 4 hours and IFN-αA 4 hours + α-CD40 4 hours, part of the NF-κB signalling pathway (list derived from KEGG database). DE genes identified with -1.5 ≤ FC ≤ 1.5 and FDR adjusted p-value < 0.01 from 10,222 present genes. Fold change of the expression of the genes in the comparison IFN-αA 4 hours + α-CD40 4 hours vs IFN-αA 4 hours indicated on the right of the heatmap. Standardised expression values were shifted to mean zero and scaled to standard deviation of one. In red bold is highlighted the gene differentially regulated with the highest fold change. In black bold is highlighted the highest Mean of expression under IFN-αA 4 hours + α-CD40 4 hours.

signalling. eCD8$^+$ DCs treated with the small molecule inhibitor alone at 50 μM or in combination with IFN-αA and α-CD40 antibody survived up to 80% (**Figure 4.21.C**). This percentage is comparable to the survival of unstimulated cells. Moreover, CD86, marker for myeloid cell activation, was up-regulated by the DCs stimulated with IFN-αA, α-CD40 antibody and CD40-TRAF6 inhibitor at a level (MFI=2068) comparable to the stimulation without inhibitor (MFI=3430) (**Figure 4.21.D**). Therefore, 50 μM of CD40-TRAF6 inhibitor for 4 hours was not toxic for the cells and did not inhibit the activation of the DCs in response to IFN-αA stimulation. The simultaneous treatment with the small molecule inhibitor targeting CD40-TRAF6 interaction, IFN-αA and α-CD40 antibody did not inhibit the amplification of *Il15* (**Figure 4.21.A**). However, given the rapid effect of CD40 engagement following DCs licensing, the signal triggered by the α-CD40 antibody treatment might be delivered before the CD40-TRAF6 inhibition could block the signalling cascade. Therefore, CD40-TRAF6 interaction was inhibited ahead of the beginning of the α-CD40 antibody treatment (**Figure 4.22**). As expected, 4 hours of IFN-αA stimulation induced *Il15* expression (**Figure 4.22.A**). Expression amplified by the addition of α-CD40 antibody treatment for the last 2 hours. The inhibition of CD40-TRAF6 interaction before CD40 engagement slightly reduced the α-CD40-triggered amplification of IFN-αA induced *Il15* expression. Indeed, under CD40-TRAF6 inhibition, IFN-αA stimulation induced *Il15* to a level 300-times higher than without IFN-αA and this level of expression was unchanged when α-CD40 antibody treatment was added. Moreover, TRAF6 binding to CD40 might play a role in the CD40-mediated *Il12b* expression as it was restrained under the inhibitor treatment (**Figure 4.22.B**). The level expression of *Il6* measured at 4 hours was diffused and the CD40-TRAF6 inhibition did not seem to have any effect on the α-CD40-triggered amplification of IFN-αA-induced expression of this cytokine (**Figure 4.22.C**). However, when the concentration of Il-6 secreted in the supernatant of the stimulated cells was measured, the inhibition of CD40-TRAF6 interaction seemed to trigger the inhibition of the amplified release of Il-6 (**Figure 4.22.D**).

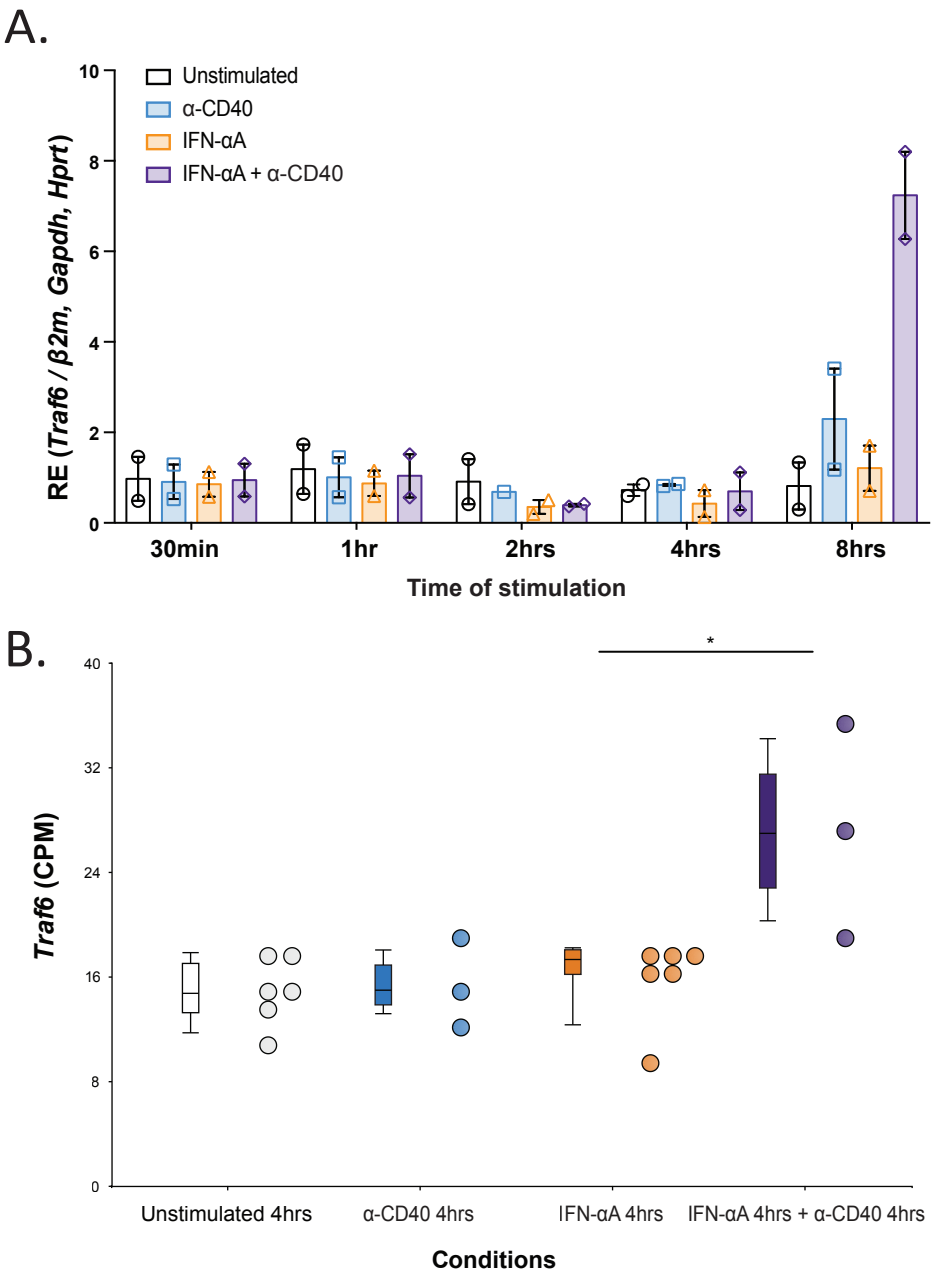
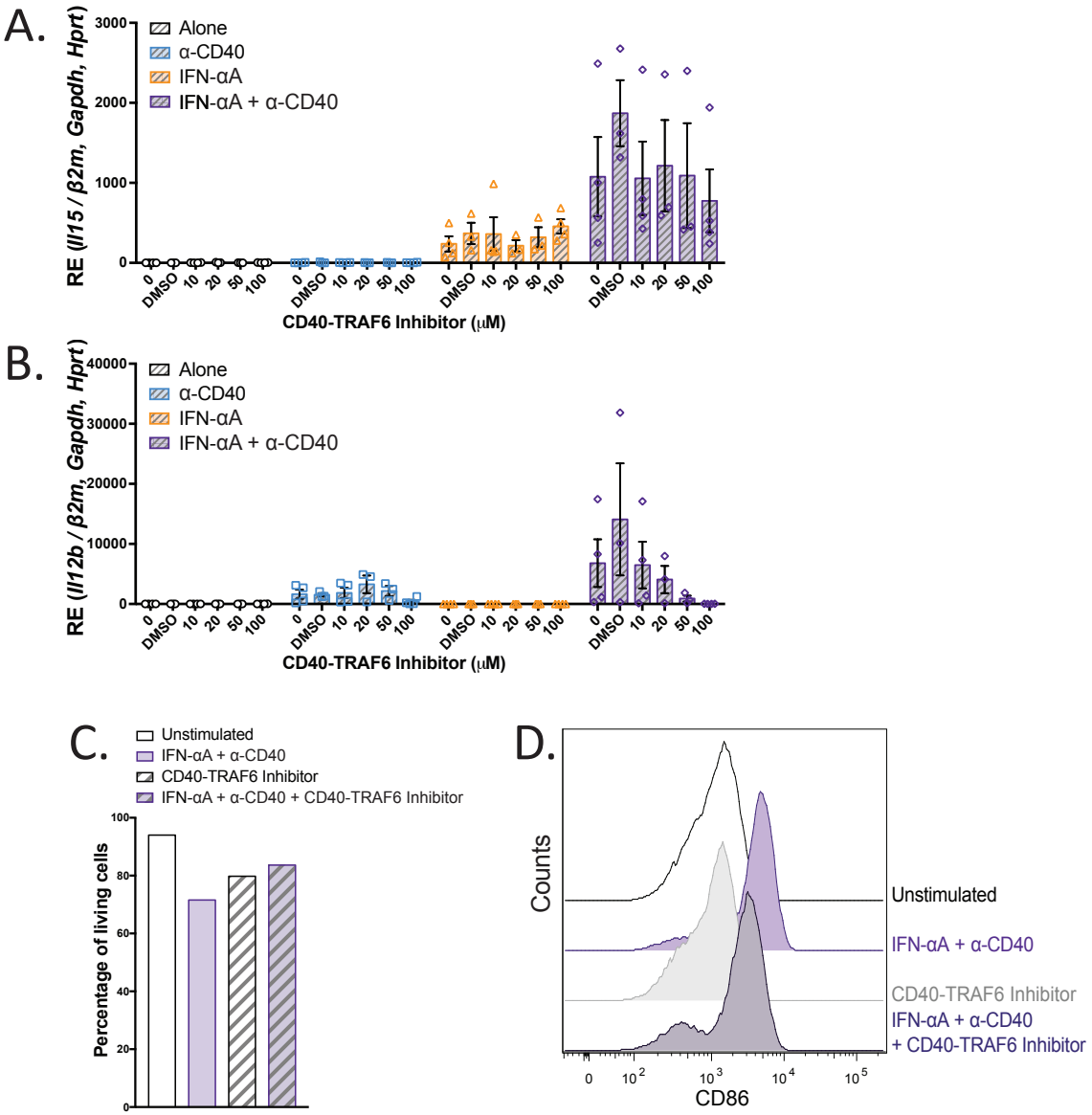



**Figure 4.20: α-CD40 and IFN-αA combination-dependent *Traf6* expression.**
eCD8⁺ DCs regulation of *Traf6* assessed via RT-PCR (Pool from 2 independent experiments) (**A.**) or via RNA sequencing analysis (Pool from 3 to 6 independent experiments) (**B.**) following the indicated timing of stimulation with media only (Unstimulated, white), α-CD40 mAb (10 µg/ml) (α-CD40, blue), IFN-αA (1000 U/ml) (IFN-αA, orange) and IFN-αA (1000 U/ml) with α-CD40 mAb (10 µg/ml) (IFN-αA + α-CD40, purple). (**A.**) Error bars represent mean +/- SEM. (**B.**) Box plot of *Traf6* expression: each dot is a sample, the line inside the box is the median, the whiskers represent the variability.
Asterisks indicate statistically significant differences between IFN-αA condition and IFN-αA + α-CD40 condition as assessed by one-way ANOVA; adjusted p-value: * p≤0.029.

To trigger NF-κB activation, TRAF6 recruits TAK1 that requires phosphorylation to be activated. In turn, TAK1 phosphorylates IKKα and IKKβ, which phosphorylate IκB for the release of p105/RelA (**Figure 1.4**) (Ghosh and Dass 2016). Therefore the canonical NF-κB pathway is subject to many phosphorylation events. Phosphorylation and dephosphorylation events were analysed via mass spectrometry following the stimulation of eCD8$^{+}$ DCs for 4 hours under different conditions. In total, 8406 phosphosites with a high localization probability (higher than 0.75) were detected from about 2901 proteins in the phosphoproteome dataset. Within these 8406 phosphosites, 181 displayed significant changes in their phosphorylation status when comparing the combination of IFN-αA with α-CD40 treatment for 4 hours to the IFN-αA stimulation alone for 4 hours (**Figure 4.23**). 118 phosphosites showed a decrease in the abundance of phosphorylation (dephosphorylation) while 63 phosphosites underwent a significant phosphorylation event when α-CD40 antibody treatment was added to IFN-αA stimulation (see Appendix **Table A.2** for a complete list of the 119 dephosphorylation events and 64 phosphorylation). Interestingly, signalling molecules and TFs from NF-κB pathway were amongst the peptide sequences with phosphorylation events. Within this group of proteins with significant changes in their phosphorylation status as consequence of the addition of α-CD40 treatment to IFN-αA stimulation, only a small proportion were also undergoing changes when α-CD40 stimulation was compared to untreated samples and none (**Figure 4.24.A**). Moreover, none of the phosphorylation and dephosphorylation events occurring with the addition of α-CD40 treatment to IFN-αA stimulation were already happening when the cells were stimulated with IFN-αA alone. Interestingly, IFN-αA stimulation triggered the significant phosphorylation of Stat1 on the Tyrosine 701 that have been associated with its activation (Hirata et al. 2013). The phosphorylation of Irf9 on Serine 136 and Serine 393 could be detected under IFN-αA alone as well. These phosphorylation events on Irf9 have not been linked to the activation of the TF as its expression more than its post-transcriptional modifications seems to play a crucial role in IFN-αA signalling pathway (Nan et al. 2018). However, none of these phosphorylation




**<u>Figure 4.21</u>: No effect of CD40-TRAF6 inhibition on survival and activation.**
**(A.-B.)** CD40-TRAF6 inhibitor (6877002) dose response (from 0 μM to 100 μM) (grey striped), alone for 4 hours (Alone, white), with α-CD40 mAb (10 μg/ml) (α-CD40, blue), with IFN-αA (1000 U/ml) (IFN-αA, orange) and with IFN-αA (1000 U/ml) and α-CD40 mAb (10 μg/ml) (IFN-αA + α-CD40, purple). These same conditions were performed with dimethyl sulfoxide (DMSO). Following stimulation, the regulation of *Il15* **(A.)** and *Il12b* **(B.)** was assessed (Pool from 4 independent experiments). Error bars represent mean +/- SEM. No statistical significance detected.
Percentage of living cells **(C.)** or histogram of CD86 expression **(D.)** measured following 4 hours of culture with media only (Unstimulated, white), IFN-αA (1000 U/ml) with α-CD40 mAb (10 μg/ml) (IFN-αA + α-CD40, light purple), CD40-TRAF6 inhibitor (50 μM) (6877002) (CD40-TRAF6 Inhibitor, grey) and IFN-αA (1000 U/ml) with α-CD40 mAb (10 μg/ml) and CD40-TRAF6 Inhibitor (50 μM) (IFN-αA + α-CD40 + CD40-TRAF6 Inhibitor, dark purple) (n=1).

events, Irf9 or Stat1, were increased nor decreased with the addition of α-CD40. Altogether, these observations showed a strong and specific effect of α-CD40 treatment in the context of IFN-αA stimulation at the post-transcriptional level. The STRING online tool was used to investigate the potential protein-protein interactions on the set of significantly phosphorylated proteins (**Figure 4.24.B**). While the phosphorylation events occurring under α-CD40 treatment alone did not show any significant pathway enrichment, the phosphorylation events occurring following the addition of α-CD40 antibody to IFN-αA stimulation were highly enriched within several KEGG pathways. Interestingly, the Epstein-Barr virus infection pathway, was the top ranked pathway enriched from the phosphosites analysis, with an adjusted p-value of 0.00024 and was also enriched from the DE genes under the same conditions (**Figure 4.18**). Moreover, the NF-κB signalling pathway was also significantly enriched in both cases. Within the different events, the phosphorylation of Ikbkb (Inhibitor of NF-κB kinase subunit beta), also called IKKβ, was detectable (**Figure 4.23**). The phosphorylation was identified on the Serine 697 of the protein, a site that has not been linked with the activation of the kinase (Kray et al. 2005). Another phosphorylation event induced when α-CD40 treatment was added to IFN-αA was measured on NFκBie (NF-κB-inhibitor epsilon), IκBε member of the IκB family that interact with NF-κB proteins to trap them in the cytoplasm. This phosphorylation was identified on the Serine 18. The Ser-18 phosphorylation has been described to be mediated by IKKβ and to lead to IκBε proteasomal degradation in response to TNF-α and IL-1β stimulation (Whiteside et al. 1997; Viatour et al. 2005). Finally, the phosphorylation of Tnfaip3 (TNF-α-induced protein 3) on Serine 381 was significantly detectable when CD40 was stimulated for 4 hours in addition of IFN-αA. TNFAIP3, also known as A20 is a negative regulator of NF-κB pathway (Ruland 2011). Interestingly, Ser-381 phosphorylation, mediated by IKKβ as well, has been shown to be detrimental in A20 functions (Wertz et al. 2015). Therefore, several activating phosphorylation events seem to target proteins involved in a negative feedback control of the NF-κB pathway in response to α-CD40 treatment in the context of IFN-αA stimulation. In addition of these post-transcriptional modifications, the

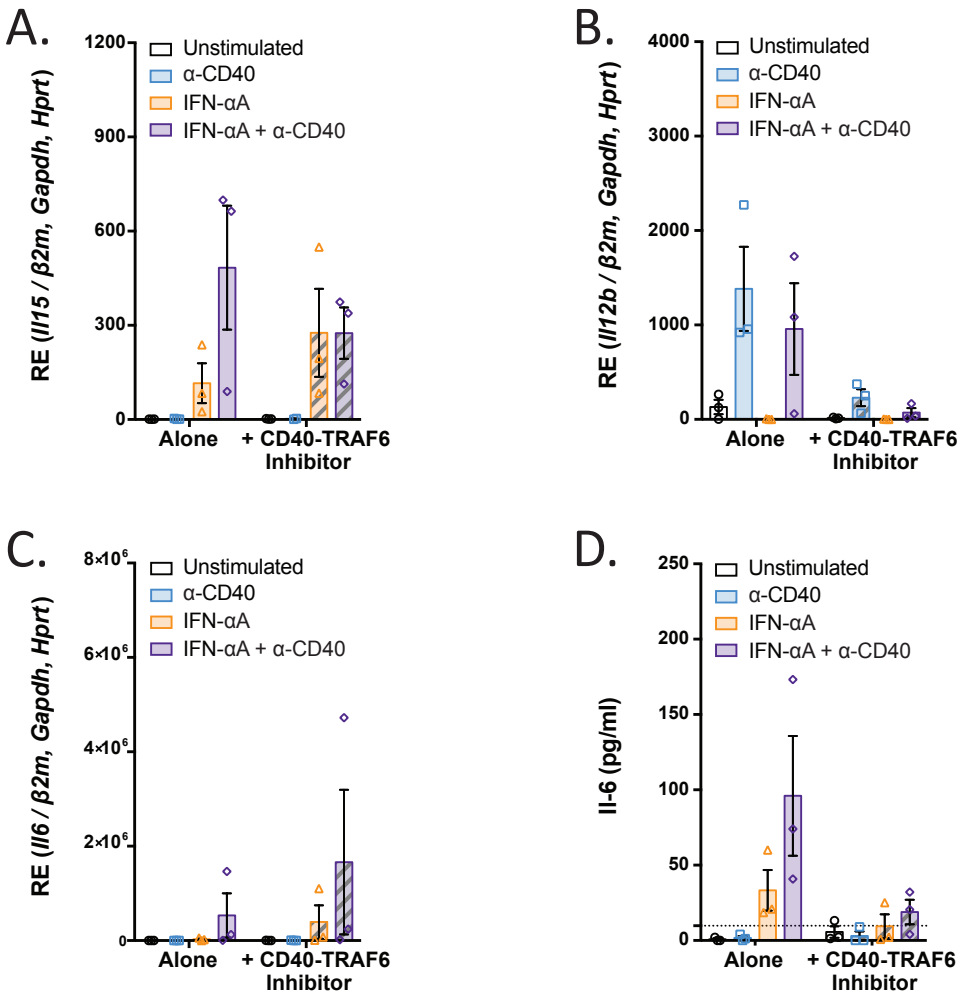



**Figure 4.22: TRAF6 binding to CD40 required for α-CD40-driven amplification of IFN-αA-induced cytokine expression.**
eCD8⁺ DCs were cultured over 4 hours with media only 4 hours (Unstimulated, white), α-CD40 mAb 2 hours (10 μg/ml) (α-CD40, blue), IFN-αA 4 hours (1000 U/ml) (IFN-αA, orange) and IFN-αA 4 hours (1000 U/ml) with α-CD40 mAb 2 hours (10 μg/ml) (IFN-αA + α-CD40, purple). These conditions were performed on their own (Alone, left, filled pattern) or in the presence of CD40-TRAF6 inhibitor 4 hours (50 μM) (6877002) (+ CD40-TRAF6 Inhibitor, right, grey striped). Following stimulation, the regulation of *Il15* **(A.)**, *Il12b* **(B.)**, *Il6* **(C.)** was assessed (Pool from 3 independent experiments), as well as the secretion of Il-6 **(D.)** with the dotted line indicating the limit of detection for the assay performed (Pool from 3 independent experiments). Error bars represent mean +/- SEM. No statistical significance detected.

feedback control of NF-κB pathway also depends on the regulation of expression of these proteins. For instance, the expression of IκBα and IκBε is induced in response of NF-κB signalling pathway (Kearns et al. 2006). *Nfkbie* (IκBε) (**Figure 4.25.A**) and *Nfkbib* (IκBβ) (**Figure 4.25.B**) progressively increased longer the α-CD40 treatment was applied to IFN-αA stimulation, significantly expressed under 4 hours of both stimuli. On the other hand, *Nfkbia* (IκBα) (**Figure 4.25.C**) and *Tnfaip3* (A20) (**Figure 4.25.D**) were rapidly and significantly induced under IFN-αA with α-CD40 treatment for only 15 minutes or 30 minutes of CD40 stimulation and their expression slightly decreased after 4 hours of stimulation. Interestingly, none of these regulators are significantly induced by IFN-αA stimulation alone. Instead, they seem to follow the regulation of specific genes as described in **Figure 4.14**, requiring both signals IFN-αA and α-CD40 in combination to be expressed.

A group of TFs belonging to NF-κB signalling pathway was highlighted as potentially involved in the amplified genes regulation. Importantly, the motifs recognised by these TFs were absent from the genes up-regulated under IFN-αA and not amplified. The analysis of gene regulation and phosphorylation events suggested a tight regulation of NF-κB pathway. However, further analysis, especially on the protein synthesis and degradation will be required to confirm this involvement.

#### 4.2.2.4 Mechanism of α-CD40 and IFN-αA induced specific gene expression

As described above, the expression of a group of genes were induced by stimulation followed a distinct pattern of regulation. One group of genes, which will refer to as “specific” (**Figure 4.14.C**), were not induced by IFN-αA stimulation or α-CD40 treatment alone. They only got induced when the cells were stimulated by both IFN-αA and α-CD40. To investigate the mechanism behind this particular pattern of expression, the potential TFs involved in the

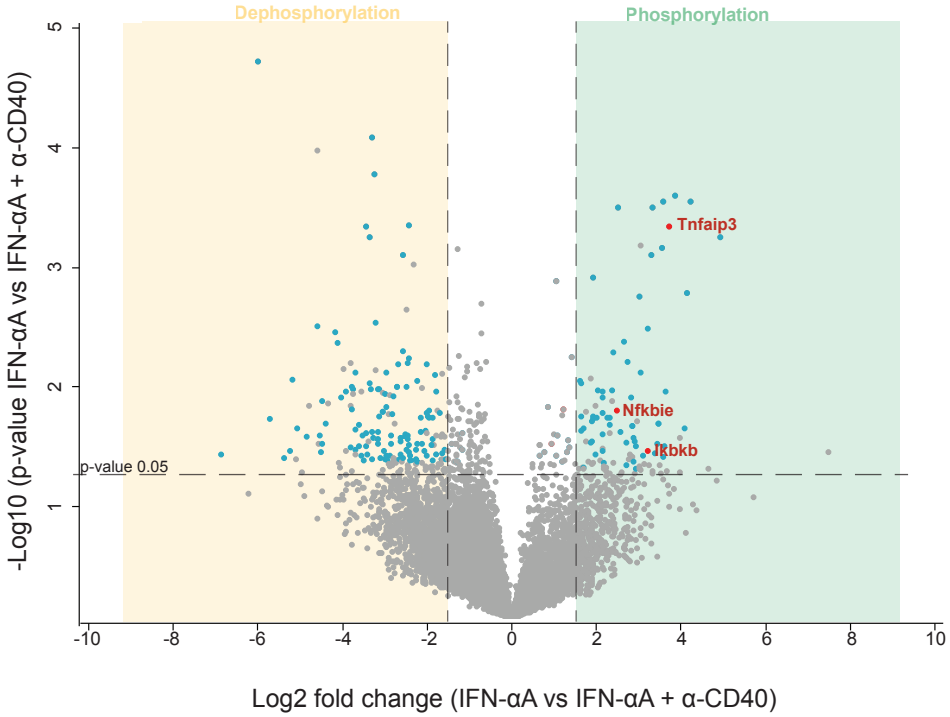
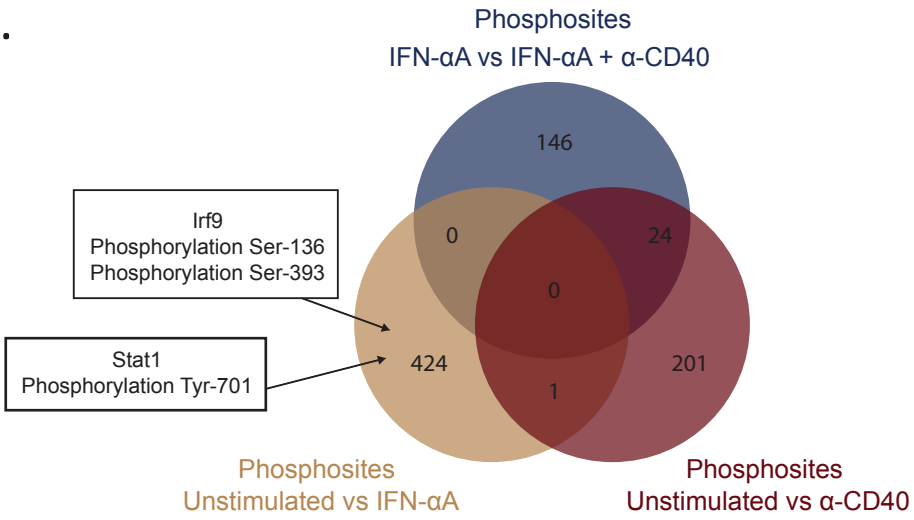



**Figure 4.23: Control of NF-κB signalling pathway via post-transcriptional modifications.**
Volcano plot of the magnitude and significance of differential phosphopeptide abundance in IFN-αA vs IFN-αA + α-CD40. Each dot represents a protein. Phosphopeptide undergoing phosphorylation (on the right, green background) or dephosphorylation (on the left, orange background) were considered as significant with a p-value ≤ 0.05, with a probability of localization > 0.75 and exhibiting -1.5 ≤ FC ≤ 1.5 (blue dots). Proteins highlighted in red known as involved in the NF-κB signalling pathway.

expression of the specific genes were analysed at 30 minutes (**Figure 4.26.A**) or 4 hours (**Figure 4.26.B**) of α-CD40 treatment. Here again, the number of specific genes induced with only 15 minutes of CD40 stimulation was too restricted to conduct a satisfactory GO-term of pathway analysis. Utilizing PBP analysis to identify TFs being responsible for the gene expression of the "specific" gene signature, it was observed that the top ranked clusters motifs were different to the TF motifs predicted for the amplified genes. Indeed, the cluster motifs with a NES greater than 7, obtained from both sets of specific genes, 30 minutes and 4 hours of α-CD40 antibody treatment, mainly contained NF-κB related TFs. Stat6 and Irf1 were the only transcriptional regulators highlighted belonging to the Stat and Irf families. Therefore, compared to the analysis of IFN-αA-induced genes and amplified genes, the clusters of TFs predicted for the regulation of "specific" genes induced by the combination of IFN-αA and α-CD40 treatment did not include most of the IFN-related TFs such as Irf3, Irf7, Irf9 or Stat1/2. The NF-κB pathway seemed again to be involved in the expression of these specific genes. Following the hypothesis that as the patterns of expression of amplified and specific genes differ, the mechanisms behind them could be different, the data mining for the transcriptional regulators prediction was different. As IFN-αA stimulation alone or α-CD40 treatment alone are not efficient in triggering this pattern of expression, the hypothetic mechanism could require the expression of a transcriptional regulator triggered by CD40 stimulation in the context of IFN-αA signalling. To investigate this hypothesis, the TFs enriched from the specific genes prediction were firstly filtered to investigate the ones expressed by the eCD8$^+$ DCs (**Figure 4.26.C**). These TFs were considered as TF candidate for the regulation of specific genes and were further analysed taking into account significant fold changes in their expression under IFN-αA with α-CD40 as well as their overall expression level. Following this strategy, the potential TFs were obtained from the prediction of specific genes induced with 30 minutes of α-CD40 antibody treatment (**Figure 4.27.A**). As the expression of those TFs might be regulated early, the IFN-αA alone condition was compared to IFN-αA with α-CD40 from 15 minutes to 30 minutes. This filtering strategy lead to 5 TFs which might be the master

A.




B.

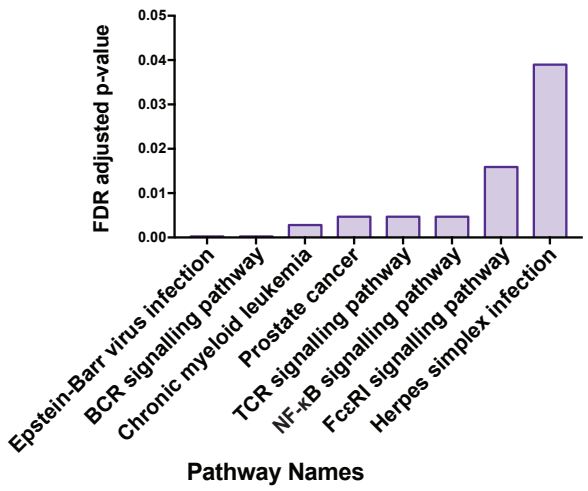
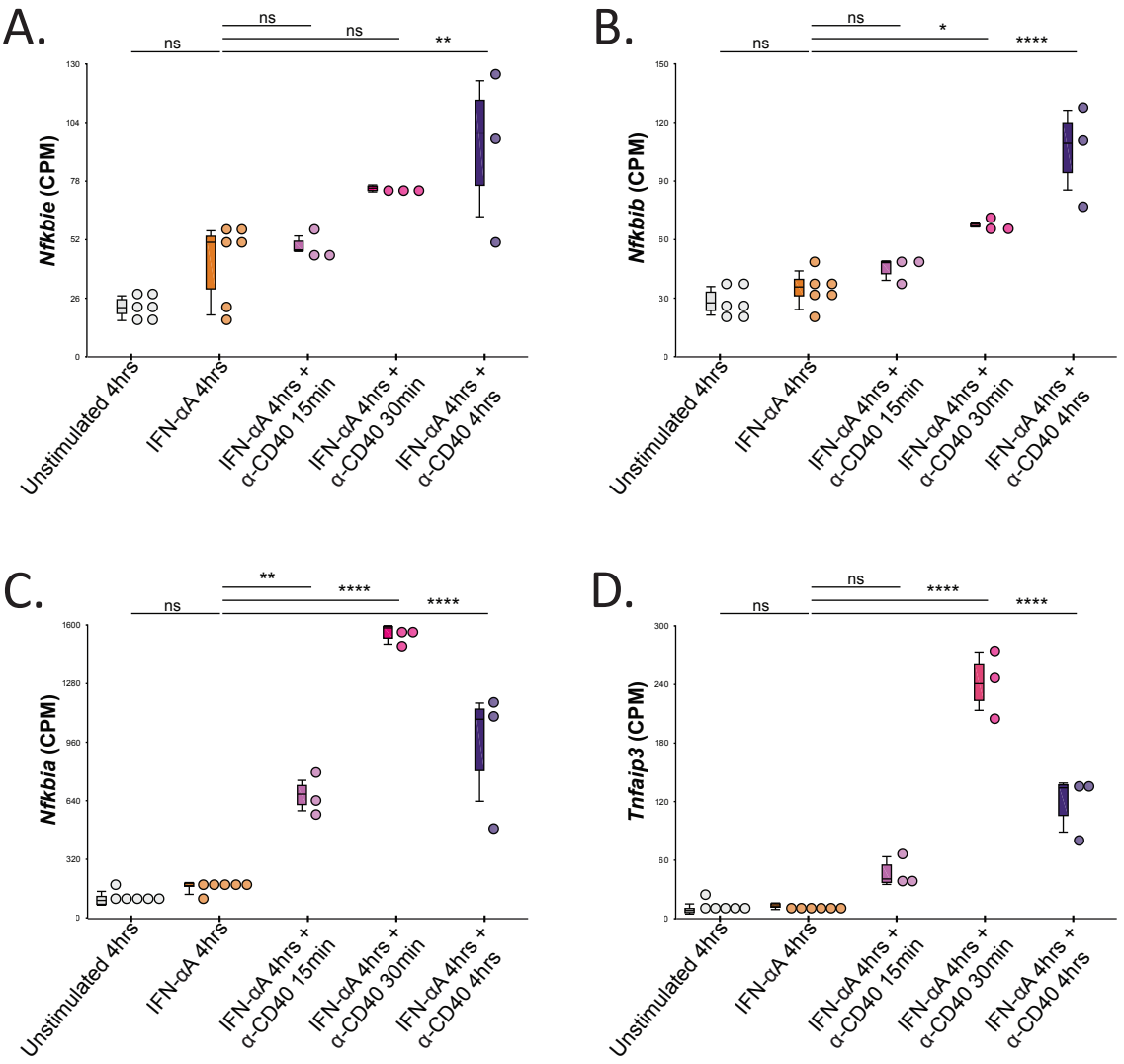



**Figure 4.24: Phosphorylation events specific to IFN-αA + α-CD40 condition.**
**(A.)** Venn diagram of phosphosites detected between IFN-αA 4 hours vs IFN-αA 4 hours + α-CD40 4 hours; Unstimulated vs α-CD40 4 hours and Unstimulated vs IFN-αA 4 hours.
**(B.)** KEGG pathway enrichment analysis performed via STRING on all proteins undergoing a significant phosphorylation event in IFN-αA vs IFN-αA + α-CD40.

regulators of IFN-αA and α-CD40 combination DC programming at 30 minutes: Jun, Junb, Fos, Fosb and Irf1. Therefore, those proteins were part of the predicted and expressed TFs and were significantly DE under IFN-αA with α-CD40 antibody for 15 and 30 minutes. Next, the proportion of specific genes potentially targeted by these TFs was assessed (**Figure 4.27.B**). Junb and Fosb were only capable of binding a small portion of those genes. However, Jun/Fos and Irf1 could target about 28 % of the genes. About 9 % of them were only targeted by Jun and Fos while Irf1 alone could bind 23 % of the genes. Therefore, altogether, more than 60 % of the specific genes were induced following 30 minutes of CD40 stimulation.

The same strategy was used to investigate, the potential TFs involved in the expression of the specific genes induced with 4 hours of α-CD40 antibody treatment (**Figure 4.28.A**). The expression of the potential TFs, predicted and expressed by $eCD8^+$ DCs, was assessed. Again, the IFN-αA alone condition was compared to IFN-αA with α-CD40 treatment from 15 minutes to 30 minutes for TFs expressed early enough to be involved in the gene regulation. However, this time, these proteins were further filtered for the ones with sustained expression until 4 hours of α-CD40 treatment. The analysis identified 3 TFs which might guide the specific IFN-αA and α-CD40 combination DC programming at 4 hours: Mxd1, Rel and Irf1. When the proportion of specific genes potentially targeted by these TFs was assessed, more than 40 % of the specific genes induced under 4 hours of α-CD40 treatment could be recognised by Irf1 (**Figure 4.28.B**). Interestingly, Rel was predicted to bind motif(s) on all of these genes and 28 % more. Thus, Rel and Irf1 could potentially target more than 70 % of the specific genes 4 hours while the portion of Mxd1 targeted genes was negligible. Interestingly, *Fos* (**Figure 4.29.A**) and *Jun* (**Figure 4.29.B**) were significantly up-regulated after only 15 minutes of α-CD40 treatment. On the other hand, the up-regulation of *Irf1* (**Figure 4.29.C**) and *Rel* (**Figure 4.29.D**) was slightly delayed, occurring after 30 minutes of α-CD40 treatment. All together, the strategy used for the investigation of the regulators




**<u>Figure 4.25:</u> Gene regulation of proteins involved in the negative feedback control of NF-κB signalling pathway.**
Regulation of *Nfkbie* **(A.)**, *Nfkbib* **(B.)**, *Nfkbia* **(C.)** and *Tnfaip3* **(D.)** expression assessed following the indicated stimulation (Pool from v3 to 6 independent experiments). Each dot is a sample, the line inside the box is the median, the whiskers represent the variability. Asterisks indicate statistically significant differences between Unstimulated condition and IFN-αA condition or between IFN-αA condition and all IFN-αA + α-CD40 conditions as assessed by one-way ANOVA; adjusted p-value: ns = non significant; * p≤0.028; ** p≤0.006; **** p<0.0001.

of the specific genes pointed towards a possible early involvement of Fos and Jun, a late involvement of Rel and a sustained involvement of Irf1.

Taken together, the results indicate a potential requirement for TRAF6 for the α-CD40-driven amplification of IFN-αA-induced mRNA expression. TRAF6 could then trigger the activation of the NF-κB pathway, most likely supported by the analysis of the TFs potentially involved in the regulation of amplified genes presented here. The phosphorylation and gene regulation of different proteins known to be involved in the negative feedback control of NF-κB pathway were observed, corroborating this hypothesis. On the other hand, the specific genes could be regulated in a dynamic manner with Fos/Jun and Irf1 involved in the early response and, Irf1 and Rel involved in the late response to α-CD40 treatment.

## 4.3 Discussion

This chapter investigated the kinetics and molecular mechanisms underlying the gene regulation induced under IFN-αA and α-CD40 stimuli. Stimulated DCs displayed precise dynamic and functional organisations of their responses. Importantly, 1 to 2 hours of IFN-αA stimulation was required to induce DCs cytokines and chemokines expression. While the pre-treatment with α-CD40 antibody did not modify this minimum requirement, the activated $eCD8^+$ DCs exhibited the ability to respond to the CD40 stimulation in a surprisingly rapid manner. Indeed, once the IFN-mediated program was initiated, 15 to 30 minutes of CD40 ligation efficiently induced the amplification of gene expression and cytokine secretion. Analysing the changes triggered by the stimulations at the whole transcriptome level highlighted a dynamic enrichment of biological functions. Mostly, genes involved in immune responses were enhanced by the α-CD40 antibody treatment added to IFN-αA in sustained fashion while transcriptional activity was rapidly promoted. Moreover, transcription factor prediction and phosphoproteomic analyses revealed specific NF-κB signalling

A. All specific genes 30 min (43 genes)

| Cluster | Transcription Factors | NES |
|---|---|---|
| M1 | Nf-κb1, Nf-κb2, Bcl3, E2f1, Rela, Relb, Irf1,... | 8.688 |
| M2 | Nf-κb1, Nf-κb2, Bcl3, E2f1, Rela, Relb, Stat6,... | 7.613 |
| M3 | Ets1, Zfp354c, Ing4, Gli3, Runx1,Zbtb14, Elf5 | 5.821 |

B. All specific genes 4 hrs (111 genes)

| Cluster | Transcription Factors | NES |
|---|---|---|
| M1 | Nf-κb1, Nf-κb2, Bcl3, E2f1, Rela, Relb, Stat6,... | 7.950 |
| M2 | Pds5a | 3.990 |
| M3 | Lhx3, Pou1f1, Msx1,... | 3.687 |

C.

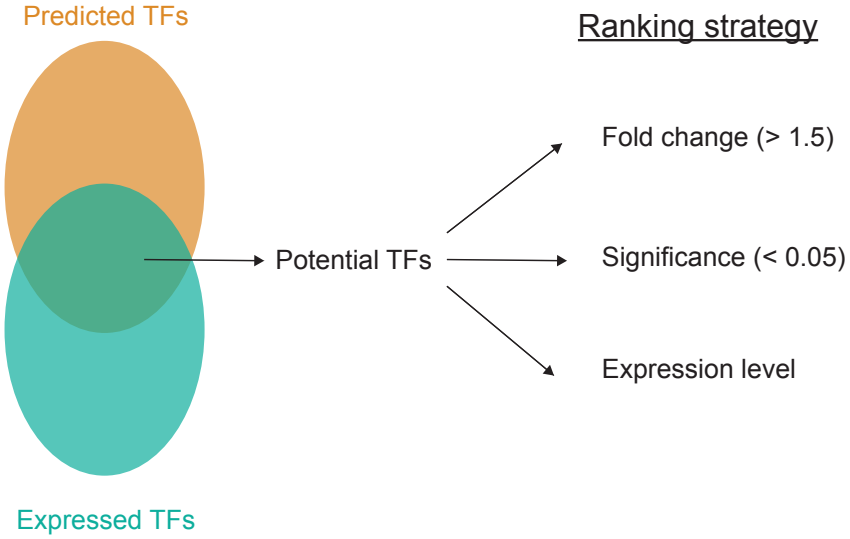



**Figure 4.26: Transcription factors binding prediction specific genes.**
**(A.)** Top three of the TFs motifs clusters enriched for amplified 30 minutes genes signature as presented in **Figure 4.15.C**.
**(B.)** Top three of the TFs motifs clusters enriched for amplified 4 hours genes signature as presented in **Figure 4.15.C**.
**(C.)** Strategy for specific genes expression TFs prediction analysis.

events as potential key regulators of the mechanisms responsible for the α-CD40-mediated amplification phenomenon. Finally, an intriguing pattern of gene regulation requiring the combination of both IFN-αA and CD40 signals was highlighted. The rapid and late regulation of these specific genes could involve the sequential and respective involvement of different transcriptional regulators.

Focusing on the precise kinetics of α-CD40-triggered amplification of a few cytokines and chemokines, our results revealed the rapid amplification of *Il15*, *Il6* and *Ccl4* expression. Interestingly, the secretion of Il-6 and Ccl4 followed a similar pattern with, Ccl4 secretion matching the kinetics of mRNA expression. The precise timing required for mRNA transcription and subsequently translation into protein remains unclear and is most likely conditioned by the cell type and the signal received. However, using a fluorescent-based system to follow the mRNA production, Ben-Ari et al. observed a doxycycline-induced *β-actin* expression starting from 20 minutes with a peak at 1 hour following treatment. Interestingly, they also highlighted the discrepancy between this timing requirement for transcription and the protein translation occurring earlier. They hypothesized a presence of existing mRNAs in the cytoplasm, potentially ready for translation before the presence of newly transcribed mRNAs (Ben-Ari et al. 2010). Moreover, once the proteins are formed, their secretion into the supernatant of cells is quick (Rivera et al. 2000). These studies can explain the fast amplification of Ccl4 chemokine secretion following α-CD40 antibody treatment. Intriguingly, Il-6 and Ccl4 secretion appeared to reach immediately a plateau phase. Both molecules have been implicated in chronic inflammation and autoimmunity (Tanaka et al. 2016; Jones et al. 2018; Ahmad et al. 2019; Kang et al. 2019). Our results could highlight an intrinsic mechanism of control of the cytokines and chemokines concentration released by the cells in response to IFN-αA and α-CD40 combined signalling.

As for *Il15*, *Il6* and *Ccl4*, the whole transcriptomic analysis highlighted the rapid regulation of a number of genes. The investigation of transcription

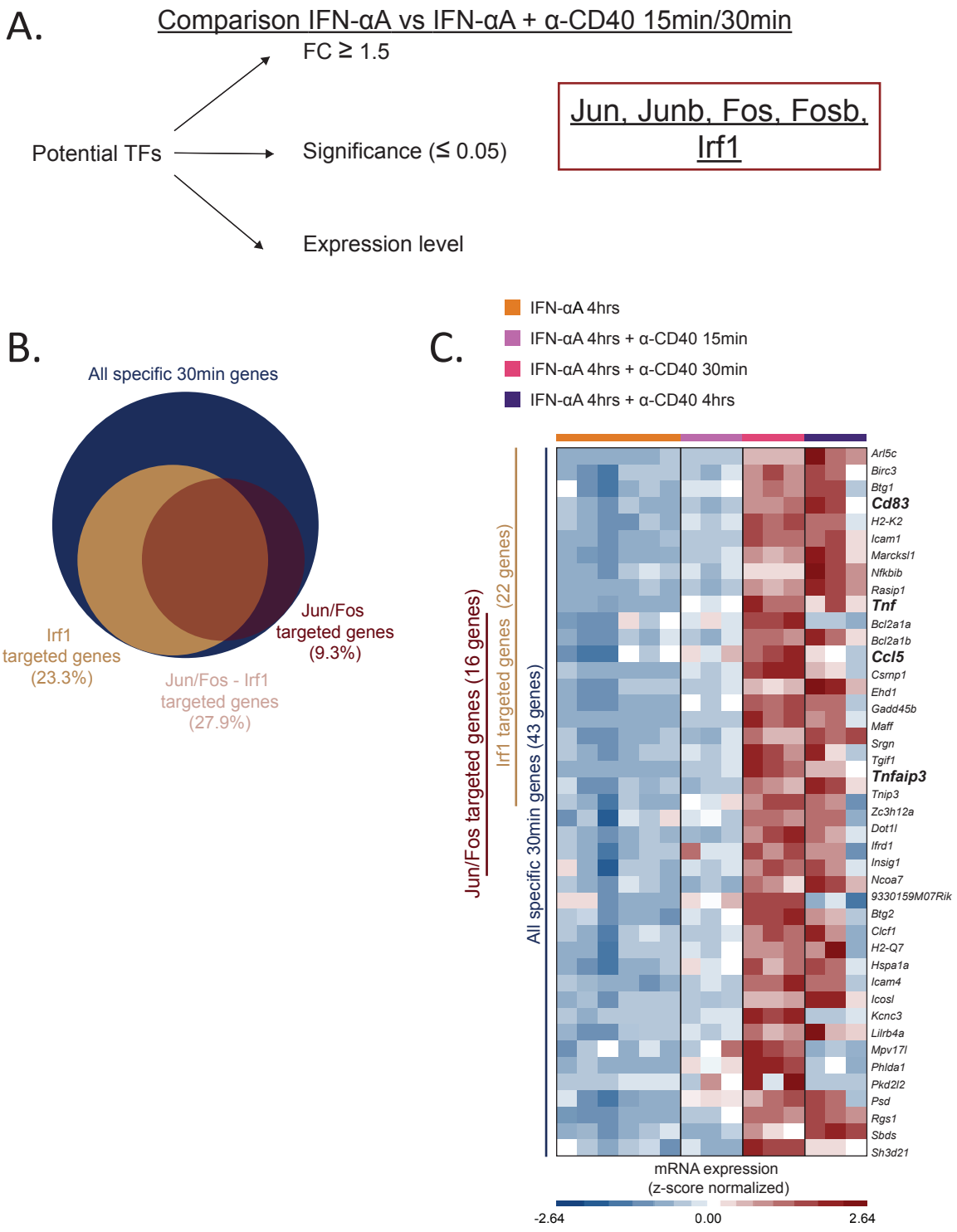



**Figure 4.27: Potential transcription factors for specific 30 minutes gene expression.**
**(A.)** Results of the analytic strategy.
**(B.)** Venn diagram of specific 30 minutes genes targeted by Irf1 and/or Jun/Fos.
**(C.)** Heatmap of the specific genes between IFN-αA 4 hours and IFN-αA 4 hours + α-CD40 30 minutes. Predicted TF indicated on the right. Standardised expression values were shifted to mean zero and scaled to standard deviation of one.

regulators potentially involved in the gene regulation following α-CD40 treatment revealed a possible role for the NF-κB signalling pathway. The genes induced with IFN-αA and not affected by the addition of α-CD40 did not contain motifs that would allow the binding of the NF-κB TFs. Therefore, the presence or the absence of a motif that NF-κB TFs can recognise could dictate which genes are amplified within the set of IFN-αA-triggered genes. In the hypothetical mechanism concluded from this study, NF-κB signalling pathway could be responsible for the amplification of IFNαA-triggered genes in addition of Irf9 involvement. Such a cooperation between NF-κB and IRFs has been observed previously with the transcriptional regulation of CD83 depending on the synergistic activation of IRFs, including IRF1, and NF-κB TFs (Stein et al. 2013). In addition to this gene amplification pattern, some genes such as *Tnf*, *Cd83* and *Ccl5* were rapidly induced in a specific manner, requiring both α-CD40 and IFN-αA stimuli. Hypothetically, IFN-αA-induced Irf1 could rapidly be recruited and cooperates with Fos and Jun to induce the rapid expression of specific genes. Importantly, while most of Irf TFs were up-regulated by IFN-αA and unchanged following the addition of α-CD40 antibody, Irf1 was the only TF clearly enhanced by CD40 stimulation with an up-regulation occurring after 15 to 30 minutes only. Importantly, the TFs Irf1 and Rel were highlighted as key candidates in the late regulation of the specific genes, such as *Cxcl16*, *Il15ra*. Therefore, the α-CD40-mediated amplification of Irf1 and Rel expression might allow the subsequent expression of the specific genes (**Figure 4.30**). Under this hypothetical mechanism, the early up-regulation of the specific genes *Jun* and *Fos* remains unclear. Both were part of the group of genes displaying a rapid up-regulation in response to α-CD40 antibody treatment in the context of IFN-αA stimulation. Those genes have been previously characterised as immediate early (IE) genes in the context, for instance of liver regeneration. IE genes are regulated without requiring *de novo* protein synthesis (Fausto 2000). *Fos* is one of the most described IE genes. This rapid expression is explained by the organisation of its promoter with paused RNA polymerase II on the transcription start site (TSS) and the chromatin constitutively in an open structure (Senecal et al. 2014). Moreover, its promoter also displays a sustained level of histone 3

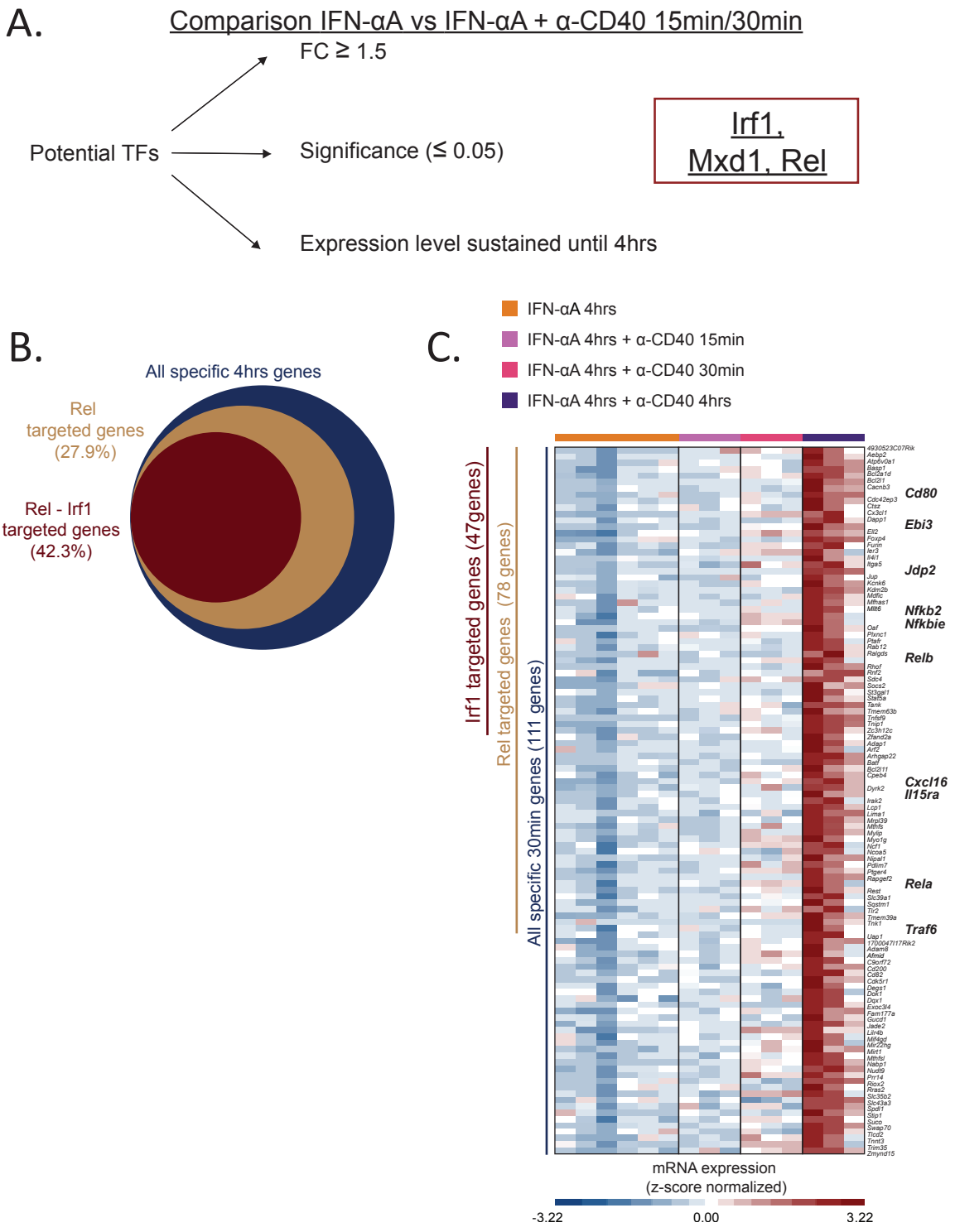
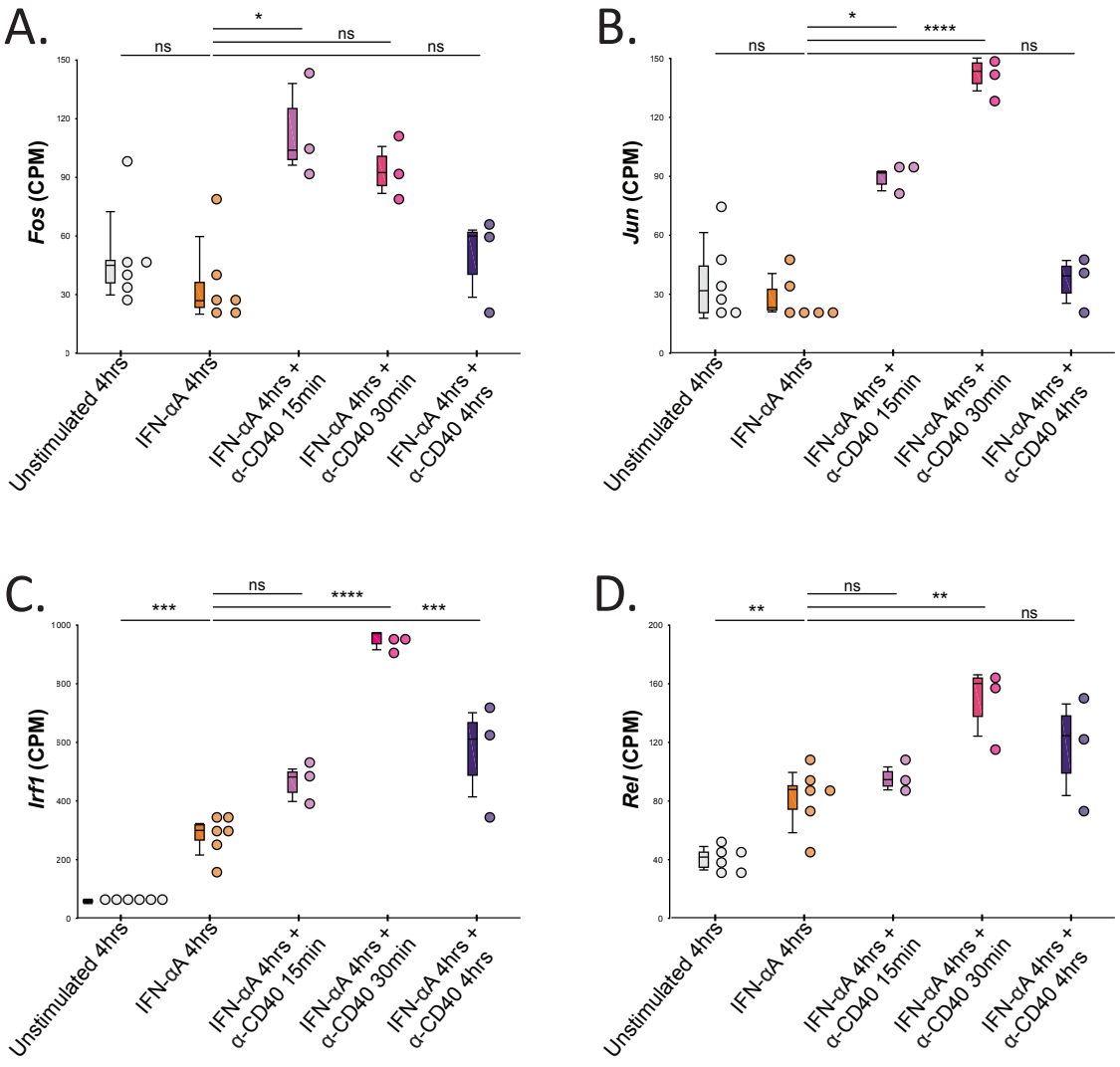



**Figure 4.28: Potential transcription factors for specific 4 hours gene expression.**
**(A.)** Results of the analytic strategy.
**(B.)** Venn diagram of specific 4 hours genes targeted by Irf1 and/or Relb.
**(C.)** Heatmap of the specific genes between IFN-αA 4 hours and IFN-αA 4 hours + α-CD40 4 hours. Predicted TF indicated on the right. Standardised expression values were shifted to mean zero and scaled to standard deviation of one.

acetylation (H3Ac), histone modification that enhances and is responsible for TF recruitment and *Fos* transcription via MAPK/Erk signalling pathway activation in response to stimulus (O'Donnell et al. 2008; Fowler et al. 2011). However, as specific genes, *Fos* and *Jun* regulation also depends on an unknown IFN-αA-related factor.

If the NF-κB potentially played a major role in CD40 signalling under the investigated conditions, its activation must be controlled. This pathway requires the phosphorylation, ubiquitination and degradation of the inhibitory IκB proteins. The IKK complex, formed by homodimers or heterodimers of IKKα and IKKβ and NEMO/IKKγ is responsible for the phosphorylation of IκB, with IKKβ playing a crucial role in canonical NF-κB activation pathway and NEMO associated to the recruitment of kinases activating IKKα/β. The activation of those IKKα/β proteins necessitates their phosphorylation on Ser-176/177 by Protein Kinases C (PKC) and TAK1, allowing their subsequent autophosphorylation on Ser-180/181 (Tojima et al. 2000; Kray et al. 2005; Zhang et al. 2014). The phosphoproteomics analysis under the addition of α-CD40 treatment to IFN-αA stimulation highlighted the phosphorylation of IKKβ on Ser-697. While it has not been linked to the kinase activation, this amino acid is localised in a serine-rich region (aa 643-735) between the NEMO binding domain and the helix loop helix domain (Israël 2010). Interestingly, phosphorylation events in the Serine-rich region, including Ser-697, and the NEMO binding domain have been associated with the negative regulation of IKKβ functions. Three members of the IκB family are known: IκBα, IκBβ and IκBε. Their degradation, detrimental step for the NF-κB activation, occurs following different kinetics. For instance, IκBα and IκBβ are both degraded more rapidly than IκBε (Ruland 2011). As described previously, NF-κB moves into the nucleus and is exported to the cytoplasm in an oscillatory dynamic (Zambrano et al. 2016; Lane et al. 2017). This oscillatory behaviour depends on the inhibitory mechanisms targeting NF-κB: IκBα and IκBε can enter the nucleus, interact with NF-κB and return it to the cytoplasm. The expression of both inhibitors is induced in response to NF-κB signalling pathway activation leading




**<u>Figure 4.29</u>: Regulation of the expression of TFs potentially involved in specific genes.**

Regulation of the expression of *Fos* **(A.)**, *Jun* **(B.)**, *Irf1* **(C.)** and *Rel* **(D.)** assessed following the indicated stimulation (Pool from 3 to 6 independent experiments). Each dot is a sample, the line inside the box is the median, the whiskers represent the variability. Asterisks indicate statistically significant differences between Unstimulated condition and IFN-αA condition or between IFN-αA condition and all IFN-αA + α-CD40 conditions as assessed by one-way ANOVA; adjusted p-value: ns = non significant; * p≤0.03; ** p≤0.005; *** p≤0.0005; **** p<0.0001.

to a feedback control of the NF-κB activation (Hoffmann et al. 2002; Hayot and Jayaprakash 2006). While IκBα is thought to be the main coordinator of NF-κB oscillations, its expression was rapidly induced, from 15 to 30 minutes of α-CD40 treatment under IFN-αA stimulation. Moreover, IκBε, associated to the decrease of the oscillations amplitude, was up-regulated later, following 4 hours of CD40 stimulation (Kearns et al. 2006). Interestingly the stimulation of CD40 receptor in the context of IFN-αA stimulation triggered IκBε Ser-18 phosphorylation known to lead to IκBε proteasomal degradation (Whiteside et al. 1997; Viatour et al. 2005). These two opposite phenomenons could participate in a balance between activation and control of NF-κB responses taking place late following CD40 stimulation. One of the targets of TNFAIP3, or A20, deubiquitinase is RIP1. Ubiquitinated RIP1 is activated and is involved in the recruitment of IKK. Therefore, the activation of A20 leads to the inhibition and degradation of RIP1 eventually inhibiting IKK complexe (Ruland 2011). Phosphorylation of Ser-381 is required for A20 activation and was triggered by α-CD40 treatment in the context of IFN-αA stimulation (Wertz et al. 2015).

Taken together these results highlight a potential early involvement of the MAPK pathway and a key role for NF-κB signalling pathway in the α-CD40-mediated responses in the context of IFN-αA stimulation. This NF-κB pathway is tightly controlled, either via dynamic up-regulation of inhibitors IκBs associated to the oscillatory NF-κB responses; or via post-transcriptional modification potentially resulting in negative feedback control. Interestingly, both early and late specific or amplified responses require the involvement of Irfs which would explain the absence of gene regulation following CD40 stimulation only.

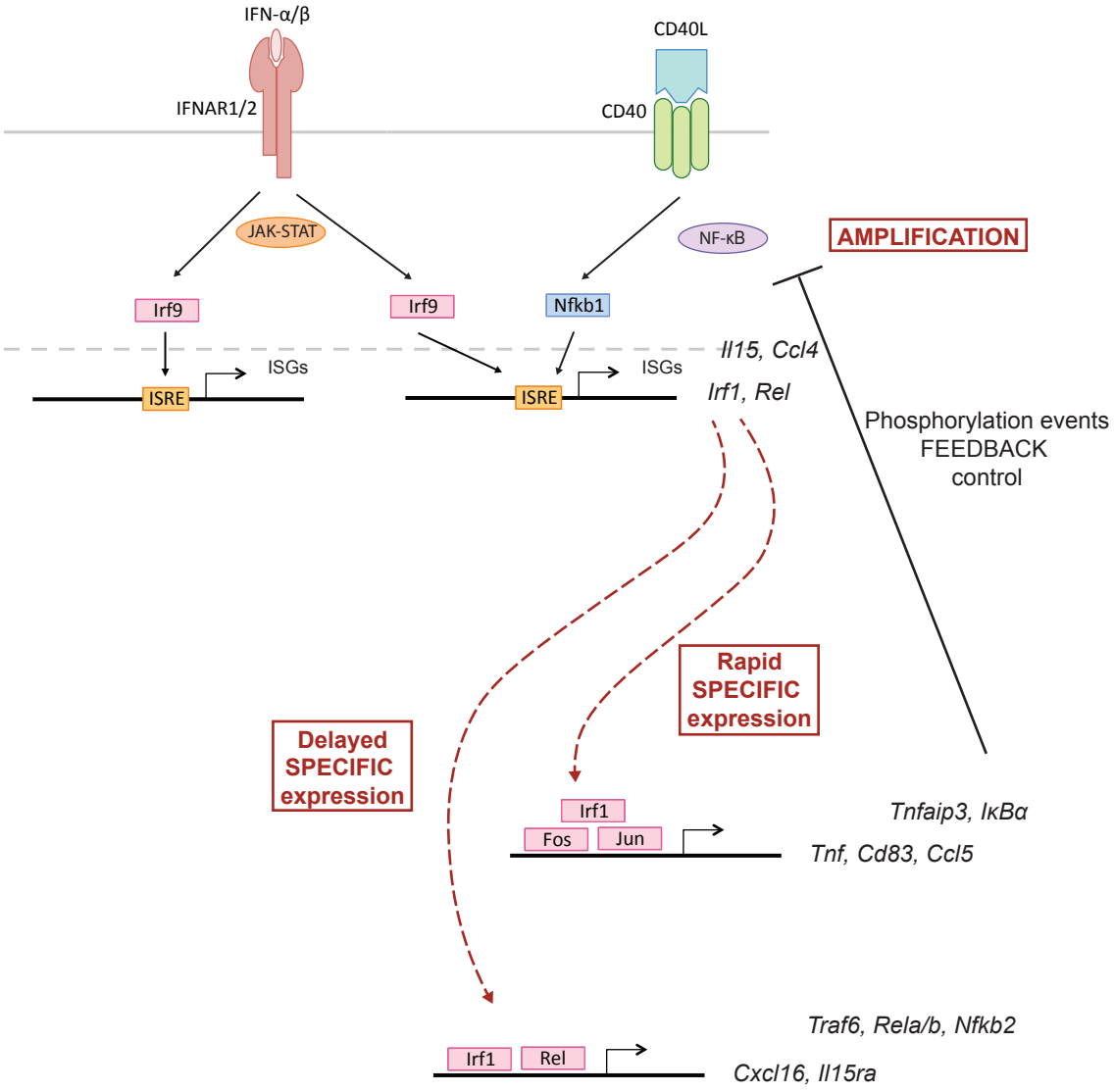



**Figure 4.30: Proposed mechanisms of synergy between α-CD40 and IFN-αA stimuli.**
IFN-α/β stimulation triggers ISGs expression via the JAK/STAT signalling pathway resulting in the activation of IRF9 transcription factor. Following the stimulation of CD40 receptor, the amplification of IRF9-regulated genes has been observed. This mechanism could involve the NF-κB signalling pathway and lead to the amplification of various genes including *Irf1* and *Rel*. *Irf1* being up-regulated via IFN-αA on its own could rapidly be involved in the early specific genes regulation, in cooperation with *Jun* and *Fos*. Subsequently, amplified *Irf1* and *Rel* could induce the late specific response. Interestingly, both of these responses target genes playing a role in the negative feedback control of NF-κB signalling pathway.

# Chapter 5

## Discussion

Naïve $CD8^+$ T cells require priming by DC to eliminate intracellular pathogens and tumours. This involves T cell receptor (TCR)-mediated antigen recognition and depends on the integration of contextual cues released by tissue damage associated with the antigenic encounter. These cues are transmitted to the DC by innate signals, such as type I interferon (IFN-α/β), and are subsequently communicated from the DC to the T cells via co-stimulatory molecules and cytokines (Prilliman et al. 2002). Interestingly, $CD4^+$ T cells also play an important role in this and this involves CD40L/CD40 interactions (Ridge et al. 1998; Schoenberger et al. 1998; Smith et al. 2004; Williams and Bevan 2007; Rajasagi et al. 2009; Zhu et al. 2015; Ghosh and Dass 2016; Ahrends et al. 2017). However, precisely how innate signals and T cell help are integrated and ultimately regulated the provision of cytokines and chemokines remains unclear. The work summarized in this thesis sheds new light on these important questions, revealing an intriguing interplay between innate triggers and T cell help.

The first aim of this thesis was to establish an *in vitro* model that allows the investigation of how T cell help shapes the responsiveness of DC. BM-derived equivalent of $CD8^+$ DCs ($eCD8^+$ DCs) were stimulated with an innate signal and treated with a α-CD40 antibody to mimic T cell help. In this set up, the stimulation of CD40 enhanced the expression and secretion of cytokine and chemokine, such as Il-15, Il-6, Tnf or Ccl4/5 in response to various innate signals including the danger signal IFN-αA or the TLR3, 4, 9 agonists, poly(I:C), LPS and CpG respectively. The IFN-αA stimulation associated with an isotype antibody did not result in the enhancement of the DCs responses, indicating that specific stimulation of CD40 was responsible for this amplification phenomenon. Similar responses were observed when we used antigen-activated $CD4^+$ T cells rather than the α-CD40 antibody. This supports the conclusion that our model faithfully reproduces the effect of T cell help through CD40-CD40L interactions.

Various kinetics of DCs responses resulted from precise dynamics of

stimulation. Stimulating CD40 receptor prior to any innate stimuli did not enable the DCs to amplify their responses to IFN-αA. Instead, DCs required innate stimulation for about 1 to 2 hours and only then became receptive to α-CD40 signal. Once activated, DCs could respond to CD40 ligation in a remarkably rapid manner, within minutes. Indeed, only 15 to 30 minutes of CD40 stimulation efficiently enhanced the gene expression and protein secretion induced by IFN-αA stimulation. CD40L expression on T cells is transient and regulated (van Kooten and Banchereau 2000). The interactions between $CD4^+$ T cell and DCs are short (Hor et al. 2015). Therefore the binding ligand-receptor is limited to a precise window of time. The rapid induction of CD40 signalling matches with helper T cells competent of shaping DCs priming ability in a fast and efficient manner without necessity of long-lasting cell to cell interactions.

Relying on the precise kinetics of DC responses to IFN-αA and α-CD40 stimulation, the investigation of gene regulation at whole transcriptome level revealed three intriguing patterns of expression. As expected from previous work conducted in the team (Greyer et al. 2016) and from qPCR experiments, the expression of some genes up-regulated under IFN-αA stimulation could be significantly enhanced with the addition of α-CD40 antibody treatment. This amplification of IFN-αA-induced expression was the first pattern. This ability of two stimuli acting in synergy to increase the responses triggered by a solely stimulus has been previously suggested. LPS pre-treated macrophages trigger enhanced CpG-mediated IL-6 and TNF production (De Nardo et al. 2009). Similarly, DCs pre-treated with CpG display amplified IL-12 production in response to LPS stimulation. Several pieces of evidence presented here excluded the possibility that amplification of IFN-αA-mediated responses was the result of a CD40-driven additional IFN-α/β secretion, acting in an autocrine manner to boost the signalling pathway. Indeed, blocking the IFNAR receptor during CD40 stimulation did not abrogate the amplification of innate responses. Furthermore, a large portion of IFN-αA-regulated genes were unaffected by the addition of α-CD40 antibody treatment dismissing the α-CD40-mediated IFN-α/β secretion as solely responsible for the enhancement of DCs responses. These

genes represent the second pattern of expression. Interestingly, this amplification phenomenon did not affect all IFN-αA-induced genes, as some of these genes could not be amplified even with longer duration of CD40 stimulation. These results revealed a specific effect of CD40-mediated enhancement targeting a precise set of genes only. Finally, the third pattern of expression observed following IFN-αA and α-CD40 stimulations was the "specific" pattern. The genes following this pattern were not induced by IFN-αA stimulation alone or by α-CD40 antibody treatment only but required the combination of both stimuli to be up-regulated. We demonstrated that IFN-αA stimulation was responsible for an up-regulation of various co-stimulatory molecules including CD40. The increase of the surface expression of this receptor could explain the regulation of these specific genes. IFN-αA stimulation would only be required indirectly to increase the DCs ability to receive the CD40L signal. Therefore CD40 stimulation would be sufficient for the expression of these genes. However, we were able to detect a basal expression of CD40 without stimulation as well as a slight up-regulation of the surface expression of this receptor without any stimulation most likely due to the culture conditions. When $eCD8^+$ DCs were stimulated with α-CD40 antibody alone, this basal expression was not sufficient to induce gene regulation. Therefore, this particular pattern of expression requires the combination of factors induced by the two different signalling pathways. It is tempting to speculate that such interplay might arise from the independent induction of individual transcription factors that then heterodimerize to induce transcription of these specific genes. The amplification model is not the only model that have been proposed to explain the respective involvement of $CD4^+$ T cell help and innate signals in $CD8^+$ T cell priming. An alternative model suggests that the result of the combination of help signal and innate signal is different from the result of the innate signal alone. Therefore, $CD4^+$ T cell help could enhance, in terms of quantity, the innate signal responses or would shape the quality of these responses. While those two models might have been seen as incompatible (Borst et al. 2018), our results bring a new dimension into the contribution of help in the context of CTL priming with a quantitative amplification of IFN-αA-

induced responses associated with the establishment of a specific response to the combinatorial signals.

The analysis of up-regulated genes exhibited an enrichment of immune response functions in a dynamic manner from 15 minutes to 4 hours of α-CD40 treatment. Moreover, genes involved in the regulation of transcriptional activity were rapidly enhanced suggesting the early organisation of mechanisms responsible for the amplification. The data mining of the genes regulated allowed us to propose a potential model for the response to CD40 stimulation. In the case of amplified genes, IRF9 being the main candidate as transcription regulator of IFN-αA-induced responses, our findings indicate its cooperation with another regulator triggered by α-CD40 antibody treatment. NF-κB signalling pathway was then highlighted as enriched in our conditions and potentially involved in the regulation of the group of amplified genes only. Such cooperation between IRFs and NF-κB TFs has been previously observed in response to virus infection. In humans, IRF3 and p65/Rela co-activate and recruit the RNA polymerase II (Freaney et al. 2013). In addition, when both pathways were activated via the same stimulus, the gene expression in response to IRF3 translocation into the nucleus was delayed and NF-κB translocation triggered rapid gene regulation (Zhao et al. 2013). This observation corroborates our results were IRF9-induced responses were slow but the effect of CD40 stimulation on the regulation of the same genes would occur rapidly. The regulation of the specific genes, requiring the combination of IFN-αA and α-CD40 antibody treatment together likely followed a different mechanism. The prediction of TFs potentially involved in the specific genes expression showed a possible dynamic organisation of transcription regulators. Indeed, Jun and Fos, were identified for the regulation of the early specific genes while Rel was identified for the later expression. In both cases, Irf1 was suggested as a potential co-regulator acting in a sustained manner. IRF1 is thought to be expressed at a low basal level and induced by IFN-γ and/or TNF-α stimuli via the involvement of STAT1 and NF-κB TFs (Kröger et al. 2002; Michalska et al. 2018). Moreover, IRF1 interacts with other TFs in order to

activate the transcription of ISGs. In addition of the IFN-αA-driven up-regulation of *Irf1* expression our results highlighted a strong enhancement of this TF expression after only 30 minutes of CD40 stimulation and amplification detectable after 4 hours of treatment. Interestingly, IRF1 half-life is only of 30 minutes implying direct correlation between mRNA expression and protein synthesis (Kröger et al. 2002). This observation strongly suggests a key role for IRF1 in the transmission of the help signal, in a sustained manner.

While our results provide novel insights into help mechanisms within the DCs, more work is necessary to further validate our hypotheses. For instance, NF-κB signalling pathway heavily relies on protein trafficking to the nucleus and protein degradation in response to post-transcriptional modifications (Oeckinghaus et al. 2011). The phosphoproteomics analysis suggests tight regulation of the NF-κB signalling pathway, however the phosphorylation events resulting in the activation of this pathway most likely occur earlier in transient manner and therefore investigations at earlier time points are needed. In addition of the investigation of the protein synthesis and TFs trafficking in response to stimuli, epigenetic modifications necessitate further investigation. Histone acetylation is for instance critical for transcriptional control. Acetylation of histones leads to a change in the chromatin conformation, rendering it open and thus allowing the binding of TFs. We hypothesise that the up-regulation of *Irf1*, one of the key candidates TF highlighted in our work, would eventually result in its protein synthesis and this will be important to test. Moreover, IRF1 is involved in the recruitment of acetyltransferases at the promoter of target genes (Marsili et al. 2004).

While the study presented herein focused on the molecular mechanisms underlying α-CD40-amplification of IFN-αA-induced genes and the genes positively regulated in response to their combinatorial stimulation, our transcriptomic dataset offers interesting patterns of expression potentially governed by specific mechanisms. Our study focused for instance on gene expression being induced by IFN-αA stimulation and enhanced with the addition

of α-CD40 treatment. However, some genes followed an opposite regulation and were downregulated under IFN-αA stimulation and further downregulated with CD40 stimulation in addition. Furthermore, a group of genes was progressively downregulated when α-CD40 treatment was added to IFN-αA stimulation. Although these genes were not linked to a specific function, with only a few of them such as *Lin54*, *Ccne1/2* or *Tgfb3* related to cellular senescence (data not shown), further analysis would provide insight into what mechanisms are responsible for this specific gene downregulation. Moreover, our results highlighted a group of genes linked to the mRNA stability, upregulated only after 30 minutes of α-CD40 treatment added to IFN-Aα stimulation and subsequently actively downregulated in our conditions.

Our team previously demonstrated that the cytokines and chemokines produced by DCs are dictated by the innate stimuli they receive (Greyer et al. 2016). The observation of an amplification process confined to a portion of IFN-αA-induced genes reveals a new layer in the DCs complex ability to decipher and assimilate signals they integrate to provide precise responses towards T cell priming and the promotion of immune responses. Altogether, the results presented in this thesis provide an insight in the mechanisms allowing DCs to regulate their responses to stimuli. Using CD40L-CD40 interaction blockade has been observed to inhibit autoimmune disease development as well as increase the number of islet transplant acceptance in diabetic animals. Direct antibody blockade, targeting the trimerization of CD40L or inhibition of CD40L mRNA transcription are all various strategies for CD40 signalling inhibition either currently used or proposed for autoimmune disease treatment (Howard and Miller 2004). Promising advances on allograft tolerance have been achieved. Prior to organ transplantation, blocking anti-CD40L is administrated in addition to donor alloantigen. DC maturation is prevented but the alloantigen can still be presented by non-matured DCs, thus leading to an abortive priming alloantigen specific T cells and creating an environment tolerant for the allograft (Elgueta et al. 2009). Although CD40 signalling has been extensively studied, particularly in respects to B cells, the details of this signalling in DCs and its involvement in an

adequate and powerful CTL priming still needs to be clarified. Precisely targeting the CD40 signalling and its nuances is an exciting prospect for autoimmune diseases and graft rejection treatments. The efficiency of vaccines relies on the use of potentially inflammatory adjuvants (Garçon et al. 2011). Antibodies targeting CD40 as adjuvant have been studied in the context of vaccination against Influenza A and have shown an enhancement of the immune responses (Hatzifoti and Heath 2007). Understanding how and when T helper signals can improve DCs ability for CTL priming would benefit vaccination efficacy and limit potential side effects related to adjuvants.

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# CURRICULUM VITAE

## Education

| | |
|---|---|
| 2016-current | Jointly awarded PhD position: Bonn, Germany & Melbourne, Australia Research and Graduate training group (Bo&MeRanG)<br>- Sammy Bedoui, Dep. of Microbiology and Immunology, University of Melbourne, Peter Doherty Institute, Melbourne, Australia<br>- Susanne V. Schmidt, Eicke Latz group, Institute of Innate Immunology, Rheinische-Friedrich-Wilhelms University of Bonn, Bonn, Germany<br>Project: **The role of T cell help in shaping dendritic cell function** |
| 2013-2015 | Master of Immunology, University Aix-Marseille, France, with Honors |
| 2011-2012 | Bachelor of Cellular Biology and Physiology, University Science and Technology, Lille, France, with Honors |
| 2009-2011 | Faculty of medicine Henri Warembourg, University Science and Technology, Lille, France |
| 2008-2009 | High school graduation in science, France, with Honors |

## Research experience

| | |
|---|---|
| 06/2012-08/2012 | Center of Infection and Immunity Lille (CIIL), France<br>Supervised by Dr C. Duez in A. Tsicopoulos Laboratory<br>**Initiation to work on biology and immunology** |
| 05/2013-06/2013 | Center of Infection and Immunity Lille (CIIL), France<br>Supervised by Dr C. Duez in A. Tsicopoulos Laboratory<br>**Implication of NK cells in the regulation of pulmonary immunity in presence of micro-organisms** |
| 03/2014-07/2014 | Center of Immunology, Marseille-Luminy (CIML), France<br>Supervised by Dr M. Bebien in T. Lawrence Laboratory<br>**Mechanisms of IFN-I induction on macrophages by *Group A Streptococcus*** |

09/2014-02/2015 Center of Immunology, Marseille-Luminy (CIML), France
Supervised by Dr E. Tomasello in M. Dalod Laboratory
**Regulation of pDC effector functions by IFN-I**

03/2015-05/2015 Harvard Medical School, Boston Children's Hospital, US
Supervised by J. Chang in M. Carroll Laboratory
**Role of Conduits-forming Fibroblastic Reticular Cells in the Peyer's patches**

## Oral Presentations

***Dissecting the molecular mechanisms underpinning T cell help for Dendritic Cell***

2017 Bonn & Melbourne Research and Graduate training group Retreat

2017 Postgrad Association for Students in Microbiology and Immunology Biennial Student Retreat

2017 IgV (The Immunology Group of Victoria) Scientific Meeting

2017 Australasian Society for Immunology (ASI) Annual Scientific Meeting

2018 Doherty Institute Immunology theme – Data Presentation Series

2018 Australasian Society for Immunology (ASI) Annual Scientific Meeting

2019 Bonn & Melbourne Research and Graduate training group Retreat

## Poster Presentations

***Dissecting the molecular mechanisms underpinning T cell help for Dendritic Cell***

2017 Australian Society for Medical Research, Student Symposium

2017 Victorian Infection & Immunity Network (VIIN), Young Investigator Symposium

2017 Doherty Institute Inaugural Research Day

2018 European Congress of Immunology (ECI)

2019 Australasian Society for Immunology (ASI) Annual Scientific Meeting

# APPENDIX

**Table A.1: List of genes within SOM clusters (part 1).**

| Cluster ID (Row – Column) | Genes |
|---|---|
| Cluster 1 (1 – 1) | *Bcl2a1a* |
| | *Ccl5* |
| | *Cd274* |
| | *Cd69* |
| | *Chd7* |
| | *Cxcl10* |
| | *Egr3* |
| | *Fosb* |
| | *Grasp* |
| | *Id1* |
| | *Kcnc3* |
| | *Kdm6b* |
| | *Ldlr* |
| | *Nr4a1* |
| | *Phlda1* |
| | *Skil* |
| | *Zfand5* |
| | *Zfp36l1* |
| Cluster 2 (1 – 2) | *A3galt2* |
| | *Arl5b* |
| | *Clcf1* |
| | *Csrnp1* |
| | *Dusp2* |
| | *Irf1* |
| | *Nlrp3* |
| | *Pim1* |
| | *Rnf19b* |
| | *Sh3d21* |
| | *Socs3* |
| | *Tgif2* |
| | *Trib1* |
| | *Zc3h12a* |
| Cluster 3 (1 – 3) | *Ccl4* |
| | *Cd40* |
| | *Cdkn1a* |
| | *Dcbld2* |
| | *Dusp5* |
| | *Gadd45b* |
| | *H2-K2* |
| | *H2-Q7* |
| | *Icam4* |
| | *Ifrd1* |
| | *Maff* |
| | *Mybpc3* |
| | *Nfkbia* |
| | *Nfkbid* |
| | *Rasgef1b* |
| | *Rel* |
| | *Sowahc* |
| | *Taf7* |
| | *Tgif1* |
| | *Tnf* |
| | *Tnfaip3* |

| Cluster ID (Row – Column) | Genes |
|---|---|
| Cluster 4 (1 – 4) | *Atf3* |
| | *Btg1* |
| | *Dot1l* |
| | *Ets2* |
| | *Etv3* |
| | *Herpud1* |
| | *Hspa1a* |
| | *Hspa1b* |
| | *Hspbap1* |
| | *Icam1* |
| | *Itpkc* |
| | *Ncoa7* |
| | *Snhg15* |
| Cluster 5 (1 – 5) | *Bcl2a1b* |
| | *Birc3* |
| | *Cd83* |
| | *Gpr132* |
| | *Klf6* |
| | *Lilr4b* |
| | *Rgs1* |
| | *Slc35b2* |
| | *Stx11* |
| | *Tnip3* |
| | *Traf1* |
| Cluster 6 (2 – 1) | *A930037H05Rik* |
| | *Btg2* |
| | *Egr1* |
| | *Gm18853* |
| | *Ier2* |
| | *Jun* |
| | *Mpv17l* |
| | *Pkd2l2* |
| Cluster 7 (2 – 2) | *Ccl3* |
| | *Cited2* |
| | *Dusp1* |
| | *Gdap10* |
| | *Insig1* |
| | *Nfkbiz* |
| | *Nr4a3* |
| | *Pmaip1* |
| | *Ppp1r15a* |
| | *Zfp36* |
| Cluster 8 (2 – 3) | *Casz1* |
| | *Junb* |
| | *Mxd1* |
| | *Npas2* |
| | *Psd* |
| | *Tagap* |
| Cluster 9 (2 – 4) | *Ell2* |
| | *Ier3* |
| | *Il2rg* |
| | *Lilrb4a* |
| | *Marcksl1* |
| | *Mcl1* |
| | *Serpinb9* |
| | *Tent5c* |

| Cluster ID (Row - Column) | Genes |
|---|---|
| Cluster 10 (2 – 5) | *9330159M07Rik* |
| | *Afmid* |
| | *Arl5c* |
| | *Cacfd1* |
| | *Cx3cl1* |
| | *Icosl* |
| | *Itga5* |
| | *Myo1g* |
| | *Nfkb2* |
| | *Nfkbie* |
| | *Pdlim7* |
| | *Prr14* |
| | *Rasip1* |
| | *Rela* |
| | *Srgn* |
| | *Tnnt3* |
| | *Trim35* |
| Cluster 11 (3 – 1) | *Armcx6* |
| | *Arntl* |
| | *Frmd4a* |
| | *Ifi44* |
| | *Ifit3* |
| | *Iigp1* |
| | *Olr1* |
| | *Rin2* |
| | *Rnf214* |
| | *Slfn9* |
| | *Stat1* |
| | *Tgtp2* |
| | *Treml2* |
| Cluster 12 (3 – 3) | *Fos* |
| | *Tulp2* |
| Cluster 14 (3 – 4) | *4930523C07Rik* |
| | *Arhgef3* |
| | *Axl* |
| | *Ccnd2* |
| | *Gm15708* |
| | *Clic4* |
| | *Dyrk2* |
| | *Inpp5b* |
| | *Ncf1* |
| | *Ptger4* |
| | *Sdc4* |
| | *Serpinb6b* |
| | *Tlr2* |
| | *Zc3h12c* |
| | *Zmynd15* |

**Table A.1: List of genes within SOM clusters (part 2).**

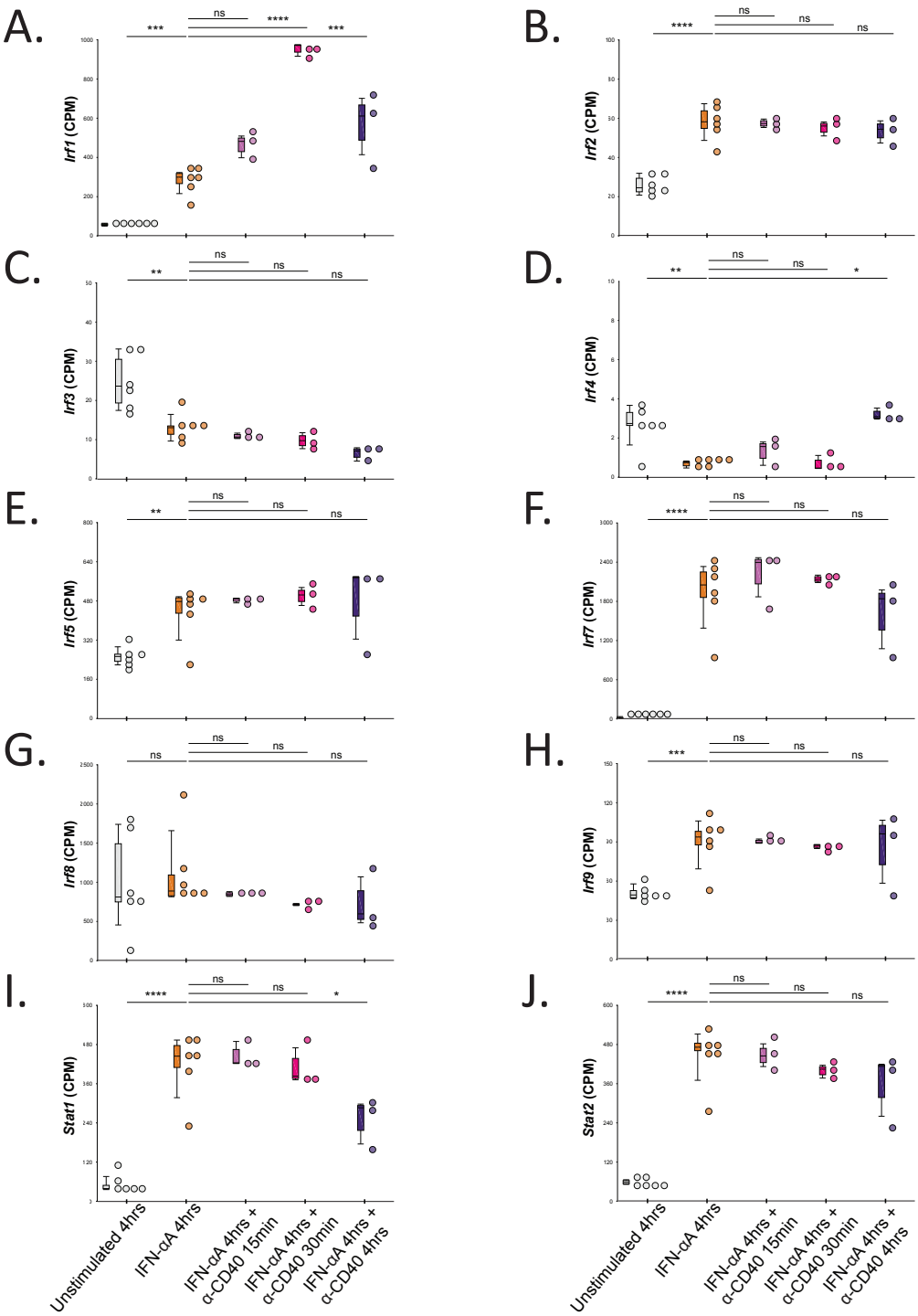
| Cluster ID (Row – Column) | Genes |
|---|---|
| Cluster 15 (3 – 5) | *Bcl2a1d* |
| | *C9orf72* |
| | *Cpeb4* |
| | *Cxcl16* |
| | *Ehd1* |
| | *Furin* |
| | *Hivep1* |
| | *Il15* |
| | *Kcnk6* |
| | *Mapk6* |
| | *Mdm2* |
| | *Mir22hg* |
| | *Mirt1* |
| | *Mthfs* |
| | *Nabp1* |
| | *Nfkbib* |
| | *Pvr* |
| | *Relb* |
| | *Sbds* |
| | *Slc2a6* |
| | *Suco* |
| | *Tmem123* |
| | *Tmem39a* |
| | *Tnfaip2* |
| Cluster 16 (4 – 1) | *Arhgap12* |
| | *Ccne2* |
| | *Dmpk* |
| | *Flt4* |
| | *Gnptab* |
| | *Gpsm2* |
| | *Hbp1* |
| | *Lims1* |
| | *Lin54* |
| | *Lpin2* |
| | *Mcm10* |
| | *Mcm6* |
| | *Mxi1* |
| | *Npat* |
| | *Ppm1k* |
| | *Smad1* |
| | *Smarca5-ps* |
| | *St8sia1* |
| | *Tgfb3* |
| | *Tpr* |
| | *Traf3ip3* |
| | *Trim34a* |
| | *Trim56* |
| | *Usp1* |
| | *Wdr62* |
| | *Zcchc24* |

| Cluster ID (Row – Column) | Genes |
|---|---|
| Cluster 18 (4 – 3) | *Atp6v0a1* |
| | *Ctsz* |
| | *Irf4* |
| | *Jade2* |
| | *Ncoa5* |
| | *Plxnc1* |
| | *Rab12* |
| | *Rras2* |
| | *Socs2* |
| | *Tank* |
| | *Zfp280b* |
| Cluster 19 (4 – 4) | *Basp1* |
| | *Dqx1* |
| | *Irak2* |
| | *Lcp1* |
| | *Mtf1* |
| | *Nipal1* |
| | *Pik3r5* |
| | *Poglut1* |
| | *Spdl1* |
| | *Stat5a* |
| | *Tmem63b* |
| | *Tnip1* |
| | *Traf6* |
| Cluster 20 (4 – 5) | *1700047I17Rik2* |
| | *Adam8* |
| | *Bcl2l11* |
| | *Cd200* |
| | *Cdc42ep3* |
| | *Cdk5r1* |
| | *Cflar* |
| | *Ebi3* |
| | *Fam177a* |
| | *Mif4gd* |
| | *Mllt6* |
| | *Mthfsl* |
| | *Mylip* |
| | *Nudt9* |
| | *Oaf* |
| | *Pi4k2b* |
| | *Rap1b* |
| | *Rest* |
| | *Sema6d* |
| | *Slamf7* |
| | *Slc22a21* |
| | *Slc39a1* |
| | *Sqstm1* |
| | *Tnfsf15* |
| | *Tnfsf9* |
| | *Zfand2a* |

| Cluster ID (Row – Column) | Genes |
|---|---|
| Cluster 21 (5 – 1) | *4921531C22Rik* |
| | *A130010J15Rik* |
| | *Akap17b* |
| | *Arl4a* |
| | *B4galt4* |
| | *C430042M11Rik* |
| | *Cbll1* |
| | *Ccne1* |
| | *Degs2* |
| | *Ell3* |
| | *F630111L10Rik* |
| | *Fam111a* |
| | *Hap1* |
| | *Kbtbd7* |
| | *Mitd1* |
| | *Nedd9* |
| | *Ogfrl1* |
| | *Pou6f1* |
| | *Rab19* |
| | *Rmi2* |
| | *Slc30a1* |
| | *Tent5a* |
| | *Tex9* |
| Cluster 22 (5 – 2) | *Ap3m2* |
| | *C920021L13Rik* |
| | *F420014N23Rik* |
| Cluster 23 (5 – 3) | *Batf* |
| | *Cptp* |
| | *Cst7* |
| | *Dapp1* |
| | *Dok1* |
| | *Exoc3l4* |
| | *Gucd1* |
| | *Il27* |
| | *Parp3* |
| | *Ralgds* |
| | *Riox2* |
| | *Slc43a3* |
| | *Tlcd2* |
| | *Tnk1* |

**Table A.1: List of genes within SOM clusters (part 3).**

| Cluster ID (Row – Column) | Genes |
|---|---|
| Cluster 24 (5 – 4) | *Arl4c* |
| | *Cacnb3* |
| | *Cd82* |
| | *Cdk6* |
| | *Degs1* |
| | *Dennd4a* |
| | *Fam84b* |
| | *Foxp4* |
| | *Hmga1* |
| | *Hsd17b11* |
| | *Hsf2* |
| | *Il4i1* |
| | *Jdp2* |
| | *Jup* |
| | *Kdm2b* |
| | *Lima1* |
| | *Mdfic* |
| | *Ptafr* |
| | *Rapgef2* |
| | *Rcsd1* |
| | *Rhof* |
| | *St3gal1* |
| | *Stip1* |
| | *Txndc17* |
| Cluster 25 (5 – 5) | *Adap1* |
| | *Aebp2* |
| | *Arf2* |
| | *Arhgap22* |
| | *Bcl2l1* |
| | *Cd80* |
| | *Cldn1* |
| | *Dnase1l3* |
| | *Emp3* |
| | *Il15ra* |
| | *Kpna3* |
| | *Mfhas1* |
| | *Mrpl39* |
| | *Nub1* |
| | *Rnf2* |
| | *Slc30a4* |
| | *Stx6* |
| | *Swap70* |
| | *Uap1* |
| | *Zswim4* |




**<u>Figure A.1</u>: Regulation of the expression of *Irfs* and *Stat1/2*.**
Regulation of the expression of *Irf1* **(A.)**, *Irf2* **(B.)**, *Irf3* **(C.)**, *Irf4* **(D.)**, *Irf5* **(E.)**, *Irf7* **(F.)**, *Irf8* **(G.)**, *Irf9* **(H.)**, *Stat1* **(I.)** and *Stat2* **(J.)** assessed following the indicated stimulation. Each dot is a sample, the line inside the box is the median, the whiskers represent the variability. Asterisks indicate statistically significant differences between the indicated conditions as assessed by one-way ANOVA; adjusted p-value: ns = non significant; * p≤0.047; ** p≤0.007; *** p≤0.0004; **** p<0.0001.

**Table A.2: List of phosphorylation and dephosphorylation events under IFN-αA+α-CD40 4 hours condition compared to IFN-αA 4 hours condition (part 1).**
Significant events were selected with a p-value ≤ 0.05 and a probability of localisation > 0.75.
Events presented from the lowest p-value (high significance) to the highest p-value (lower significance).

Phosphorylation events:

| -Log10 (p-value IFN-αA vs IFN-αA+α-CD40) | Log2 FC (IFN-αA vs IFN-αA + α-CD40) | Protein | Localisation (Amino acid – position) |
|---|---|---|---|
| 3.5296 | 3.8498 | Crebbp | Ser1077 |
| 3.477 | 3.57649 | Rlim | Ser194 |
| 3.47627 | 4.20114 | Gab2 | Ser402 |
| 3.43593 | 2.51402 | Ampd2 | Ser45 |
| 3.4323 | 3.31568 | Spn | Ser139 |
| 3.27709 | 3.69652 | Tnfaip3 | Ser381 |
| 3.18662 | 4.92123 | Myo1g | Ser998 |
| 3.09868 | 3.54325 | Tmpo | Ser291 |
| 3.0363 | 3.28189 | Coro1a | Ser426 |
| 2.84376 | 1.92042 | Pcyt1a | Ser347 |
| 2.7145 | 4.12413 | Taf3 | Ser90 |
| 2.68712 | 3.00416 | Cenpe | Ser2423 |
| 2.4238 | 3.21354 | Lrmp | Ser373 |
| 2.30787 | 2.64733 | Gcc2 | Ser341 |
| 2.22294 | 2.37997 | Inpp5d | Ser173 |
| 2.14658 | 2.71468 | Akap9 | Ser3694 |
| 2.05209 | 3.02263 | Zbp1 | Ser384 |
| 1.97866 | 1.5995 | Tex2 | Ser195 |
| 1.95929 | 1.64142 | Atrip | Ser102 |
| 1.90297 | 2.0261 | Ahnak | Ser5596 |
| 1.8993 | 2.35394 | Krit1 | Ser276 |
| 1.89513 | 3.6341 | Kifap3 | Ser60 |
| 1.89103 | 2.1418 | Lmnb2 | Ser402 |
| 1.84581 | 2.82309 | Ogfrl1 | Ser65 |
| 1.84269 | 2.14502 | Huwe1 | Ser1368 |
| 1.77473 | 1.97334 | Pde4dip | Ser195 |
| 1.73577 | 2.46296 | Nfkbie | Ser18 |
| 1.71399 | 2.13797 | Cep128 | Ser33 |
| 1.6851 | 1.90442 | Ssrp1 | Ser657 |
| 1.68335 | 1.61834 | Spag9 | Ser561 |
| 1.67788 | 2.24202 | Ccdc88a | Ser1584 |
| 1.67536 | 1.99893 | Srpk1 | Ser335 |

| | | | |
|---|---|---|---|
| 1.67155 | 2.29818 | C7orf50 homolog | Ser180 |
| 1.6664 | 1.91294 | Card11 | Ser448 |
| 1.65114 | 1.88712 | Zmym4 | Ser242 |
| 1.62434 | 3.46781 | Plekho2 | Thr251 |
| 1.61453 | 2.31649 | Myo9a | Ser1300 |
| 1.60525 | 2.84119 | Snap29 | Ser65 |
| 1.59179 | 1.68009 | Secisbp2l | Ser900 |
| 1.58785 | 4.0828 | Mtdh | Ser297 |
| 1.57018 | 1.63469 | Prrc2a | Thr782 |
| 1.55297 | 2.57025 | Zc3h18 | Ser45 |
| 1.53482 | 2.14485 | Arfgap2 | Ser431 |
| 1.51213 | 2.85533 | Ahctf1 | Ser1928 |
| 1.47783 | 2.90321 | Palld | Ser1146 |
| 1.47592 | 1.8848 | Smg9 | Ser53 |
| 1.47293 | 2.90789 | Mvb12a | Ser168 |
| 1.46621 | 2.70381 | Pcf11 | Ser495 |
| 1.46415 | 1.86075 | Ndrg1 | Thr328 |
| 1.45371 | 3.43613 | Rb1 | Ser31 |
| 1.43485 | 2.91031 | Tpd52 | Ser145 |
| 1.4348 | 3.60622 | Jdp2 | Thr148 |
| 1.4001 | 3.2158 | Ikbkb | Ser697 |
| 1.39384 | 2.13993 | Card10 | Ser678 |
| 1.37489 | 3.38107 | Tbc1d10b | Ser693 |
| 1.36774 | 1.9717 | Sh3bp4 | Ser240 |
| 1.35576 | 3.09447 | Srrm2 | Ser433 |
| 1.35087 | 2.83804 | Cbx8 | Ser229 |
| 1.3489 | 3.57887 | Macf1 | Ser7295 |
| 1.32642 | 2.7496 | Nedd4 | Ser309 |
| 1.31837 | 2.07825 | Sipa1l1 | Ser1116 |
| 1.30778 | 1.81134 | Sos2 | Ser1283 |
| 1.30431 | 2.87349 | Hnrnpm | Ser574 |

**Table A.2: List of phosphorylation and dephosphorylation events under IFN-αA+α-CD40 4 hours condition compared to IFN-αA 4 hours condition (part 2).**

Dephosphorylation events:

| -Log10 (p-value IFN-αA vs IFN-αA+α-CD40) | Log2 FC (IFN-αA vs IFN-αA + α-CD40) | Protein | Localisation (Amino acid – position) |
|---|---|---|---|
| 4.65002 | -6.02586 | Son | Ser2047 |
| 4.02031 | -3.32748 | Pikfyve | Ser475 |
| 3.70601 | -3.26485 | Rexo1 | Ser353 |
| 3.28061 | -2.45748 | Rsf1 | Ser401 |
| 3.27111 | -3.45232 | Mki67 | Thr1952 |
| 3.18406 | -3.38134 | Ell | Ser300 |
| 3.03041 | -2.5885 | Cep128 | Ser1058 |
| 2.46856 | -3.22592 | Baiap2 | Ser493 |
| 2.44423 | -4.60781 | Nop56 | Thr546 |
| 2.3904 | -4.19358 | Son | Ser2049 |
| 2.2987 | -4.14234 | Ubap2l | Ser625 |
| 2.23136 | -2.5985 | Rpsa | Ser43 |
| 2.17236 | -2.45818 | Fermt3 | Ser8 |
| 2.1325 | -2.48194 | Top2a | Ser1211 |
| 2.1272 | -2.01409 | Hist1h1d | Thr18 |
| 2.1248 | -2.6917 | Grap2 | Ser41 |
| 2.05429 | -2.97466 | Hnrnpf | Ser187 |
| 2.05098 | -3.7054 | Phip | Ser1283 |
| 2.03173 | -1.82344 | Nadk | Ser50 |
| 1.99501 | -5.20582 | Rreb1 | Ser1452 |
| 1.98133 | -2.24405 | Pnn | Ser441 |
| 1.96722 | -3.36034 | Nfic | Ser339 |
| 1.9361 | -2.49673 | Cdk13 | Ser664 |
| 1.93325 | -2.72872 | Gm20431;Ube2v1 | Ser370 |
| 1.92983 | -3.78564 | Fxr2 | Ser603 |
| 1.91494 | -3.13332 | Fam126a | Ser465 |
| 1.91426 | -3.76288 | Mbd2 | Ser410 |
| 1.91425 | -3.33242 | Phip | Ser1281 |
| 1.91038 | -3.1707 | Nmd3 | Thr470 |
| 1.8972 | -1.80362 | Hist1h1e | Thr18 |
| 1.89123 | -3.94332 | Tns3 | Ser332 |
| 1.87531 | -3.00582 | Atxn2 | Ser834 |
| 1.85233 | -2.79783 | Cad | Thr1037 |
| 1.84707 | -4.04609 | Cdyl | Ser83 |
| 1.819 | -4.49519 | Rcsd1 | Ser378 |

| | | | |
|---|---|---|---|
| 1.76248 | -2.9672 | Rcsd1 | Ser177 |
| 1.7497 | -3.77992 | Fcho2 | S394 |
| 1.73218 | -1.97058 | Rock2 | S1374 |
| 1.73021 | -3.06876 | Rsrc2 | Thr219 |
| 1.71646 | -2.01245 | Diap3;Diaph3 | Ser1072 |
| 1.71232 | -1.70057 | Itgb3 | Thr778 |
| 1.70767 | -2.83158 | Sub1 | Ser92 |
| 1.68618 | -3.15368 | Rbl1 | Ser959 |
| 1.68158 | -2.00377 | Arhgap21 | Ser875 |
| 1.6757 | -2.17686 | Ppih | Ser6 |
| 1.6747 | -1.87852 | Atm | Ser1987 |
| 1.67352 | -1.95414 | Lrrc40 | Ser37 |
| 1.66428 | -5.72701 | Lrrc25 | Ser227 |
| 1.62752 | -4.41118 | Rif1 | Ser1565 |
| 1.61313 | -3.61108 | Hsd17b4 | Ser3 |
| 1.58239 | -5.07196 | Rgs14 | Ser203 |
| 1.57318 | -3.71659 | Srrm2 | Ser2070 |
| 1.56518 | -2.03763 | Srrm2 | Ser946 |
| 1.5564 | -3.32773 | Cic | Ser778 |
| 1.55621 | -3.17743 | Sptbn1 | Ser2163 |
| 1.55296 | -2.16765 | Prkd2 | Thr715 |
| 1.54998 | -3.47008 | Eef1g | Thr46 |
| 1.54118 | -2.54248 | Twistnb | Thr226 |
| 1.53469 | -1.64387 | Mki67 | Ser128 |
| 1.53107 | -2.86663 | Wdr90 | Ser20 |
| 1.52732 | -3.53792 | Thrap3 | Ser320 |
| 1.52651 | -2.46248 | Dennd1b | Ser395 |
| 1.52316 | -4.54814 | Wdr44 | Ser563 |
| 1.52038 | -4.85644 | Ddi2 | Ser121 |
| 1.51522 | -2.54608 | Mki67 | Ser2934 |
| 1.50966 | -2.64157 | Pbrm1 | Ser636 |
| 1.50691 | -3.2781 | Chaf1a | Ser173 |
| 1.50038 | -2.46659 | Plekhg3 | Ser502 |
| 1.49929 | -3.18726 | C2cd2l | Ser660 |
| 1.49784 | -1.8793 | Irf8 | Ser232 |
| 1.49405 | -3.20341 | Nap1l1 | Ser69 |
| 1.48127 | -2.63757 | Mki67 | Thr797 |
| 1.46249 | -2.85549 | Ik | Ser225 |
| 1.46134 | -3.16853 | Irf8 | Ser162 |
| 1.45939 | -2.13223 | Tpm4 | Ser179 |
| 1.4575 | -2.23491 | Iws1 | Thr672 |
| 1.45688 | -4.49456 | Vim | Ser412 |
| 1.4475 | -2.43856 | Gpalpp1 | Ser249 |

| | | | |
|---|---|---|---|
| 1.44614 | -1.89761 | Lpin2 | Ser144 |
| 1.43701 | -3.46722 | Tfeb | Ser167 |
| 1.43593 | -3.61993 | Rftn1 | Ser198 |
| 1.43033 | -3.81911 | Eif4b | Ser93 |
| 1.41316 | -2.28094 | Cdca2 | Ser758 |
| 1.41043 | -1.61421 | Atad2 | Ser317 |
| 1.40896 | -3.70831 | Parp12 | S273 |
| 1.4072 | -2.5126 | Rptor | Ser859 |
| 1.40053 | -5.24351 | Pak1 | Thr184 |
| 1.39784 | -2.08432 | Peak1 | Ser1206 |
| 1.39441 | -1.51608 | Mcm3 | Ser253 |
| 1.39391 | -3.03028 | Prkag2 | Ser90 |
| 1.38431 | -2.44391 | Phf10 | Ser326 |
| 1.38389 | -4.53788 | Fgd3 | S589 |
| 1.38231 | -1.64932 | Osbpl11 | Ser192 |
| 1.37185 | -6.8898 | G3bp1 | S231 |
| 1.37139 | -3.43056 | Raly | Thr268 |
| 1.36795 | -2.6349 | Arhgap11a | Ser284 |
| 1.36738 | -1.79681 | Cdca7l | Thr153 |
| 1.36272 | -2.88557 | Fubp1 | Ser410 |
| 1.36233 | -2.96947 | Pcyt1a | Ser315 |
| 1.35907 | -2.40717 | Lmnb1 | Thr400 |
| 1.35833 | -3.57857 | Bcl2l13 | Thr342 |
| 1.35532 | -2.0161 | Asap2 | Ser722 |
| 1.3437 | -3.10828 | Otud4 | Ser1016 |
| 1.33899 | -2.59814 | Map3k4 | Ser424 |
| 1.33587 | -5.39164 | Sash3 | Ser42 |
| 1.33217 | -1.57546 | Phf2 | Ser899 |
| 1.33216 | -3.30801 | Isy1 | Ser222 |
| 1.3277 | -2.26973 | Pqbp1 | Ser245 |
| 1.32723 | -2.66356 | Map7d1 | Ser296 |
| 1.32651 | -3.01051 | Fam21 | Ser1173 |
| 1.32635 | -2.99368 | Rabep1 | Ser410 |
| 1.32464 | -1.67302 | Rhbdf2 | Ser113 |
| 1.32006 | -1.87869 | Shkbp1 | Ser621 |
| 1.31779 | -2.41728 | Hmga1 | Thr53 |
| 1.31721 | -2.36837 | Bag6 | Ser1121 |
| 1.31594 | -2.95905 | Rnf113a2 | Ser85 |
| 1.31459 | -3.51151 | Lrch4 | Ser281 |
| 1.30511 | -2.24882 | Tnks1bp1 | Ser713 |