# Expression of TRIM Genes in Different Immune Cells and Mechanism of Regulation of Their Expression: Implications for the Immune Response to Pathogens

## Thesis

UNRESTRICTED

# "Expression of TRIM genes in different immune cells and mechanism of regulation of their expression: implications for the immune response to pathogens"


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## Abstract

The tripartite motif (TRIM) proteins are important in a variety of cellular functions including antiviral activity. We systematically analyzed mRNA expression of representative TRIMs in primary mouse macrophages, myeloid and plasmacytoid dendritic cells, and a selection of CD4+ T cell subsets. These cells have different effector functions in innate and adaptive immune responses, to a large extent due to the different patterns of cytokines that they produce. Here, we defined four clusters of TRIM genes based on their selective expression in these cell subsets. The first group of TRIMs was preferentially expressed in CD4+T cells and contained the COS-FN3 motif. Additional TRIMs were identified that showed up-regulation in macrophages and dendritic cells upon influenza virus infection in a type-I IFN dependent manner suggesting that they may play a role in anti-viral responses. However, stimulation of macrophages and mDC with LPS and double stranded RNA also led to type-I IFN dependent up-regulation of these TRIM genes, suggesting that their expression is not directly regulated by the virus, and that they may have broader functions in innate immune responses. In support of the proposed role of TRIMs in anti-viral responses, a subset of the type-I IFN dependent TRIMs mapped to mouse chromosome 7, syntenic to human chromosome 11 where TRIMs such as TRIM5, shown to have anti-viral activity, are localized. Consistent with these findings, up-regulation of the same TRIM genes in human macrophages was mainly observed under conditions which resulted in the induction of IFNβ (in this case by LPS and IFNγ stimulations), as observed by reanalysis of a previously published microarray study. Within the group of TRIMs induced by viruses in macrophages and dendritic cells via a type-I IFN dependent mechanism we distinguish two clusters on the basis of TRIM expression in CD4+ T cells. A fourth group of TRIMs was constitutively expressed in

plasmacytoid dendritic cells independently of viral infection or signalling through the type-I IFN receptor. Our findings on expression and regulation of TRIMs may help to develop potential strategies for determining functions of this diverse family of molecules in immune cells.

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## List of abbreviations

| | |
|---|---|
| AP-1 | Activator protein 1 |
| APC | antigen presenting cell |
| APL | acute promyelocytic leukaemia |
| B | B-box domain |
| BM | bone marrow |
| CAL | influenza virus strain A/New Caledonia/20/99 |
| CBP | CREB-binding protein |
| CC | coiled-coil domain |
| Chr | chromosome |
| cPML | cytoplasmic isoform of the promyelocytic leukaemia protein |
| DC | dendritic cell |
| cDC | conventional DC |
| mDC | myeloid DC |
| pDC | Plasmacytoid dendritic cell |
| dsRNA | double stranded RNA |
| EAE | Experimental autoimmune encephalitis |
| EIAV | Equine infectious anaemia virus |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ELK-1 | Member of the ETS family of transcription factors |
| FITC | Fluorescein isothiocyanate |
| FLT3 | Fms-like tyrosine kinase-3 |
| FLT3L | Fms-like tyrosine kinase-3 ligand |
| FN | Fibronectin |
| FV1 | Friend-virus susceptibility gene |
| GAS | Gamma-activation sequences |
| GFP | Green fluorescent protein |
| GMCSF | Granulocyte macrophage–colony stimulating factor |
| HIV | Human immunodeficiency virus |
| HFV | Human foamy virus |
| HP1 | Heterochromatin protein-1 |
| HSV | Herpes simplex virus |

| | |
|---|---|
| IFN | Interferon |
| IFNAR1 | Type-I IFN receptor |
| Ig | Immunoglobulin |
| IKK- | IkappaB kinase |
| IL | Interleukin |
| IMDM | Iscove's modified Dulbecco's medium |
| IRAK4 | IL-1R-associated kinase 4 |
| IRF | Interferon regulatory factor |
| ISG | Interferon stimulated gene |
| ISGF3 | Interferon stimulated gene factor 3 |
| ISRE | Interferon stimulated response elements |
| JAK-1 | Janus activated kinase-1 |
| LCMV | Lymphocytic choriomeningitis virus |
| LPS | Lipopolysaccharide |
| LTR | Long terminal repeat |
| MAC | Macrophage |
| MAL | MyD88 adaptor-like protein |
| MAPK | Mitogen-activated protein kinase |
| MAVS | Mitochondrion antiviral signalling protein |
| MCSF | Macrophage–colony stimulating factor |
| MDA5 | Melanoma differentiation factor-5 |
| MEF | Murine embryonic fibroblast |
| MEFV | Mediterranean fever |
| MHC | Major histocompatibility complex |
| MLV-N | Murine leukaemia virus, N-tropic |
| MyD88 | Myeloid differentiation primary-response gene 88 |
| NB | Nuclear bodies |
| NDV | Newcastle disease virus |
| OAS | 2'5'-oligoadenylate synthetase |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate Buffered Saline |
| PCR | polymerase chain reaction |
| PFV | Prototypic foamy virus |

| | |
|---|---|
| PI3K | Phosphoinositide-3 kinase |
| PKR | Protein kinase R |
| PML | Promyelocytic Leukaemia protein |
| Poly I:C | Polyriboinosinic:polyribocytidylic acid |
| PP2Ac | Protein phosphatase 2A |
| PR8 | Influenza virus strain A/Puerto Rico/8/34 |
| PRR | Pattern recognition receptors |
| R | RING domain |
| RAR | Retinoid acid receptor |
| RBCC | RING domain, B-box domain, coiled-coil domain |
| RIG-I | Retinoic acid inducible gene-I |
| RT-PCR | Real Time-PCR |
| SOCS | Suppressor of cytokine signalling |
| SARA | Smad anchor for receptor activation |
| SIV | Simian immunodeficiency virus |
| SIVmac | Macaque simian immunodeficiency virus |
| ssRNA | Single stranded RNA |
| STAT | Signal transducers and activators of transcription |
| TAB1/2 | TAK-binding protein 1/2 |
| TAK1 | Transforming-growth-factor-β-activated kinase |
| TCR | T cell receptor |
| TGFβ | Transforming growth factor-β |
| Th | CD4+ T helper cell |
| TIF | Transcriptional intermediary factor |
| TIR | Toll/IL-1 receptor |
| TLR | Toll like receptors |
| TNFα | Tumour necrosis factor -α |
| TRAF6 | TNF receptor-associated factor 6 |
| TRAM | TRIF-related adaptor molecule |
| Treg | Regulatory T lymphocyte |
| TRIF | TIR-domain-containing adaptor protein inducing IFNβ |
| TRIM | Tripartite motif |
| Tyk2 | Tyrosine kinase-2 |

| | |
|---|---|
| VSV | Vesicular stomatitis virus |
| WT | Wild type |

# Chapter 1:

# Introduction

## 1.1. Perspective

During the evolution of multicellular organisms, defence against microbial infections has been a universal requirement for adaptation. In this process of co-evolution between host and invader organism, a complex relationship has evolved in which both organisms compete for survival. From the point of view of the host organism, many gene products and entire pathways involved in host defence appear to be of ancient origin and are found in organisms evolutionarily distant, from humans to insects, and even in plants. Many of the proteins involved in these pathways remain highly homologous, indicating the essential role that these proteins play in response to pathogens and suggesting similar functions during evolution.

An ancient family of proteins that has been suggested to be a component of the innate immune response is the family of Tripartite motif (TRIM) containing proteins which can be found in all metazoans, from nematodes and invertebrate organisms, such as flies and worms, to primates. The large number of TRIM genes in higher eukaryotes suggests a rapid evolution of this family by gene duplications. This family of proteins is characterized by the conserved molecular structure of the tripartite motif. The striking conserved pattern, combination, and order of the domains, strongly suggests that this minimal structure was selectively maintained to carry out a specialized basic function common to all tripartite motif proteins. While the basic domain structure has been maintained, the sequences in the C-terminal region have rapidly evolved to acquire novel specificity and assume new physiological functions. TRIM proteins have been suggested to be involved in a wide range of molecular functions, from transcriptional regulation to post-translational modifications that may result in different cellular functions from apoptosis to cell differentiation, development, oncogenesis, signalling and immune responses. Since

the discovery of TRIM5α as a restriction factor to HIV-1 infection, there has been increasing interest in studying TRIMs as a family of molecules with anti-viral function. Moreover, the fact that many TRIMs have also been shown to be involved in immune response mechanisms like signal transduction pathways leading to production of cytokines, suggests that TRIMs may be an important component of the immune response in general, not only against viruses. The search for a common function in all TRIM proteins has been the focus of intense research, but much of this has been limited to the use of cell lines and over-expression assays which can lead to inaccurate or misleading interpretations and conclusions. Also, most of the studies on TRIMs as anti-viral effectors have been focused on their possible molecular mechanism of direct interaction with viruses; however, recent evidence suggests that TRIMs may act indirectly against viruses by playing important roles in the signalling cascades that lead to production of cytokines known to play a role in anti-viral responses. For this aspect it is important to consider the physiological conditions under which these molecules may be expressed and therefore primary cells are needed for these studies. We have used a systematic approach to determine the expression of representative TRIMs in a broad number of primary immune cells that produce different cytokines upon stimulation in an attempt to understand firstly, how expression of TRIM molecules is regulated and secondly, to obtain potential leads as to their function. The search for common patterns of gene expression that may help to predict the function of a large number of proteins, the TRIM family, will be the focus of this thesis.

## 1.2. Structure of TRIM proteins: The Tripartite motif (TRIM)

The tripartite motif (TRIM) or RBCC protein family was originally described as a group of proteins that contain a RING finger (R), one or two B- boxes (B1, B2) and a coiled-coil domain (CC) (**Figure 1.1A**) [1-4]. The N-terminal domain of almost all TRIM proteins consists of the RING finger domain which is composed of 40-60 amino acids that bind two zinc atoms in a unique cross-braced metal ligation scheme. This domain has a consensus sequence of a Cys-$X_2$-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys/His-$X_2$-Cys-$X_{(4-48)}$-Cys-$X_2$-Cys where X can be any amino acid, although there are clear preferences for particular residues at certain positions (**Figure 1.1B**)[3, 5]. The RING finger domain is probably the most extensively studied, and there are mainly two different functions assigned to it. Some studies have shown this domain to be involved in protein-protein interactions [6, 7] while others have shown that some TRIMs mediate E3 ubiquitin ligase activity by interacting with and promoting E2-dependent ubiquitin conjugation [6, 8]. An increasing number of TRIMs are being found to mediate protein ubiquitination and therefore it has been suggested that TRIM proteins may represent a novel subclass of E3-ubiquitin ligases [8]. This is highlighted by the fact that recent findings [9] have shown protein ubiquitination to be important not only as a mechanism for proteasome mediated protein degradation, but also as part of non-proteolytic pathways including cytokine induced signalling or signal transduction pathways resulting in cytokine production [10](e.g. activation of IKK depends on ubiquitination of TRAF6 by a non-proteolytic mechanism [11]; activation of RIG-I depends on its degradation-independent ubiquitination by TRIM25 [12].

Following the RING domain is the B-box domain, which exists in two different forms; B1 and B2. These domains consist of different types of cysteine-

histidine zinc-binding motifs of about 40 amino acids and share a similar pattern of cysteine and histidine residues (shown in **Figure 1.1B**) [8, 13]. The B-boxes are found exclusively in TRIM proteins and are an important determinant of the family, but no specific function has yet been attributed to these domains. It has been suggested that since the overall architecture of the RBCC/TRIM motif is highly conserved, the three dimensional structure of the motif may be important for protein-protein interactions [14, 15]. The best evidence so far are the molecular structures of the B1 and B2 domains of TRIM18/MID1, which have been recently solved [16, 17]. The molecular structure of the B-box 1 was found to share some conserved structural features with the RING domain, suggesting that the B-box1 might also possess E3 ubiquitin ligase activity [17]. Moreover, the structure of the B-box2 adopts a RING-like structure and can also bind two zinc atoms with the same cross-braced pattern observed for the B-box1 and RING domains suggesting that B-box1 and B-box2 are indeed similar and may have evolved from a common ancestor with the RING domain [16]. Further evidence for the possible role of the B-box domain in protein-protein interactions is that the B-box1 domain of TRIM18/MID1 was found to interact specifically with the alpha 4, the catalytic subunit of the protein phosphatase 2A (PP2Ac). This complex is required for the targeting of PP2Ac for proteasome-mediated degradation [18]. In addition, mutational deletion studies of the B-box of one of the TRIM members, the TRIM27/RFP protein, have shown it to be important for facilitating the formation of homodimers through the coiled-coil region, and mutations of the B-box affect the sub-cellular compartmentalization of TRIM27 in various cell lines [19].

The third characteristic motif of the TRIM proteins is the coiled-coil (CC) domain, a region predicted by bioinformatics to be a typical hyper-helical structure.

This region is formed by multiple α-helices and invariably follows the B-box2 in the entire set of TRIM proteins [20-22]. This CC motif has been shown to be necessary and sufficient for homo-dimerization in a large number of TRIM proteins (TRIM1, 3, 5, 6, 8, 9, 10, 11, 18, 21, 23, 24, 25, 26, 27, 29, 31, 32) [19, 20, 23]. On the other hand, only a few of them can form heterologous complexes using this domain (TRIM1/TRIM18; 19/27; 23/29; 23/31)[20]. Furthermore, mutation and deletion experiments revealed that the CC domain is responsible for promoting homo-oligomerization and therefore formation of distinct sub-cellular structures that can identify cellular compartments [20].

### 1.3. The C-Terminal region of TRIMs

An interesting aspect of the TRIM proteins is that the common RBCC feature is followed by one or more specific C-terminal domains that can determine, at least in part, the function of the protein by recruiting unique partners. Some of these domains include NHL (defined by sequence homologies with Ncl-1, HT2A, Lin41 proteins), ARF/SAR (found in the ADP ribosylation factor and Secretion associated and Ras related proteins), WD40 (repeats to form β-propeller structures), Fibronectin III (FN3), B30.2, plant homeodomain (PHD), Filamin, Bromodomain (BROMO), meprin and TRAF homology domain (MATH), and the recently described COS domain [22]. **Figure 1.2** shows a schematic representation of the TRIM family members sub-grouped based on their C-terminal domain composition. The B30.2 domain is the most common since it is present in 40 of the 66 human TRIMs and it is believed to be involved in protein-protein interactions and/or RNA binding [22]. This domain is of special interest since it has been implicated in the virus restriction specificity of certain TRIMs [22, 24].

### 1.3.1. The B30.2 domain

The B30.2 domain was originally identified as a protein domain encoded by a single exon within some genes in the major histocompatibility complex (MHC) region of human chromosome 6p21.3 [25]. This domain is composed of about 200 amino acids and comprises a combination of a PRY motif followed by a SPRY motif (**Figure 1.3A**) [26]. The SPRY and B30.2 domains can be found not only in TRIM proteins but also in 10 additional protein families encoded in the human genome including the immunoglobulin super-family and negative regulators of the JAK/STAT pathway (SOCS-box SSB). These domains cover a wide range of functions, including regulation of cytokine signalling (SOCS), RNA metabolism, intracellular calcium release, immunity to retroviruses, as well as regulatory and developmental processes (**Figure 1.3B**) [26]. This evolutionary adaptation, from SPRY to B30.2 and subsequent expansion of the number of genes with this domain, mimics that of some immune receptors, after the emergence of the adaptive immune system [27], and has led some to suggest that the B30.2 domain may have been selected as a component of immune defence [26]. Experimental evidence is consistent with a broad role for the B30.2 domain in innate immune recognition of retroviruses since B30.2 mutagenesis studies, as well as sequence analysis of TRIM5α from related primates, suggested that the differences in anti-viral activities are defined by patches in the B30.2 domain [28]. This is also supported by the fact that a single amino acid substitution (R332P) in the B30.2 domain of the human TRIM5α can confer the ability to restrict HIV-1 (**Figure 1.3C**)[24], suggesting that small changes during evolution may have extreme effects on our susceptibility to cross-species infection. However, it is also important to consider that there is evidence suggesting that the B30.2 domain is important for other cellular functions

besides anti-viral functions. Examples of this are the mutations in the B30.2 domains of TRIM18/MID1 associated with Optiz syndrome [29], and of TRIM20/MEFV, associated with Familial Mediterranean fever [30](**Figure 1.3C**). Moreover, the fact that the B30.2 domain may be involved in protein-protein interactions that can lead to activation of signal transduction pathways, but on the other hand can also function as a docking site for pathogen products make B30.2 containing proteins very versatile in their functions.

### 1.3.2. TRIM transcript variants and isoforms

An important and challenging aspect which may have relevance to the function of TRIMs is the fact that many of the family members produce alternatively spliced transcripts that result in isoforms. The isoforms of a single TRIM protein, generated by alternative splicing, usually share the same RBCC motif but differ in their C terminus, potentially allowing them to recruit different sets of proteins. An example is the human TRIM19/PML protein which has numerous transcript variants leading to 7 different isoforms detected in cells [31]. The RBCC motifs of TRIM19 mediate nuclear body formation and thus are essential for its growth suppressing, apoptotic and anti-viral activities, but particular TRIM19/PML isoforms can mediate specific interactions with other proteins, potentially giving diverse functions [15]. Another example of the functional difference of TRIM isoforms is the case of the anti-viral activity of TRIM5α. This protein is the product of the TRIM5 gene which has a total of 5 isoforms. TRIM5α is the only isoform that contains the B30.2 domain required for viral restriction [24, 32]. It is also important to note that the TRIM5γ and TRIM5δ isoforms, which lack the B30.2 domain, can act as dominant negatives to TRIM5α by formation of non-functional dimers [33, 34]. To understand the potential

role of the TRIM proteins it is necessary to identify all the possible isoforms and the conditions under which each one of them is expressed.

### 1.4. TRIM Function

As mentioned above, TRIMs have been reported to be involved in multiple functions, from transcriptional regulation to post-translational modifications, signalling, cell differentiation, cancer, apoptosis, neuronal function and anti-viral functions [5, 8, 15, 22]. There is increasing evidence that many of these cellular functions may be related to their potential role as E3 ubiquitin ligases. However, in many cases their mechanism of action is still poorly defined. Many TRIMs have been proposed to have important functions but only due to correlations or associations with certain diseases. Nevertheless this information may be useful in pointing at possible functions of TRIMs that should be further explored. Some of these functions are outlined below.

#### 1.4.1. Ubiquitin ligases

Ubiquitination is a post-translational modification thought to be used mainly to control protein levels by targeting proteins to the proteasome for degradation. This process involves at least three classes of enzymes. The E1 ubiquitin-activating enzyme binds ubiquitin to form an intermediate that then transfers the activated ubiquitin to the cysteine of an E2 ubiquitin enzyme. An E3 ubiquitin enzyme interacts directly with the E2 and the substrate protein and transfers ubiquitin to a lysine residue of the target protein [35]. In the case of TRIM proteins, a model has been suggested (shown in **Figure 1.4A**) where the RING domain binds to the ubiquitin-conjugating enzyme (E2) while the B-box/coiled-coil region forms the

pocket to recruit the substrate (S) [8]. The ubiquitin is then transferred from the E2 to the substrate (**Figure 1.4A**) [8]. The modified target is then directed towards proteasome-mediated degradation or other non-proteolytic pathways. Interestingly, recent studies have shown that protein ubiquitination is important not only for protein degradation [9] but also for regulation of protein activity, sub-cellular localization, control of gene transcription [36] or signal transduction pathways (e.g. TRIM25)[12]. Taken together, these results suggest a mechanism by which TRIM family members may exert their wide variety of cellular functions (summarized in **Figure 1.4B**) [8].

### 1.4.2. Cellular localization and compartmentalization

Some TRIMs have been previously characterized in terms of their sub-cellular localization and their capacity to form or associate with specific compartments, such as nuclear bodies (TRIM19, TRIM24 and TRIM27) or microtubules (TRIM1, 9, 18, 36, 42, 46, and 67). In an extensive study of TRIM cellular localization, Reymond *et al.* [20] investigated the sub-cellular localization of a large number of TRIM proteins in living cells using green fluorescent protein (GFP) technology and found discrete cytoplasmic or nuclear structures. The TRIMs found in the cytoplasm were either associated with filaments or concentrated in the form of cytoplasmic bodies, occasionally located around the nucleus. Nuclear TRIM proteins (TRIM8, 19, 30 and 32) localized mostly to structures called nuclear bodies (NB) where TRIM19 is the main component. The members of the bromodomain-containing subfamily (TRIM24, 28, and 33) were found associated with specific chromatin regions, consistent with the proposed role of this domain in transcriptional regulation [37]. Although this study gives a good insight on possible localization patterns of TRIMs and therefore may pinpoint putative cellular functions, however, it

has to be carefully interpreted since the use of GFP-TRIM fusion proteins and over-expression assays can result in formation of protein aggregates or non-specific binding that could lead to misinterpretations. Based on the TRIM localization patterns observed, the authors concluded that many TRIM proteins define novel sub-cellular compartments and suggested that the TRIM motif may be responsible for the "compartmentalization" of other proteins thus revealing a novel cellular function [20]. Interestingly, a different study identified a sub-group of TRIMs that share an identical domain arrangement (RBCC-COS-FN3-B30.2 domains; TRIM1, 9, 18, 36, 42, 46, 67) and co-localize to the microtubules. Binding to the microtubules is mediated by the COS domain, suggesting basic functional similarities for TRIMs sharing this same domain organization [38]. Therefore cellular localization and protein homology may help to predict possible cellular functions.

### 1.4.3. Transcriptional activities

Many TRIMs have been found to be located in the nucleus and some overexpression and gene reporter studies have shown TRIMs to affect transcription of specific genes or interact with known transcription factors suggesting that TRIMs may affect transcription either directly or indirectly. It is important to note that TRIMs involved in transcription can also be classified as functionally important in signalling pathways since many of these TRIMs interact with signalling adaptor molecules or are targets of post-translational modifications important during signalling. Although not fully characterized, TRIM14/PUB, TRIM27/RFP, and TRIM45 have been suggested to play repressive roles in transcriptional regulation. Overexpression of TRIM45 in COS-7 cell lines has been shown to inhibit the transcriptional activities of ElK-1 and AP-1, suggesting that TRIM45 may act as a

transcriptional repressor in mitogen-activated protein kinase (MAPK) signalling pathways [39]. Using a luciferase-based assay it was shown that TRIM14/PUB inhibits the transcriptional activity of PU.1 [40], a member of the Ets family of transcription factors, and plays critical roles in the development of haematopoietic cells such as macrophages and B cells [41, 42]. In keeping with this, TRIM14 is predominantly expressed in haematopoietic tissues, specifically in cells where PU.1 is also expressed [40]. On the other hand, TRIM27/RFP's repressive role on transcription has been associated with cancer when the TRIM motif is fused with the tyrosine kinase domain of the RET protein by chromosomal translocation [43]. TRIM27 was suggested to repress transcription by forming a more potent transcriptional repressor complex with methyl-CpG binding domain proteins (MBD proteins) which are involved in histone deacetylase-dependent transcriptional repression [44]. The role of TRIM19/PML, and the bromodomain containing TRIMs (TRIM24 and TRIM28) in transcription has been more extensively characterized.

#### 1.4.3.1 TRIM19/PML as a regulator of transcription

Nuclear bodies (NB) are highly organized nuclear structures that are present in most mammalian cell nuclei and have been shown to be sites of transcriptional regulation. These structures lie near highly acetylated chromatin and many transcription factors and transcriptional regulators co-localize with TRIM19/PML in the NB [45]. One possible mechanism by which the NB and particularly TRIM19 regulates transcription is by participating in chromatin remodelling. One example is the case of the major histocompatibility complex (MHC) locus, where PML regulates transcription by interacting with special AT-rich sequence binding protein 1 (SATB1) to organize the MHC class I locus into distinct higher-order chromatin-loop

structures that can have positive or negative transcriptional activity [46]. However, TRIM19/PML has also been associated with transcriptional repression since PML-NB co-localizes with transcriptional co-repressors and heterochromatin-bound proteins such as HP1 (for heterochromatin protein-1) [45, 47]. Moreover TRIM19 can bind transcription factors and inhibit their transcriptional activity, for example, it has been suggested to act as a negative regulation of IFNγ signalling by binding to the transcription factor STAT1 [48].

#### 1.4.3.2 The bromodomain containing TRIMs: TRIM24, 28, 33, 66

TRIM24, 28 and 33 belong to a subfamily of TRIM proteins that contain a bromo-domain in the C-terminal region of the protein. The bromodomain can recognize acetyl-lysines on histones and can serve as a pivotal mechanism for regulating protein–protein interactions in numerous cellular processes including chromatin remodelling and transcriptional activation [49, 50]. Accordingly, these TRIMs associate with chromatin regions in the nucleus [20], and have been shown to play positive and negative roles in transcriptional processes. TRIM24 (also called TIF-1α), a nuclear protein kinase [51], can regulate transcriptional activity of some nuclear receptors including the retinoid receptors (RXRs and RARs), while bound to their ligands [52]. TRIM24 forms complexes with TRIM28 and certain Kruppel-associated box (KRAB) motif-containing zinc finger repressors to inhibit transcription by a mechanism involving histone deacetylation and chromatin remodelling [53]. Although TRIM33, a protein involved in erythroid differentiation, has also been shown to have some silencing activity of gene promoters, the mechanism seems to be independent of KRAB-motif containing repressors or the

chromatin remodelling protein HP1 [54]. TRIM66 (also called TIF1δ), the last member of this subfamily of bromodomain containing TRIMs, has also been shown to have a deacetylase-dependent transcriptional repression activity [55]. This TRIM can form homodimers and can bind the HP1 indicating that it may function in a similar way to TRIM24 and TRIM28. However, it is different to the other TIF1 members in that its expression is largely restricted to the testis [55]. Interestingly and of relevance to my study, there are known cases of these Bromodomain containing TRIMs involved in regulation of cytokine gene transcription [56, 57]. Although these bromodomain-containing TRIMs share common structural and functional features and all seem to have negative regulatory functions, to date only TRIM28/ KAP1 (also called transcriptional intermediary factor: TIF-1β) has been suggested to be a negative regulator of type-I IFN dependent transcription [56](discussed in more detail later). Additionally, TRIM28 can bind histone methyltransferases [58] which methylate histones to inhibit transcription, supporting its role as inhibitor of transcription. Interestingly, this negative role of TRIM28 on gene transcription has important implications in silencing of retroviral transcription. Replication of murine leukaemia virus (MLV) is restricted in embryonic carcinoma and embryonic stem cells where TRIM28 forms a complex with histone methyltransferases, histone deacetylase and HP1 family members to methylate histone H1 and promote chromatin condensation [59, 60]. This report highlighted the importance of TRIMs as potential viral restriction factors using transcriptional mechanisms to inhibit viral replication.

### 1.4.4. TRIMs associated with disease; Cancer and autoimmunity

Since the function of TRIMs is so diverse and in many cases non-redundant, it is not surprising to find that mutations in particular TRIM genes have been associated with a variety of diseases. One may hypothesize that TRIMs with important functions in cell cycle, apoptosis or cell differentiation may be involved in cancer and tumour suppressor activities, while TRIMs found to be important in signal transduction pathways or transcriptional regulation of genes related to the immune response may be linked to autoimmunity or susceptibility to viral infections. The importance of TRIM function in specific biochemical processes is highlighted by the fact that mutations, lack of expression, or inactivation of TRIM function has been associated with a variety of tumours and may lead to cancer. However, although many TRIMs have been associated with tumour formation, only a few of them have been extensively characterized. The following are some examples of TRIMs that have been shown or suggested to have some involvement in cancer.

#### 1.4.4.1. TRIMs in disease: TRIM19//PML and tumour suppression

The best example and probably most extensively studied TRIM implicated in cancer is the promyelocytic leukaemia protein (PML or TRIM19) which was originally discovered in patients suffering from the haematopoietic malignancy, acute promyelocytic leukaemia (APL). This disease is associated with a reciprocal chromosomal translocation of human chromosomes 15 and 17 resulting in PML-retinoid acid receptor α (RARα) fusion protein [61-63]. APL is characterized by a block in differentiation of promyelocytes. Unlike in normal cells where PML localizes to the nuclear bodies (NB), PML is dispersed in the nucleus and the

cytoplasm in APL cells [64, 65]. Studies using PML deficient mice have shown an impaired capacity for terminal maturation of their myeloid cells [66]. Some experiments on the role of PML in cell growth control have been carried out using cell lines derived from PML deficient mice and have revealed tumour suppressor and pro-apoptotic functions for PML [67]. Moreover, it has also been shown that PML is involved in p53-dependent apoptosis [68] and in the regulation of gene expression [45, 69]. However, PML deficient mice do not develop spontaneous tumours, indicating that inactivation of PML is not the only requirement for development of APL in mice. Like other TRIMs, TRIM19/PML is involved in other cellular functions in addition to the ones leading to APL. PML is an essential component of the nuclear bodies (NB) and PML-NBs have been implicated in the induction of cellular senescence, inhibition of proliferation, maintenance of genomic stability and antiviral responses [31]. Since the number of studies on PML in relation to immune regulation are very limited and mostly using cell lines or over-expression analysis, the dominant function of TRIM19/PML remains unclear.

#### 1.4.4.2. TRIMs in disease: TRIM25/EFP and breast cancer

TRIM25/EFP was first described as a protein regulated by estrogen and suggested to be an estrogen-responsive transcriptional regulator [70]. Later it was implicated in promoting breast tumours because TRIM25/EFP is responsible for the ubiquitin-dependent proteolytic inactivation of 14-3-3sigma, a negative regulator of the cell cycle [71]. Reduction of 14-3-3sigma is common in breast cancer and its expression is induced by p53 after DNA damage [72]. TRIM25 is essential for estrogen-dependent cell proliferation and organ development since TRIM25/EFP deficient mice display underdeveloped uteri and reduced estrogen responsiveness

[71]. However, TRIM25 can promote a switch from estrogen-dependent to estrogen-independent proliferation of breast cancer cells suggesting that expression and activity of TRIM25 must be tightly regulated. Interestingly, as mentioned in previous sections, TRIM25 has also an important role in the innate immune system by participating in the signal transduction pathway to induce IFNβ [12](discussed more below). This indicates the importance of having mechanisms of tight regulation of TRIM gene expression to maintain a balance between required effector TRIM protein functions and disease.

### 1.4.4.3. TRIMs in disease: TRIM24 and cancer

Another well-characterized TRIM associated with cancer is TRIM24 (or Tif1α) which is also known to act as a transcriptional regulator and has been shown to interact with many proteins involved in chromatin structure [51, 53, 73, 74]. TRIM24, a ligand-dependent nuclear receptor, functions in mice as a liver-specific tumour suppressor. In TRIM24 deficient mice, hepatocytes fail to execute proper cell cycle withdrawal during the neonatal-to-adult transition and continue to cycle in adult livers, resulting in cellular alterations that progress toward metastatic hepato-cellular carcinoma (HCC) [75]. Interestingly, TRIM24 has been shown to share similar activities with TRIM19/PML and can also form fusion genes implicated in cancer. The TRIM24 gene fuses to a truncated B-Raf gene in murine hepatocellular carcinoma [76], and with a truncated RET tyrosine kinase proto-oncogene in thyroid papillary carcinoma [77]. TRIM24 also maps to a region in human chromosome 7q32-34 which is a region frequently lost in myeloid disorders [78-80].

#### 1.4.4.4. TRIMs in disease: other TRIMs associated with cancer

Other TRIMs reported to be associated with cancer are TRIM8, 13, 27, 29, 32, 35, 36. However, many of these TRIMs have been poorly characterized and some studies have only shown some correlation of expression with tumour formation. TRIM29 has been proposed to be a marker of lymph node metastasis in gastric cancer since some patients have shown TRIM29 expression in their lymph nodes [81]. Some other TRIMs map to chromosomal regions that are affected in tumours and therefore suggested to be tumour suppressor genes. For example, TRIM13 maps to the chromosomal region 13q14.3 where homozygous deletions are found in chronic lymphocytic leukaemia (CLL) and multiple myeloma (MM) [82]. TRIM35 inhibits cell growth, clonogenicity, and tumourigenicity when its expression is enforced in HeLa cells. TRIM35 is located on chromosome 8p21, a region also implicated in numerous leukaemias and solid tumours [83]. Another example is TRIM36, which is located on chromosome 5q22.3, at a region with frequent DNA alterations in different types of tumours including urological cancers. Although no mutations were found in TRIM36 of patients with prostate cancer, its expression was increased in prostate tissues of these patients suggesting that this gene might be associated with prostate tumourigenesis [84].

There are further studies associating other TRIMs with cancer. For example, TRIM32 mRNA has been reported to be highly expressed in human head and neck squamous cell carcinoma [85]. The mechanism of TRIM32 as an oncogene has been suggested to be via an interaction with Abl-interactor 2 (Abi2), which is a known tumour suppressor and an inhibitor of cell migration. It has also been shown that overexpression of TRIM32 promotes degradation of Abi2, resulting in enhancement of cell growth, transforming activity, and cell motility, whereas a dominant-negative

mutant of TRIM32, lacking the RING domain, inhibited the degradation of Abi2, supporting the role of TRIM32 as an oncogene [86]. Another example is the TRIM8/GERP human gene which maps to chromosome 10q24.3, a region showing frequent deletions in glioblastomas [87]. In support of a role of TRIM8 in cancer, a transcriptional profiling study of patients with larynx squamous cell carcinoma (LSCC), which is the most frequent neoplasm of the head and neck region, found that TRIM8 expression negatively correlated with nodal metastatic progression and was absent from adenocarcinoma, large cell carcinoma, chondrosarcoma, epithelioid carcinoma, and glioblastoma [88]. Moreover, overexpression of TRIM8 *in vitro,* showed a reduction in cell colony formation units suggesting growth suppressor functions [88]. TRIM27/RFP, also associated with cancer, was originally identified as the N-terminal fusion partner with the *RET* tyrosine kinase proto-oncogene [89].TRIM27 is differentially expressed in testicular germ cell tumours [90] and is expressed in a wide range of other tumour types [91].

#### 1.4.4.5. TRIMs in disease: TRIMs and autoimmunity

TRIM21 (also called Ro52), has been described as one of the main autoantigens in Sjögren syndrome (SS), a systemic inflammatory disease caused by the presence of autoantibodies that occur also in systemic lupus erythematosus (SLE) and rheumatoid arthritis [92]. In addition to being targeted by autoantibodies, TRIM21 forms trimers to bind IgG through its B30.2 domain [93]. Moreover, the crystal structure of TRIM21 bound to the Fc region of IgG revealed hot-spot residues in the B30.2 domain that are similar to the ones involved in the control of HIV or MLV restriction by TRIM5α, as well as mediating severe familial Mediterranean

fever by TRIM20 [94]. Autoantibodies against TRIM21 are associated with congenital heart block in the fetuses of mothers with Sjögren syndrome during pregnancy, and patient-derived monoclonal antibodies against TRIM21 have been shown to induce accumulating intracellular calcium levels in neonatal cardiomyocytes [95]. The syndrome is histologically characterized by lymphocytic infiltration into an inflammatory lesion and is also likely to be associated with malignant transformation such as lymphomas [96]. Although the causes of these diseases remain unclear, it is worth noting that a link between these pathologies and deregulation of cytokine production may also explain the pro-inflammatory effects seen, since TRIM21 has been shown to positively regulate IL-12p40 and to negatively regulate IFNβ production (discussed in more detail below) [97, 98]. Recently, TRIM59 (also called SS-56) was also described as a potential autoantigen in SS and SLE. Moreover, there was an increased correlation with the presence of antibodies against TRIM59 and the visceral complications in SLE [99].

Mutations on the B30.2 domain of TRIM20 (also known as pyrin) have been associated with a recessive inherited systemic autoinflammatory disease called Mediterranean Fever (MEFV), which is characterized by recurrent attacks of fever and synovial, or cutaneous inflammation [30]. TRIM20 has been suggested to play a role in the regulation of the systemic inflammatory response, since TRIM20 can modulate caspase-1 and IL-1β activation. Two opposing hypothesis have been suggested for the role of TRIM20 since both an inhibitory and activating role have been reported. Using mice with a targeted mutation of the pyrin gene it was proposed that TRIM20 sequesters the apoptosis-associated speck-like protein (ASC), involved in activation of caspase-1 in the inflammasome. This would result in a reduction of caspase-1 activation and a reduction in IL-1β processing [100]. Conversely, using a

different system by co-expressing TRIM20 and ASC, it was shown that TRIM20 can increase IL-1β processing. To explain this observation it was suggested that a pathogen product or a pathogen-associated molecular pattern (PAMP) might bind the C-terminal B30.2 domain of TRIM20 and might lead to caspase-1 activation [101, 102]. It is important to keep in mind that the mouse TRIM20 does not possess a B30.2 domain [103], indicating that there may be differences in the mechanism of IL-1β regulation between human and mouse.

#### 1.4.4.6. TRIMs in disease: TRIMs and genetic disorders

Mutations in TRIM18 (or MID1) have been associated with a genetic disease named Opitz G/BBB syndrome. This disease is characterized by abnormalities of the upper airways, cleft lip and palate, mental retardation, and gastrointestinal malformations [104], and additional features involving defects in development of the ventral midline. Many of the TRIM18 mutations associated with this disease are located in the B30.2 domain, attributing an essential role of this domain in TRIM18 function [105, 106]. The proposed mechanism by which TRIM18 mutations affect the differentiation of the midline involves the re-localization of TRIM18 from its normal location with the microtubules, to the cytoplasm of the cell. TRIM18 has been proposed to be required for ubiquitin-dependent degradation of the PP2A protein phosphatase, which is essential for microtubule dynamics [106]. Although poorly characterized, another TRIM described to be involved in a genetic disorder is TRIM37 (also called MUL). Mutations in the C-terminal region of TRIM37 cause a frameshift in the DNA sequence, which would be predicted to produce a truncated protein [107]. These mutations have been associated with muscle-liver-brain-eye

nanism (Mulibrey nanism), a genetic recessive disorder that is characterized by growth failure of prenatal onset, cardiomyopathy, and hepatomegaly [108, 109].

### 1.5. The innate and adaptive immune response. An overview

Defence against pathogens is initiated by antigen presenting cells (APC), including macrophages and dendritic cells (DC) which recognize molecular structures or patterns that are unique to microorganisms [110]. This recognition takes place within minutes when innate immune cells expressing pattern recognition receptors (PRR), which have a broad specificity, bind molecules that have a common structural motif or pattern. Viruses, bacteria, fungi, protozoa and parasites have a vast number of molecular signatures, from proteins and nucleic acids to components of the cell wall, such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids and cell-wall lipoproteins or fungal β-glucan. The compounds that can activate PRRs including toll like receptors (TLRs) are usually referred as pathogen-associated molecular patterns (PAMPs), and result in activation of microbicidal effector pathways, inflammation and initiation of the adaptive immune response [110]. Macrophages play a role in the early phases of the immune response by producing cytokines and killing pathogens via various mechanisms including reactive oxygen and nitrogen intermediates, as well as engulfing apoptotic cells [111]. DC continuously take up, process, and present antigens in the form of peptides in the context of the major histocompatibility complex (MHC) which are recognized by the T cell receptor (TCR) on T lymphocytes, and are fundamental APCs initiating adaptive immune responses. APCs such as DC can present peptide bound to MHC Class I to CD8+ T cells to activate proliferation and effector functions such as cytolytic activity, or MHC Class II to CD4+ T helper cells to induce proliferation or

differentiation of effector cells producing different patterns of cytokines [112-115]. A schematic representation of innate and adaptive immune cells, and the cytokines they produce is shown in **Figure 1.5.** Once activated by microbes and/or their products, DCs can activate T cells, including CD4+ T cells of the adaptive immune response, and produce factors to induce their differentiation into CD4+ T helper (Th) effector cells. These include T helper type 1 (Th1) cells producing the hallmark cytokine IFNγ, which is essential for eradication of intracellular pathogens but can also be involved in autoimmune inflammation [116, 117], or Th2 cells producing the key cytokines IL-4, IL-5 and IL-13, important for anti-helminth responses but also implicated in allergic inflammation [118, 119]. Although not covered by our study, it should not go unmentioned that a third independent effector population has been recently described, the Th17 subset, which produce the cytokine IL-17 important in the host defence against extracellular bacteria such as *Klebsiella pneumoniae* [120-122] but also involve in autoimmune inflammation [123].

During an immune response, these effector responses can be regulated by naturally occurring Foxp3+ CD25+ T regulatory cells (CD25+ Treg) that do not produce pro-inflammatory cytokines upon *in vitro* stimulation. The effector responses can also be regulated by the immuno-suppressive cytokine IL-10, which can be produced by many cells of the immune system including CD8+ T cells, CD4+ Th1, Th2, antigen-driven Treg (IL-10 Treg), as well as Foxp3+ CD25Treg under the right conditions [124](**Figure 1.5**). Intense research on the molecular mechanisms of regulation of the innate and adaptive immune systems has led to the finding of a limited number of TRIM molecules as important players in these signalling pathways. As an increasing number of TRIM molecules are discovered to have immune functions, it remains to be seen if this family of proteins indeed has been

selected through evolution to play a role in the immune system and more research is required to test their *in vivo* relevance.

### 1.5.1. The innate immune response

#### 1.5.1.1. Innate recognition of pathogens by TLRs and other PRRs

All animal cells have developed different mechanisms of defence against pathogens that prevent infection and spread. As part of this mechanism the innate immune response acts as soon as there is recognition of PAMPs of the foreign particle by the PRRs on the innate immune cells. This allows the host to activate pathways that lead to production of protective bioactive molecules. For this to happen the host organism has developed a complex system that senses different invaders to respond accordingly and specifically, to establish a limited but effective response against a particular pathogen. PRRs include members of the TLR family which can recognize patterns found in viruses, bacteria, fungi and protozoa [125]. The well-conserved features in pathogens, summarized in **Figure 1.6**, include bacterial cell-surface lipopolysaccharides (LPS) (ligand for TLR4); lipopeptides (ligand for TLR1, TLR2 and TLR6); proteins such as flagellin from bacterial flagella (ligand for TLR5); double-stranded RNA of viruses (ligand for TLR3) or the unmethylated CpG DNA of some bacteria and viruses (ligand for TLR9); and single stranded RNA (ssRNA ligand for TLR7,8) from some viruses. Interestingly, most of these TLRs, TLR3, 7, 8, 9, are localized in endosomes, since many viruses are internalized to the cell via this compartment, and this has been suggested as a mechanism for distinguishing self from non-self [126].

However, there are also viruses that can replicate in the cytoplasm and therefore cytoplasmic PRRs that are not members of the TLR family also exist,

including the melanoma differentiation factor-5 (MDA5) which recognizes dsRNA, and the retinoic acid inducible gene (RIG-I) which recognizes ssRNA or 5'phosphorylated RNA from influenza virus [127]. This indicates that compartmentalization is also an important determinant of PRR recognition and requires the co-localization of the receptor with the viral product at the precise site of replication. Importantly, activation of any of these pathways by pathogens results in the production of effector molecules that are essential to combat pathogens. Differential expression of TLR or non-TLR molecules in different populations of DC, macrophages and B cells dictate the intensity and specificity of these responses and therefore it is essential to study purified primary cell populations to understand the role of these PRRs during infection.

#### 1.5.1.2. Differential TLR expression by distinct DC populations

DC comprise several cell subsets with diverse functions and have been shown to have different capacities to direct Th1 differentiation [128]. Based on their cell surface markers and origin (e.g. spleen or bone marrow), mouse DC expressing the CD11c marker can be classified into different subsets which include splenic-$CD11c^+/CD11b^+/CD8\alpha^-$(myeloid DC), splenic-$CD11c^+/CD11b^-/CD8\alpha^+$DC, or splenic plasmacytoid DC (pDC). These pDCs express dull levels of CD11c and are $CD11b^-/B220^+/Ly\text{-}6C^+/CD62L^+$ [129-131], in addition are recognized by the antibody 120G8, which binds the antigen bone marrow stromal cell Ag 2 (BST2)[132, 133]. Bone marrow (BM) precursors can be differentiated using granulocyte-macrophage colony-stimulating factor (GM-CSF) to obtain myeloid DC (mDC) expressing CD11c, and CD11b markers. Also, Fms-like tyrosine kinase-3 ligand (FLT3-L) can stimulate development from BM of a population that is enriched

in $B220^{+}/120G8^{+}$ pDC precursor and CD11c+/CD11b+ myeloid populations which can be separated and purified by flow cytometry. These distinct DC sub-populations in mouse and human have been shown to express different levels of TLR mRNA and protein, and consequently to respond to distinct microbial products (summarized in **Figure 1.6**)[128, 134-137]. It has been demonstrated that due to high expression of TLR4 on mouse splenic DC, mDC and macrophages these cells have the ability to respond to LPS [128, 136]. Conversely, splenic and BM derived pDC, which have an important role in regulating anti-viral responses, do not respond to LPS due to an absence of TLR4 expression but are activated by CpG due to their high expression of TLR9. This differential expression affects secretion of pro-inflammatory cytokines, and therefore their capacity to induce Th effector cell development. Mouse splenic and BM derived pDC also express TLR7 and respond to ssRNA (from viruses like Influenza) to produce large amounts of IFNα/β [128, 138]. Mouse pDC are also activated by R-848, an imidazoquinoline resiquimod synthetic agonist of TLR7/8, to produce many type-I IFNs including IFNα, IFNβ, and also produce IL-12 [128, 138]. Importantly, mouse TLR9 expression is not restricted to pDC but can be found in other DC subsets as well as macrophages and B lymphocytes, whereas human TLR9 is only expressed on pDC and memory B cells [134, 138](see **Figure 1.6**).

Mouse BM derived mDC express TLR2 and respond to its ligand Pam3Cys (a synthetic lipopeptide that mimics the acetylated amino-terminus of LPS), whereas mouse pDC express no TLR2 and do not respond to this ligand. $CD8\alpha^{+}$ DC also express lower levels of TLR5 message than other spleen DC subsets but, in contrast, display the highest expression of TLR3 message, which is absent from pDC [138, 139]. TLR3 is activated by dsRNA from viruses to produce large amounts of IFNβ highlighting the importance of this cytokine to control viral infections. This is

interesting since TLR3 is widely expressed on non-pDC, and is also present at high levels on macrophages and on some non-haematopoietic cells such as epithelial cells [138, 139]. Interestingly, although the various TLRs share significant portions of their signalling cascades, each have also unique pathways and thus each ligand can induce a different cytokine response, especially given the differential expression of the receptors just described (see **Figure 1.6 and 1.7**). The pattern of cytokine production induced by TLR ligation is also partly determined by intrinsic characteristics of the specific DC population. For example, TLR9 ligation of mouse pDC leads to production of IL-12p70 as well as IFNα/β, whereas BM derived mDC produce only IL-12p70 and IFNβ but not IFNα [128]. Therefore intrinsic differences between DC populations may explain the fact that some pathogen-derived products induce different patterns of cytokine production in different DC subsets. As a consequence of this differential expression of TLRs and production of unique sets of cytokines, we postulated that this may also result in differential up-regulation of TRIM proteins in different DC populations depending on exposure to specific TLR ligands.

#### 1.5.1.3. Innate recognition of viruses

The innate response to viruses includes constitutively expressed molecules of intrinsic immunity such as the Friend-virus susceptibility gene (FV1) or TRIM5α which target incoming retroviruses; Mx proteins, which target the nucleoproteins of bunya- and orthomyxoviruses; or APOBEC-3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex), which deaminates retroviral genomes to cause viral mutants incapable of replication [140, 141]. However, these different intrinsic anti-viral proteins are not always expressed in the cells that are the

main target of a specific viral infection, in which case the virus is free to replicate. Moreover, some anti-viral mechanisms include the induction of proteins that inhibit protein translation or cause degradation of RNA which can lead to apoptosis or cell death thereby restricting the amounts of virus produced [142], however this is a mechanism undesirable in non infected cells. For this reason, cells possess other means to induce rapid expression of anti-viral molecules that may not be expressed constitutively. Studies have shown that some intrinsic, constitutive anti-viral molecules can be further induced to even higher levels after exposure to viruses (e.g. TRIM5α and Mx are highly induced by type-I IFNs). Important components of this inducible mechanism to sense viruses are the endosomal members of the TLR family, TLR3,7,8,9; as are some cytoplasmic non-TLRs including MDA5 and RIG-I. Activation of any of the TLR or non-TLR receptors, either by bacterial or viral components, results in the production of the pro-inflammatory cytokines such as IL-12, IL-6, and TNF. Most importantly, many of these pathways also induce the central anti-viral cytokines, type-I Interferons (type-I IFNs), which will be discussed next.

#### 1.5.1.4. The type-I Interferon (IFN) system

One of the most important and extensively studied groups of innate anti-viral molecules are the type-I IFNs which are a family of secreted cytokines that can elicit anti-viral effects [143]. Type-I IFNs are part of the IFN family of cytokines which also include type-II and type-III IFNs. Each of these groups is distinct and signal through different receptors. Interestingly however, their receptors trigger common downstream signalling pathways, and can regulate many of the same genes [144]. Type-I IFNs comprise a large group of genes that varies between different species and include the well defined IFN-α, and -β and other less defined IFNs such as

IFN–ω,–ε,–τ,–δ, and –κ, which all bind the same receptor [143]. In contrast to type-II IFN (IFNγ), which is predominantly made by NK cells and T cells during adaptive immune responses, IFNβ can be produced by most nucleated cells and is induced during viral infections. IFNα is produced predominantly by pDC in response to viral infections, although it can also be produced by other cells depending on the stimulatory conditions [145]. Moreover, most cells can respond to type-I IFNs through the type-I IFN receptor (IFNAR1), which can bind all type-I IFN subtypes [144]. The type-I IFNs, and in particular IFNα/β, can activate signal transduction pathways through the IFNAR1 that can establish an "anti-viral state" in target cells [146]. This includes the induction of many effector molecules that can directly inhibit viral replication, although type-I IFNs can also induce other effects including cell cycle arrest, apoptosis and have many immuno-modulatory functions [147]. Viruses are the main inducers of type-I IFNs but it has been shown that bacterial products like LPS can also lead to IFNβ production, demonstrating that type-I IFNs are important in different effector functions [148, 149].

#### 1.5.1.5. Receptors and signalling to produce type-I IFN

Upon TLR ligation by pathogen products, signalling cascades are initiated via Toll/IL-1 receptor (TIR) domain containing adaptors that recruit signalling molecules resulting in cytokine production (**Figure 1.7**). After ligand binding a conformational change in the receptor molecules brings together the TIR domains on the TLRs where the adaptor proteins can bind [150]. There are four adaptors known to regulate the recruitment of signalling molecules in the TLR pathway: myeloid differentiation primary-response gene 88 (MyD88), MyD88 adaptor-like protein (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFNβ (TRIF also

known as TICAM1), and TRIF-related adaptor molecule (TRAM also known as TICAM2) [150] (see **Figure 1.6 and 1.7**). All TLRs, except for TLR3, use MyD88, whereas TRIF is recruited only by TLR3 and TLR4, resulting after binding of their specific ligands, dsRNA (or poly I:C) and LPS, respectively [151]. The TRIF adaptor has been shown to be responsible for a MyD88-independent signalling pathway giving rise to IFNβ [152-154].

Usage of adaptor molecules by the various TLRs was elucidated by using DC and macrophages derived from mice lacking the adaptor molecules MyD88 or TRIF and testing their responses to various TLR ligands [151]. The activation of both MyD88 and TRIF upon TLR4 stimulation by LPS became clear when it was reported that murine MyD88−/− DC treated with LPS failed to produce IL-12, IL-6 or TNF, but were still able to up-regulate activation markers [155-157]. This MyD88-independent pathway of TLR4 signalling via TRIF was subsequently shown in both mDC and macrophages to lead to the activation of the IFN regulatory factor-3 (IRF-3), a critical transcription factor for induction of IFNβ [158, 159](**Figure 1.7A**). In the case of the viral products CpG and ssRNA, TLR7 and TLR9 use only MyD88. Although clear differences have been shown in the signalling pathways in macrophages, mDC and pDC, the general observation is that the MyD88-dependent pathway in macrophages and mDC triggers the release of pro-inflammatory cytokines, such as TNFα, IL-6, and IL-12p40, but also the immunosuppressive IL-10 (found as part of my research and published in [160], discussed more in results). MyD88 recruits IRAK4 (IL-1R-associated kinase 4), IRAK1, TRAF6 (TNFR-associated factor 6) and TAK1 (transforming-growth-factor-β-activated kinase), which leads to recruitment of TAB1/2 (TAK-binding protein 1/2), and activation of MAP kinases and the transcription factor NF-κB to induce pro-inflammatory

cytokine genes [147](**Figure 1.7A**). Production of IFNβ in macrophages and mDC on the other hand, can be induced by MyD88 upon treatment with CpG via IRAK1 and activation of TRAF3 instead of TRAF6 [161] (**Figure 1.7A**). In myeloid DC, it has also been shown that MyD88 can also couple to IRF5 (interferon-regulatory factor 5) to induce pro-inflammatory cytokines [162]. In addition, another study showed that IRF-5 may also be involved in the induction of IFNβ by macrophages upon viral infection. This may partially explain the observed reduction of type-I IFN levels in viral infected IRF-5 deficient mice [163]. However, it was suggested that IRF-5 is not involved in IFNβ production by fibroblasts, indicating that this effect may be cell type specific [163]. Other mechanisms for the induction of IFNβ have also been described. In mDC and macrophages, TLR9 activation can induce IRF-1 binding to MyD88 that does not require IRF-3 and/or IRF-7. IRF-1 together with MyD88 and IRAK-1 activates the IFNβ promoter [164], indicating that different IRFs can be utilized for the induction of type-I IFNs.

In pDC, signalling to induce type-I IFNs is somewhat different to other cell types (**Figure 1.7B**). pDC are extremely important during some viral infections since they can produce up to half of the circulating type-I IFNs [165]. Moreover, pDC are unique in the sense that they can readily produce large amounts of IFNα additional to IFNβ. This difference relies on the fact that pDC are unique in their high constitutive expression levels of IRF-7 which is the alternative option to IRF-3 to produce type-I IFNs [166](see **Figure 1.7 A and B**). This activation of IRF-7 occurs via a phosphatidylinositol-3 kinase (PI3K) dependent pathway upon TLR stimulation [167, 168]. As in mDC, TLR7 or TLR9 ligation leads to recruitment of Myd88 that forms a complex containing IRAK-4, IRAK-1 and TRAF6 and TRAF3 in FLT3L grown mixed pDC and mDC [161]. As in mDC, TRAF6 can activate NF-κB through

TAK1-TAB2-TAB3 and the IKK complex. However the complex containing Myd88/IRAK-1/IRAK-4/TRAF6 binds directly to IRF-7 in pDCs [166, 169, 170](**Figure 1.7B**). IRF-7 is phosphorylated by IRAK-1 and translocates to the nucleus where it can induce transcription [147]. There is also evidence that IRF-5 bound to MyD88 is also important in the production of type-I IFNs by pDC in response to TLR7 and TLR9 ligation [162, 171, 172].

The biological significance of the type-I IFN system to defend the host against viral infection is emphasized by the fact that viral products can activate different pathways to induce type-I IFNs. Moreover these signalling pathways rely on the proper localization of the viral product in the host cell to be recognized by the receptor. For example, dsRNA from some viruses can induce type-I IFN production by an endosomal pathway via TLR3 and the TRIF adaptor molecule or by a TLR independent, cytoplasmic pathway (via RIG-I/MDA5/MAVS) [147, 173, 174] (**Figure 1.7**). dsRNA (or its synthetic analog polyinosinic-polycytidylic acid [poly I:C]) which can be delivered to endosomes either by endocytosis of externally presented dsRNA or from uncoating of endocytosed viral particles, binds TLR3 which only uses the TRIF adaptor molecule to induce IFNβ and other cytokines [153, 154, 175]. The TRIF adaptor molecule can recruit TRAF3 and TRAF6 which as described for MyD88, can lead to activation of IRF-3 and NF-κB respectively for subsequent induction of their respective target genes [147, 161, 176, 177]. An alternative pathway to induce IFNβ occurs when viral dsRNA localized in the cytoplasm by uncoating, transcription or replication can be recognized by the RNA helicases MDA-5 and RIG-I [174]. Interestingly, RIG-I can also recognize ssRNA molecules containing 5'triphosphates that are present during influenza virus

replication [178]. MDA-5 and RIG-I can both activate MAVS (mitochondrion antiviral signalling protein), a mitochondrion-associated adaptor [179, 180]. MAVS can in turn recruit the same components that lead to activation of TRAF3-IRF-3 or TRAF6-NF-κB pathways [179, 180] which is similar to the one described for the TRIF pathway. Importantly, during the course of this study some members of the TRIM family were shown or suggested to be involved in some of these pathways to induce type-I IFNs. However, whether signalling through TLRs and their adaptor molecules responding to bacterial and viral products results in a direct or indirect broad up-regulation of TRIM expression is unknown and is the subject of this thesis.

#### 1.5.1.6 Signalling in response to type-I IFNs and induction of anti-viral molecules.

All the type-I IFN family of proteins including IFNα (which can be further subdivided into 13 different subtypes, IFN-α1, -α2, -α4, -α5, -α6, -α7, -α8, -α10, -α13, -α14, -α16, -α17 and -α21), IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ and IFN-ω bind a common heterodimeric cell-surface receptor, which is known as the type I IFN receptor (IFNAR1) [143](**Figure 1.7C**). The Type-I IFN receptor is composed of two subunits, IFNAR1 and IFNAR2, which are constitutively associated with the tyrosine kinase Tyk2 and the Janus activated kinase-1 (JAK1) respectively. Upon binding of type-I IFNs to their receptor, a conformational change occurs resulting in rapid phosphorylation of the cytoplasmic tail of the IFNAR1 by Tyk2 which creates a docking site for STAT2. In the conventional signalling pathway normally assumed for type-I IFN signalling, activation of JAK1 and Tyk2 results in phosphorylation of STAT2 (signal transducer and activator of transcription 2) and STAT1 to form a STAT1–STAT2 heterodimer which is translocated to the nucleus where IRF-9 is

attracted to form a complex known as ISGF3 (IFN-stimulated gene (ISG) factor 3)[147, 181]. These complexes translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA to initiate gene transcription. Importantly, this ISGF3 complex is the only complex that can specifically bind the ISREs in the promoters of certain IFN stimulated genes (ISGs) to initiate their transcription. In a recent study it was suggested that this signalling pathway requires acetylation of the ISGF3 complex by the CREB-binding protein (CBP), which is usually a nuclear protein, while it binds to the receptor [182]. In this study the authors showed that CBP acetylates IFNAR2 which in turn creates a docking site for IRF9 leading to the formation of the ISGF3 complex containing IRF9-STAT1-STAT2. It was suggested that acetylation of both IRF9 and STAT2 by CBP is critical for the activation of the ISGF3 complex and association to target genes [182]. It is also important to note that there are other member of the STAT family that can be phosphorylated and activated by Jak1 and Tyk2, in addition to STAT1 and STAT2, including STAT3 and STAT5 [181].

Importantly and of interest for this thesis is the fact that Type-II IFN (composed of IFNγ only), although signalling through a different receptor (IFNGR1 and IFNGR2) shares some features with the type-I IFN signalling, leading in some cases, to induction of common genes. However, important differences are also present which can account for the expression of specific type-I IFN inducible genes. One important difference in the type-II IFN signalling is the formation of STAT1-STAT1 homodimers. The activated STAT1 homodimer translocates to the nucleus and binds gamma-activation sequences (GAS), a distinct sequence from the ISRE. Also distinct to the type-I IFN signalling is the fact that the STAT1-STAT1 homodimer does not require IRF-9 for DNA binding [147]. In contrast to type I IFNs,

IFNγ does not induce the formation of ISGF3 complexes and thereby cannot induce the transcription of genes that have only ISREs probably accounting for some of the different genes that are inducible by type-I IFNs but not IFNγ [181]. Moreover, the activation of different STATs and formation of different combinations of STAT dimers in response to IFNγ or type-I IFNs may also account for some specific genes induced by each stimulus. In addition to the classical JAK–STAT pathway, IFNα/β can also activate the p38 mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) signalling cascades that are required for the generation of cellular responses to IFNs [167, 168, 181]. Moreover, some studies have also suggested that the NF-κB pathway is also involved in some of the biological effects of type-I IFNs [183]. Whether TRIM expression may be regulated directly or indirectly by any of these different pathways will be discussed later in the context of the result of my thesis.

#### 1.5.1.7. Biological effects of type-I IFNs and induction of anti-viral genes.

Type-I IFNs are pleiotropic cytokines that can exert anti-viral functions by different mechanisms including immunomodulatory functions, apoptosis, cell cycle arrest and the induction of a large number of anti-viral molecules. IFNα/β can up-regulate class I MHC molecules in viral infected cells and activate virus specific CD8+ T cells [184]. Using mice deficient in the IFNRA1 it was shown that Type-I IFNs are important for DC activation *in vivo* in response to TLR ligands, and a specific requirement for IFNs by pDC but not conventional DC (cDC, CD11c+/B220- DC) for migration to CD4+ T cell areas was established, probably by induction of chemokines [185]. Type-I IFN produced by pDC also plays an essential

role in activating NK cells to kill virus-infected cells [186]. IFNα/β also promotes maturation of DCs [187, 188], and at the same time can link the innate with the adaptive immune response by promoting the development of IFNγ producing Th1 cells [128, 187]. In addition to their effects on NK and T cell responses, type-I IFNs can affect humoral immunity as demonstrated by the fact that type I IFN potently enhanced the antibody response to soluble antigen, allowing for class-switching and development of immunological memory in a DC-dependent manner [189].

Additional to all these biological functions, type-I IFNs can induce an "anti-viral state" in cells exposed to IFNα/β by up-regulation of a wide number of proteins that can directly or indirectly inhibit viral replication (**Figure 1.7C**). Some of the anti-viral molecules known to be up-regulated by IFNα/β include the protein kinase R (PKR) which is activated by dsRNA and prevents transcription by phosphorylation of the transcriptional initiation factor 2 (eIF2a)[190], the 2'5'-oligoadenylate synthetase (OAS) which is also activated by dsRNA to degrade cellular and viral RNA [191, 192], and the Mx family of proteins which are GTPases that can act by recognizing nucleocapsid structures from some viruses and restricting their localization thus restricting viral replication [141, 193].

The importance of Type-I IFNs is highlighted by the fact that they induce a positive feedback autocrine loop to produce more IFNs as a consequence of the first cell targeted by viral infection. IRF-3, broadly and constitutively expressed at a low level, is activated by this autocrine loop to promote more Type-I IFN gene transcription. Activation of the IFNα/β receptors and STAT1 also induce IRF-7 which is important in this positive feedback for type-I IFN expression [194-196].

### 1.5.1.8. TRIMs in innate immune signal transduction

An increasing number of TRIMs have been reported to have some function in signal transduction pathways and many have been suggested to do so by a mechanism that involves ubiquitination. This can be either by proteasome dependent protein degradation of target signalling molecules, or using ubiquitin as a moiety that mediates ubiquitin dependent protein-protein interactions leading to activation of signalling pathways. Interestingly and of relevance for this thesis is the fact that many of these TRIM dependent signalling pathways are related to immune responses including signalling to induce cytokine production (**Figure 1.8**). For example, TRIM25/EFP has recently been reported to be involved in the RIG-I signalling pathway to induce IFNβ. It was shown that TRIM25 ubiquitinates RIG-I which is required for MAVS (mitochondrion antiviral signalling protein) binding as well as the ability of RIG-I to induce anti-viral activity against vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) by inducing IFNβ [12]. Another interesting example of the effects of ubiquitination by TRIMs in immune signalling is the case of TRIM21/Ro52 which was proposed to be involved in signalling to induce IL-12p40 production by non-proteolytic mechanism of ubiquitination of IRF-8 in macrophages stimulated with IFNγ and CpG [98]. However, in another study it was also proposed that TRIM21 can act as a negative regulator of IFNβ production by inducing IRF-3 ubiquitination and proteasome dependent degradation upon stimulation with LPS, poly I:C or infection with Sendai virus [97]. Although these two studies do not give a deep insight on the signalling mechanisms involved and both used cell lines, this suggests that the same TRIM molecule can act as a positive and negative regulator depending on the stimulatory conditions and the cells used.

Moreover this also indicates that the same TRIM molecule may be involved in signalling by both degradation and non-degradation pathway of ubiquitination.

Other studies have shown that TRIMs may be involved in signalling by non-ubiquitination pathways. This is the case of TRIM27/RFP which can be phosphorylated by IKKα,β,ε and TBK-1, members of the NF-κB signalling pathway [197]. Moreover, it was shown that TRIM27 inhibited NF-κB and/or ISRE activation mediated by these IKKs triggered when cells were stimulated with TNF, IL-1, poly I:C, or viral infection. It was also shown that the phosphorylation of TRIM27 led to IRF-3 retention in the cytoplasm suggesting that TRIM27 may sequester IRF-3. Therefore, TRIM27 negatively regulates the signalling involved in the anti-viral response by targeting the IKKs which phosphorylate IRF-3 and critically involved in virus triggered and TLR3 mediated signalling leading to induction of type-I IFNs [197].

TRIM30α has also been shown to regulate TLR signalling by a ubiquitin independent pathway. It was shown that TRIM30α interacts with the TAB2-TAB3-TAK1 adaptor-kinase complex involved in the activation of the transcription factor NF-κB. TRIM30α promoted degradation of TAB2-TAB3 and inhibited NF-κB activation induced by TLR signalling by a mechanism dependent on lysosomes but independent of proteasomes. Over-expression of TRIM30 resulted in inhibition of IL-6 and TNF production suggesting that TAB2,3 degradation results in the inhibition of NF-κB activation after TLR stimulation [198]. Interestingly, TRIM30α expression depended on NF-κB activation suggesting functions as a feedback negative regulator [198].

TRIM8/GERP, a poorly characterized member of the TRIM family has been shown to interact with the suppressor of cytokine signalling-1 (SOCS-1). SOCS

proteins are known to be important in negative regulation of cytokine signalling to control the intensity and duration of the signal [199]. Co-expression of TRIM8/GERP with SOCS-1 decreases the stability and thus the levels of SOCS-1 which correlated with decreased inhibition of IFNγ-induced JAK-STAT activation, suggesting that TRIM8/GERP may be a positive regulator of IFNγ signalling [200].

In the TGFβ signalling pathway, TRIM19 and TRIM33 have been reported to play different important roles. TGFβ family members bind to membrane serine/threonine kinase receptors that phosphorylate Smad transcription factors and in turn activate transcription of TGFβ responsive genes [201]. TRIM33/TIF1γ has been shown to interact specifically with phosphorylated Smad2/3 in haematopoietic stem cells in response to TGFβ and this results in induction of erythroid differentiation [202]. The essential role of TRIM33 during erythroid differentiation is supported by the fact that TRIM33 knockout die during early somitogenesis [203]. Conversely, the cytoplasmic isoform of TRIM19/PML (cPML) has been suggested to be involved in the TGFβ dependent- growth arrest, induction of cellular senescence and apoptosis. cPML binds to Smad2/3 and SARA (Smad anchor for receptor activation) for subsequent signalling of TGFβ target genes. TRIM19/PML knockout MEFs have impaired phosphorylation and nuclear translocation of the TGFβ signalling proteins Smad2/3, as well as impaired induction of TGFβ target genes identifying cPML as a regulator of the TGFβ signalling pathway [204].

TRIM18/MID1, involved in Optiz syndrome (a defect of the midline during embryogenesis), is an E3 ubiquitin ligase that associates with the catalytic subunit of protein phosphatase 2A and targets it for ubiquitin-dependent degradation. It has also

been shown that TRIM18 is itself a target of MAPK phosphorylation which suggests that TRIM18 may be involved in signalling pathways to produce cytokines.

At the transcriptional level, TRIM28/KAP1 (TIF-1β) has been suggested to be a negative regulator of type-I IFN induced transcription. It was shown that TRIM28/KAP1 interacts with STAT1 and negatively regulates IFN/STAT1-mediated IRF-1 gene expression in collaboration with histone deacetylase complex [56]. It was also previously shown that TRIM28/KAP1 interacts with STAT3 and negatively regulates IL-6/STAT3-mediated gene transcription suggesting that TRIM28 may act as a general transcriptional repressor for a variety of proteins involved in cytokine signal transduction pathways [57]. Interestingly, this negative role of TRIM28 on gene transcription has important implications in silencing of retroviral transcription. Replication of MLV is restricted in embryonic carcinoma and embryonic stem cells where TRIM28 forms a complex with histone methyltransferases, histone deacetylase and HP1 family members to methylate histone H1 and promote chromatin condensation [59, 60]. This report highlighted the importance of TRIMs as potential viral restriction factors using transcriptional mechanisms to inhibit viral replication.

An overview of the many TRIMs involved in signalling pathways is shown in **Figure 1.8**.

#### 1.5.1.9. Anti-viral function of TRIM proteins

The TRIM family of proteins has recently gained importance by the discovery that TRIM5α from African green monkeys and macaques acts as a restriction factor for HIV-1 [33]. Since then other members of the TRIM family have also been found to have some anti-viral function and this led to the suggestion that the entire family

of TRIMs may be a component of an innate or intrinsic immune response to viruses [22, 140, 205-207]. This mechanism of restriction of viral replication has been assumed to rely on a set of anti-viral molecules that are constitutively expressed in most cells of an organism in a species-specific manner [140]. This differs to the "inducible" type-I IFN system where the expression of anti-viral molecules increases upon exposure to viral infection [142]. This is important since most of the studies on TRIMs have been done using transformed cell lines and over-expression assays where the levels of TRIM molecules may be artificial and do not represent physiological conditions. Of note is the fact that some of the TRIM molecules found to have anti-viral activity are themselves inducible by type-I IFNs including TRIM5α [208], further supporting their important role against viral infections. Most of the viruses that have been studied and found to be affected by TRIM proteins are retroviruses, possibly because these viruses are the most widely studied [22, 209, 210]. It should be noted that the restriction activity of TRIM proteins can take place at different stages of viral replication including viral entry, transcription of viral genes or viral release from the cells [22, 210].

#### 1.5.1.9.1. TRIM5α as a species-specific restriction factor

TRIM5α is the largest isoform encoded by the TRIM5 gene and is the only isoform to possess a B30.2 domain which is responsible for its anti-HIV-1 function [24, 32]. Originally, the human TRIM5α gene (previously named REF1, [211]) was shown to encode a restriction activity to the N-tropic form of the Murine leukaemia virus (N-MLV) [211]. Later studies demonstrated that this restriction activity varied between species and viruses, as TRIM5α from African green monkeys could restrict HIV-1, HIV-2, EIAV (equine infectious anaemia virus), N-MLV and SIVmac while

TRIM5α from Macaques was only able to restrict HIV-1 [22]. Some insight has been gained on the mechanism of viral restriction. TRIM5α blocks HIV-1 replication at the stage of early entry to the cells before reverse transcription [33, 212]. Like other TRIMs, it is believed that TRIM5α possesses E3 ubiquitin ligase activity and can be auto-ubiquitinated [213, 214] leading to a rapid proteasome dependent degradation [215]. Therefore it has been suggested that TRIM5α interacts with hexameric capsids of the incoming virus and its rapid turnover by the proteasome potentially targets virions to degradation. However, proteasome inhibitors do not rescue HIV-1 infectivity suggesting that TRIM5α may act by more than one mechanism. Another simple mechanism could be the rapid uncoating of incoming HIV-capsids by TRIM5α, before the virus has the opportunity to reverse-transcribe [209].

It is important to note that, although there is some controversy as whether the TRIM5 gene exists in mouse (possible errors in the annotated mouse genome because of multiple gene duplications, unpublished observations), TRIM5α protein has not been found to be expressed in rodents, and reciprocally its phylogenetically related homologues TRIM12 and TRIM30 are only found in mouse but not in humans [216]. Although TRIM30 has been suggested to have some anti-viral activity [198, 217, 218] it remains to be established if the other relatives of TRIM5 found in mouse may also have anti-viral activity. Whether there is an alternative biological function of TRIM5α and some of its related TRIMs in non infected cells is still unknown. Therefore it is of interest to speculate that this group of TRIMs may have essential functions in the ubiquitin pathway in non infected cells while at the same time may act as restriction factors to viruses.

#### 1.5.1.9.2. TRIM19/PML anti-viral activity

As previously mentioned, TRIM19/PML which has been implicated in many functions, is an essential component of the nuclear bodies (NB) where several viruses have been observed to replicate [219, 220]. NBs are structures composed of many proteins that are found in the nucleus tightly bound to the nuclear matrix [221, 222]. NBs interact extensively with chromatin fibres in genomic regions that are transcriptionally active and therefore it has been suggested that NBs and TRIM19/PML are important in regulation of transcription [31]. Although the study of TRIM19/PML is complicated by the fact that some anti-viral molecules also associate with the NBs (e.g. Mx1, SP100, [220]), there is evidence that human TRIM19 itself can inhibit a large number of viruses including herpes simplex virus-1 (HSV-1), Ebola virus, lymphocytic choriomeningitis virus (LCMV), Lassa virus, Influenza virus, vesicular stomatitis virus (VSV), rabies virus, HIV-1, human foamy virus (HFV) [22]. However most of these studies failed to prove a mechanism of action or demonstrate a definitive direct role of TRIM19 on inhibition of viral replication. TRIM19 expression is known to be induced by type-I IFNs which also leads to an increase in size and numbers of NBs [223-225] supporting its role as anti-viral effector. Many studies have been performed by overexpression of TRIM19 or using murine embryonic fibroblasts (MEFs) from mice lacking TRIM19 and therefore the real physiological role of TRIM19 during viral infections is still unknown. Only one study has addressed the *in vivo* role of TRIM19/PML by infection of PML -/- mice infected with LCMV or VSV. This study showed that TRIM19/PML knockout mice are more susceptible to lethal immunopathology by LCMV and exhibit higher levels of VSV replication [226], however the mechanism of inhibition remains elusive. The fact that some viruses have developed strategies to

disrupt the integrity of the PML-NBs supports the role of TRIM19 and PML-NB in anti-viral function. For example, LCMV encodes an 11 kDa RING finger protein called Z protein which associates with the PML-NB and induces relocation of TRIM19/PML to the cytoplasm, where TRIM19 and the Z protein bind to the elongation factor eIF4E to inhibit translation [22, 220]. A similar mechanism has been reported for Rabies virus which expresses a phosphoprotein P, a cofactor of the viral polymerase which controls viral transcription, and sequesters TRIM19 in the cytoplasm. This P protein has been shown to inhibit the IFN signalling pathway by blocking STAT1 translocation to the nucleus [227], a mechanism that could involve TRIM19/PML since STAT1 has also been shown to interact with PML [48]. It should not go unmentioned that there are several isoforms described for TRIM19/PML, all containing the Tripartite motif but differing in the C-terminal region, which may help to explain the large range of cellular functions and interacting partners of TRIM19.

Despite the overwhelming evidence supporting the role of TRIM19 as an antiviral effector, it has been suggested that since TRIM19/PML lacks traces of evolutionary positive selection or evolutionary pressure, this TRIM may not have anti-viral function or may act by indirect mechanisms which do not include direct interactions with the pathogen [228]. Furthermore, the fact that TRIM19 is also involved in regulation of cytokine signalling (i.e. as a negative regulator of IFNγ signalling [48]) may also suggest a possible indirect mechanism of anti-viral activity. Therefore, more studies are needed to clarify the potential physiological role of TRIM19/PML as anti-viral effector, specially using relevant physiological systems including the use of primary cells and *in vivo* systems.

#### 1.5.1.9.3. Other TRIMs with suggested anti-viral functions

In addition to TRIM5α and TRIM19/PML other TRIMs have been found to interfere with viral replication including TRIM1 [229], TRIM22, TRIM32 [22], TRIM25 [12] and TRIM28 [60]. Human TRIM1 was found to restrict MLV but not HIV-1 [229]. By contrast TRIM18, a closely related protein of TRIM1 which shares about 80% homology, has not been found to restrict any virus thus far. TRIM32 has been shown to interact specifically with the transactivation of transcription (Tat) protein of HIV-1, HIV-2 and equine infectious anaemia virus (EIAV) in the nucleus of the cells [230], therefore TRIM32 may act by inhibiting transcription of viral genes. Similarly, TRIM22/STAF50, has been suggested to control levels of HIV-1 virus by down-regulating the HIV-1 Long Terminal Repeat (LTR)-directed transcription. TRIM22 has also been shown to be induced by IFNs supporting its potential role to inhibit HIV transcription [218]. TRIM45 can inhibit the transcriptional function of AP-1 and ELK-1 [39] which are transcription factors that may be used by some viruses for transcription of viral genes.

Importantly, some recent studies have shown the importance of TRIMs in regulating cytokine signalling pathways potentially involved in inhibition of viral infection. For example, TRIM25 was shown to ubiquitinate RIG-I and initiates signalling to produce IFNβ. The importance of this involvement in IFN signalling in anti-viral activity was supported by the observation of higher levels of VSV in MEFs lacking functional TRIM25 protein as compared to WT MEFs [12].

A large number of TRIMs have been tested *in vitro* by transduction assays for viral restriction activity against a selected group of GFP-labelled retroviruses including HIV-1, HIV2, SIVmac, EIAV (equine infectious anaemia virus), MLV and prototypic foamy virus (PFV). This study suggested that human TRIM6, 18, 19, 21,

and 22 lacked activity against this panel of retroviruses whereas TRIM1, TRIM5 and TRIM34 showed weak but specific inhibition of HIV-2/SIV(MAC), and TRIM34 also inhibited EIAV [210]. A different study using a larger panel of TRIMs could discriminate between viral restriction at the early stage (before viral gene transcription) or late stage of viral replication [231]. In this study, it was shown that mouse TRIM8, 10, 11, 56 and human TRIM11, 26, 31 inhibited HIV entry, while human TRIM25, 26, 62 and mouse TRIM8, 25, 31, 56 affected N-MLV entry. In terms of the inhibitory effect of viral release this analysis identified the human TRIM proteins 15, 26, 32, and the mouse TRIM proteins 11, 25, 27, 56 as factors that specifically affected HIV release from cells, but not viral gene expression [231]. Taken together, results from different studies need to be carefully interpreted depending on the biological system used, the panel of viruses tested and the stage of viral replication. Nevertheless these studies denote the difficulty of working with large number of viruses and TRIM molecules and support the increasing evidence that TRIMs may act broadly as anti-viral molecules.

### 1.5.2. The adaptive immune response

In addition to the role of DC and macrophages during an innate immune response, DCs are fundamental in stimulating the adaptive immune response by acting on B and T cells. The interactions between DCs and CD4 T cells determine the fate of an immune response to pathogenic microbes and are dependent on the maturation and differentiation status of DCs and cytokines [232]. In immature state, DCs are unable to stimulate T cells because they lack the co-stimulatory molecules to signals for T-cell activation, such as CD40 and CD86 [232]. DCs are specialized to capture antigens and these antigens are able to induce full maturation and

mobilization of DCs. Immature DCs presenting endogenous antigenic peptides complexed to MHC class I molecules to CD8 T cells with cytotoxic capacity to eliminate infected cells and attack transplants and tumour cells [232]. On the other hand, DC up take antigens from incoming pathogens, process them and present them in the context of MHC class II molecules to CD4 T helper cells. This interactions lead to up-regulation of co-stimulatory molecules, for example CD40L on the T cell that binds CD40 on the DC, which in absence results in anergy [233]. Activation of naïve CD4+ T cells requires signals through both TCR and co-stimulatory molecules such as CD28 [234, 235], and antigen specific signalling is achieved by interaction of MHC bound to a peptide and the TCR.

DCs have also major effects on B cell growth and immunoglobulin secretion. The antibody response depends on B cells and DCs. DCs activate and expand T-helper cells, which in turn induce B-cell growth and antibody production. On the other hand DCs stimulate the production of antibodies directly and the proliferation of B cells that have been stimulated by CD40L on activated T cells. DCs also orchestrate immunoglobulin class-switching of T-cell-activated B cells [236].

Engagement of TLRs on DCs by TLR ligands such as LPS or CpG also leads to up-regulation of MHC, co-stimulatory molecules and cytokines [232]. Naïve CD4+ T cells producing the cytokine IL-2 clonally expand and differentiate into at least two subsets, Th1 and Th2 populations, important in mediating the development of the host-protective response and are distinguished by the cytokine profiles and their capacity to protect the host against intracellular pathogens or helminth infections respectively [116-118, 237, 238](see **Figure 1.5**). Another cell subset, the Th17 subset, which produce the cytokine IL-17 important in the host defence against extracellular bacteria such as *Klebsiella pneumoniae* [120-122], can also develop.

Intense research on the molecular mechanisms of the general immune response has revealed some members of the TRIM family to play a role in signal transduction pathways, however their potential role in the development of these Th1 and Th2 subsets remains elusive.

### 1.5.2.1 The Th1 response

In response to viruses, intracellular pathogens and bacteria, DCs secrete IL-12 cytokine which directs Th1 cell differentiation. Th1 cells produce IFNγ and TNFα and play a central role in cell-mediated immunity important for eradication of intracellular pathogens such as bacteria, parasites, yeasts and viruses in part through the activation of the microbicidal activity and cytokine production of macrophages [116, 117]. Alongside a Th1 response is common to observe the production of opsonizing IgG2a antibodies, as well as the activation of natural killer (NK) cells and cytotoxic $CD8^+$ T cells expressing IFNγ, perforin and granzymes, which also serve to eradicate pathogens [116-118, 237, 238]. Importantly, if uncontrolled, Th1 cells can mediate immunopathology and have also been implicated in autoimmune diseases such as type-I diabetes and multiple sclerosis [239]. The differentiation pathway for the development of this Th1 response can be mediated by a number of factors, including the dose and the affinity of the peptide antigen–TCR interaction [128] and co-stimulatory interactions between cell-surface molecules [240-243]. The mechanism of IFNγ production by Th1 cells has been shown to be selectively mediated by a transcription factor termed T-bet (T-box expressed in T cells) which is induced in Th1 cells but not Th2 [244]. T-bet acts by re-modelling and activating the IFNγ gene, inducing expression of IL-12Rβ2 to augment Th1 signal, and interacts with the Th2 transcription factor GATA3 to inhibit Th2 signalling [244, 245]. IL-12,

produced by appropriately activated macrophages and DC, is crucial for the development of Th1 responses by activation of the transcription factor STAT4 through the IL-12 receptor (IL-12R, consisting of IL-12Rβ1 and IL-12Rβ2) [246]. IL-18 synergizes with IL-12 to stimulate high levels of IFNγ required to eradicate intracellular pathogens [247, 248]. In humans it has been shown that in addition to IL-12, IFNα can also activate STAT4 to generate Th1 responses, although this was not observed in mice [249]. To date, there has not been any TRIM member described to have a selective role in the Th cell differentiation. Although expression of some members of the TRIM family has been shown to be up-regulated upon addition of IFNγ to the cells in culture, none of these TRIMs have been shown to have any specific role in Th1 cells and their expression and specific role in these cells is as yet unknown.

#### 1.5.2.2. The Th2 response

In contrast to the Th1 response to intracellular pathogens, infections with multicellular parasites, such as helminthes, generally lead to differentiation of Th2 cells, which produce IL-4, IL-5 and IL-13 and when uncontrolled contribute to eosinophilic inflammation and allergic reactions [118, 119]. A Th2 response is primarily responsible for activation and maintenance of mast cells, eosinophils and B cell growth factors [237] and is characterized by high IgG1 and IgE antibody isotypes that can instruct a humoral-mediated response to eradicate nematodes, helminths and other extra-cellular pathogens [118, 237, 250]. Binding of IL-4 to its receptor activates the JAK–STAT6 and IRS2 signalling pathways [251]. IL-4 can promote the growth or differentiation of Th2 by up-regulating the transcription factor GATA-3 [252-254], a zinc finger transcription factor that remodels the IL-4 locus allowing

transactivation of IL-4, IL-5, IL-3, and inhibits Th1 development by blocking the Th1 cytokine IFNγ via direct down-regulation of STAT4 expression [255, 256]. Importantly, differentiation of Th1 and Th2 cells can be inhibited by TGFβ [257].

It will be of interest to determine whether TRIMs may also be differentially expressed in these subsets.

#### 1.5.2.3. Regulatory T cells (Tregs)

T regulatory cells (Treg) are $CD4^+$ T cells that inhibit immunopathology and autoimmune diseases and have suppressive effects on CD4 or CD8 T cell proliferation and many immune responses including those against infectious pathogens [124, 258]. The best defined is characterized by the expression of the forkhead/winged helix transcription factor Foxp3 important for their development and function [259]. Other cells have also been suggested to have regulatory functions that do not express Foxp3 but produce inhibitory cytokines such as IL-10. These cells inhibit naive T-cell proliferation *in vitro* and autoimmune pathologies such as experimental autoimmune encephalitis (EAE) *in vivo*, an inflammatory central nervous system (CNS) pathology [124, 258, 260, 261]. However, it should be noted that IL-10 was originally described as a cytokine produced by Th2 cells, and it is now clear that it is produced by many other cells, including Th1 cells, B cells, macrophages and DC [262, 263] in addition to regulatory T cells [124, 258].

As mentioned above, Tregs and/or IL-10 and TGFβ are involved in inhibiting hyper-reactive responses like autoimmune diseases and inflammatory bowel disease, but they are important also in regulating innate and adaptive immune responses to invading pathogens to avoid host damage but as a penalty may result in chronic disease. In this respect, nothing is known about the function of TRIM proteins in the

development or function of these cellular subsets. It will be interesting to see if IL-10 can also induce or inhibit expression of certain TRIM proteins in specific cellular subsets or in cells that produce IL-10.

#### 1.5.2.4. Proposed roles of TRIMs in T cells

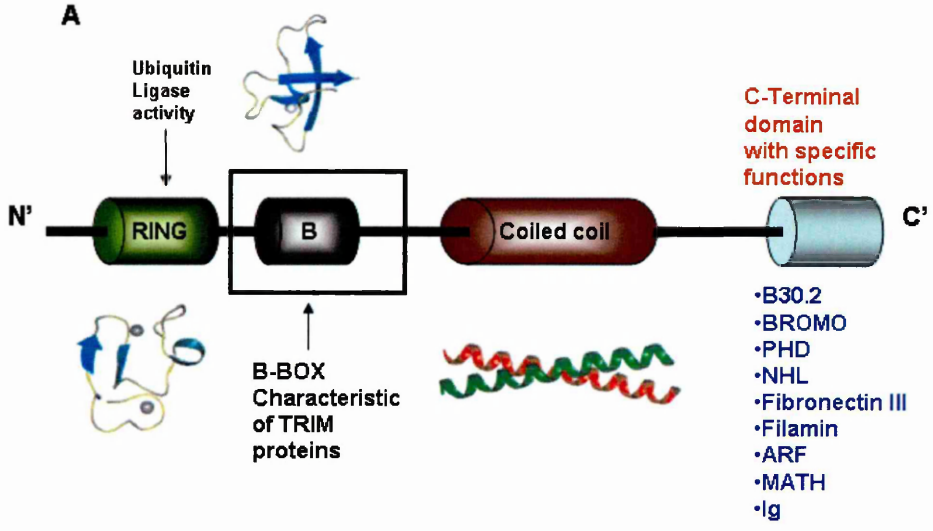
It has been suggested that TRIM proteins can have an effect in activation of T cells and could be involved in changing the fate of the immune response. For example, expression of TRIM8 decreases the repression of IFNγ signalling mediated by SOCS-1 [200]. Therefore, it is of interest to determine whether expression of TRIM8 is restricted to a specific cell type and whether its expression is up-regulated in response to a specific stimulus. Another example of TRIM function in T cells is the finding that over expression of TRIM21/Ro52 in Jurkat T cells resulted in enhanced IL-2 production following CD28 stimulation. Furthermore, transfection of anti-Ro52-specific small RNA duplexes partially blocked the expression of TRIM21/Ro52 in Jurkat T cells, and resulted in decreased IL-2 production via the CD28 pathway [264].

Many viruses target T cells because they are essential in achieving effector responses to protect from pathogens. Thus it would be expected that T cells should have a mechanism to block infection of certain viruses and bacteria and in the same way viruses have evolved mechanisms to evade this possible line of defence. TRIMs are an excellent candidate for this function since they have the capability of interacting with many proteins and form high molecular weight complexes in specific compartments where they can target intracellular pathogens and viruses. Moreover, their potential ubiquitin ligase activity that can target proteins for degradation is another way of blocking successful infection of pathogens. Induction of TRIM

proteins in response to cytokines can be a specific way of regulating immune responses depending on the type of pathogen and the type of immune response required.

### 1.6. Aims of this Thesis

In this study we have used a systematic approach to determine the expression of representative TRIMs in a broad number of primary immune cells in an attempt to understand firstly, how TRIM molecules are regulated and secondly, to obtain potential leads as to their function. We hypothesized that the patterns of TRIM mRNA expression in effector cells of the immune system, which produce different cytokines, may provide information to help delineate their potential function in innate and adaptive immune responses.




**B**

Consensus sequences:

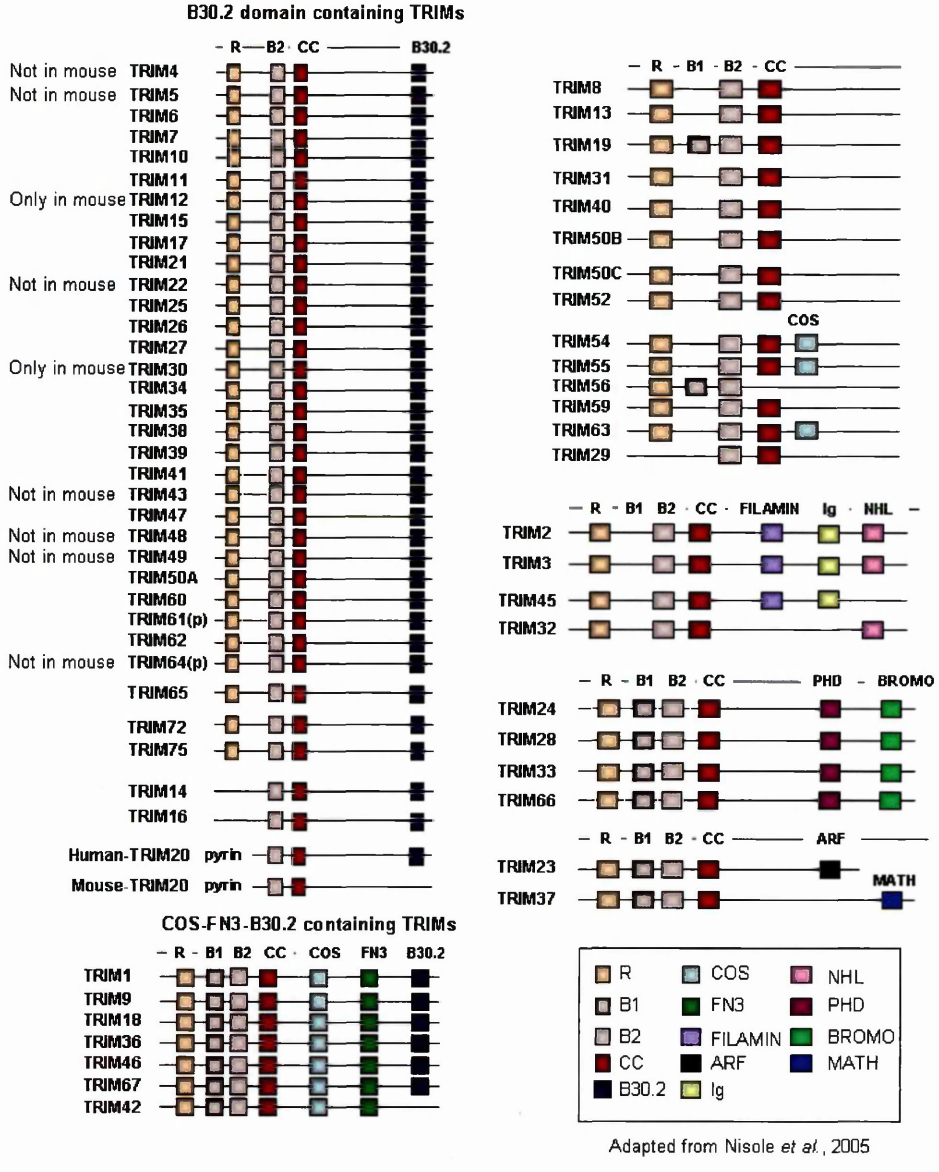
RING: $C\text{-}x_2\text{-}C\text{-}x_{11\text{-}16}\text{-}C\text{-}x\text{-}H\text{-}x_2\text{-}C\text{-}x_2\text{-}C\text{-}x_{7\text{-}74}\text{-}C\text{-}x_2\text{-}[CD]$

B-box1: $C\text{-}x_2\text{-}C\text{-}x_{7\text{-}12}\text{-}C\text{-}x_2\text{-}C\text{-}x_4\text{-}C\text{-}x_2\text{-}[CH]\text{-}x_{3\text{-}4}\text{-}H\text{-}x_{4\text{-}9}\text{-}H$

B-box2: $C\text{-}x_2\text{-}H\text{-}x_{7\text{-}9}\text{-}C\text{-}x_2\text{-}[CDHE]\text{-}x_4\text{-}C\text{-}x_2\text{-}C\text{-}x_{3\text{-}6}\text{-}H\text{-}x_{2\text{-}4}\text{-}[CH]$

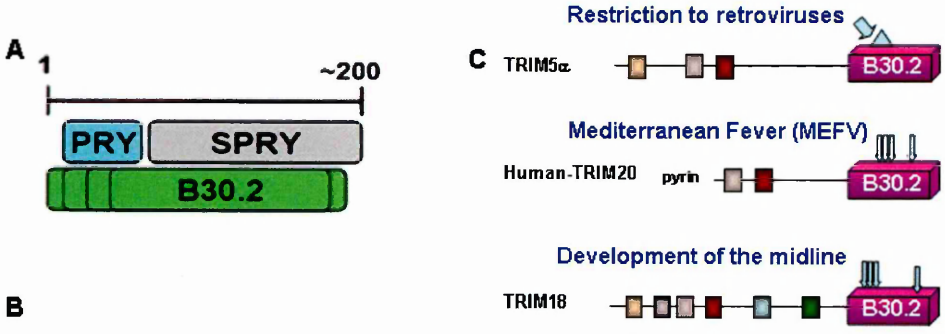
Adapted from: Jensen, *et al.*, 2001; Meroni and Diez-Roux, 2005

**Figure 1.1. Schematic representation of the RBCC/TRIM motif structure.**
**A)** Representation of the Tripartite motif (TRIM) composed of a RING finger domain (R) proposed to have ubiquitin ligase activity, followed by a B-box domain (B), the defining domain of the family, and the coiled-coil motif (CC), an hyper-helical structure predicted by bioinformatics. The different domains found in the C-terminal region of TRIM proteins are listed below a generic C terminal domain. Abbreviated as: **BROMO**, bromodomain; **MATH**, meprin and TRAF homology domain; **NHL**, NHL repeat; **PHD**, plant homodomain; **ARF**, ADP ribosylation factor; and **Ig**, immunoglobulin family. The three-dimensional structural representations of the three domains are shown next to the schematic representation. **B)** The consensus sequences for the RING domain and the two types of B-box domain (B-box1 and B-box2) are shown: C, cysteine; X, any amino acid; H, histidine; D, aspartic acid; E, glutamic acid. Adapted from [8, 15].




Adapted from Nisole *et al.*, 2005

**Figure 1.2. The Tripartite motif (TRIM) family of proteins.**
All the TRIM proteins described in humans and mouse to date are represented schematically with their conserved domains. The TRIM proteins are subdivided by their homology domains in the C-terminal region of the protein. **R**, RING domain; **B**, B-box domain; **CC**, Coiled-coil domain; BROMO, bromodomain; MATH, meprin and TRAF homology domain; NHL, NHL repeat; PHD, plant homodomain; p, predicted from computational analysis. The TRIMs not found in humans or mice are indicated. Adapted from [22].

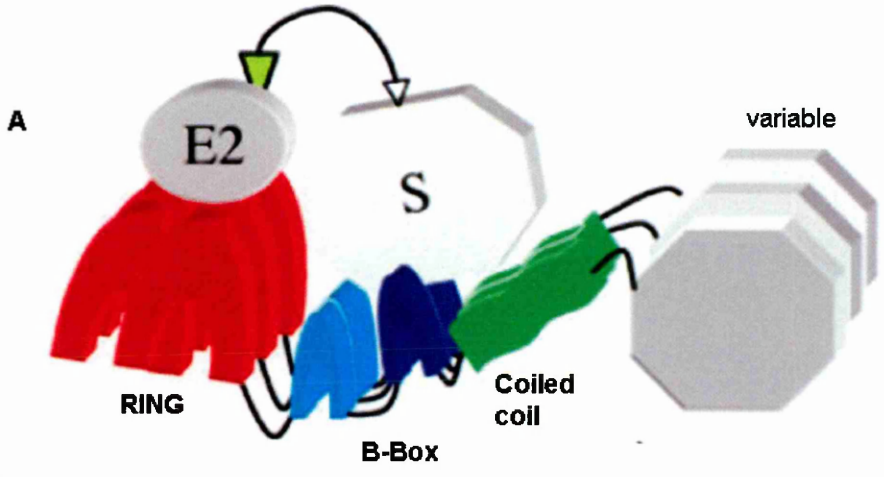



B

| Family (structure) | Function |
|---|---|
| **TRIM**<br>RBCC-B30.2 | E3 ubiquitin ligases, Viral restriction,Signaling |
| **BTN**<br>IgV-IgC-TM-B30.2 | Cell surface receptor glycoproteins of the immunoglobulin super-family.Resemble B7 co-stimulatory receptors. Role in immune regulation? |
| **SS-B**<br>SPRY-SOCS | Related to SOCS box proteins. Involved in down-regulation of JAK/STAT signaling pathways |
| **hnRNP**<br>SAP-SPRY | Family of ubiquitously expressed RNA binding proteins |
| **RyR** | Calcium release channel localized in the endoplasmic reticulum |
| **RanBPM**<br>SPRY-LisH-CTLH | Ran binding protein. Ran is a small GTPase and member of the Ras superfamily involved in nuclear import/export |
| **DDX1**<br>DAED-SPRY-DEAD-HELICASE | A member of DEAD box family. Putative RNA helicases that function in all aspects of RNA metabolism |
| **HERC1**<br>RCC1-SPRY-WD40-RCC1-HECTc | A large protein related to chromosome condensation regulator. |
| **ASH2**<br>PHD-SPRY | Member of the trihoraxgroup of transcriptional regulators of Hox genes |
| **FLJ14800**<br>SPRY-SPRY | uncharacterized |
| **KIAA1972**<br>SPRY-RING | uncharacterized |

Adapted from:
Rhodes *et al.*, 2005.
Je-Sung Woo, *et al.*, 2006

**Figure 1.3. Schematic representation of The B30.2 domain structure and function.**
**A)** The B30.2, a domain of about 200 amino acids, is composed of PRY and SPRY motifs. **B)** Table of the different families of proteins containing B30.2 or SPRY domains and their possible functions. **C)** Schematic representation of the mutations found in the B30.2 domains of TRIM5α, TRIM20 and TRIM18. Mutations are indicated by the light blue arrows. One mutation in the B30.2 domain of human TRIM5α results in restriction activity to HIV-1. Mutations in the B30.2 domain of TRIM20 are associated with Mediterranean fever (MEFV). Mutations in the B30.2 domain of TRIM18/MID1 are associated with Optiz G/BBB syndrome. Adapted from [26, 265].




B

| Function | Anti-viral | Cell differentiation | Neural function | Transcriptional regulation | Signalling Pathways | Apoptosis | Tumor or cancer |
|---|---|---|---|---|---|---|---|
| | TRIM1 | TRIM10 | TRIM2 | **TRIM14** | **TRIM8** | **TRIM19** | **TRIM8** |
| | TRIM5 | TRIM16 | TRIM3 | **TRIM19** | TRIM1 | **TRIM20** | TRIM13 |
| | **TRIM19** | **TRIM19** | TRIM9 | TRIM24 | TRIM18 | TRIM32 | **TRIM19** |
| | TRIM22 | TRIM54 | TRIM11 | **TRIM27** | **TRIM19** | TRIM35 | TRIM29 |
| | **TRIM25** | | | **TRIM28** | **TRIM20** | | TRIM24 |
| | **TRIM28** | | | TRIM33 | **TRIM21** | | **TRIM25** |
| | **TRIM30** | | | **TRIM45** | TRIM23 | | **TRIM27** |
| | TRIM32 | | | | **TRIM25** | | TRIM32 |
| | **TRIM45** | | | | **TRIM27** | | TRIM35 |
| | | | | | **TRIM30** | | TRIM36 |
| | | | | | **TRIM33** | | |

Adapted from: Meroni and Diez-Roux, 2005

**Figure 1.4. Model of TRIM-mediated protein ubiquitination and proposed molecular functions of TRIMs.**
**A)** Ubiquitin transfer from TRIM proteins to the substrate. The RING domain (depicted in red) binds to the E2 ubiquitin ligase carrying ubiquitin (shown as a green triangle). The B-box domain (depicted in blue) of the TRIM proteins interacts directly with a substrate protein (S). Then ubiquitin is transferred to a lysine residue in the substrate (depicted with an arrow). **B)** The many diverse functions of TRIM proteins. Summary of the TRIMs known or suggested to have a specific function. TRIMs with a possible link to immune function are shown in red. Adapted from [8].

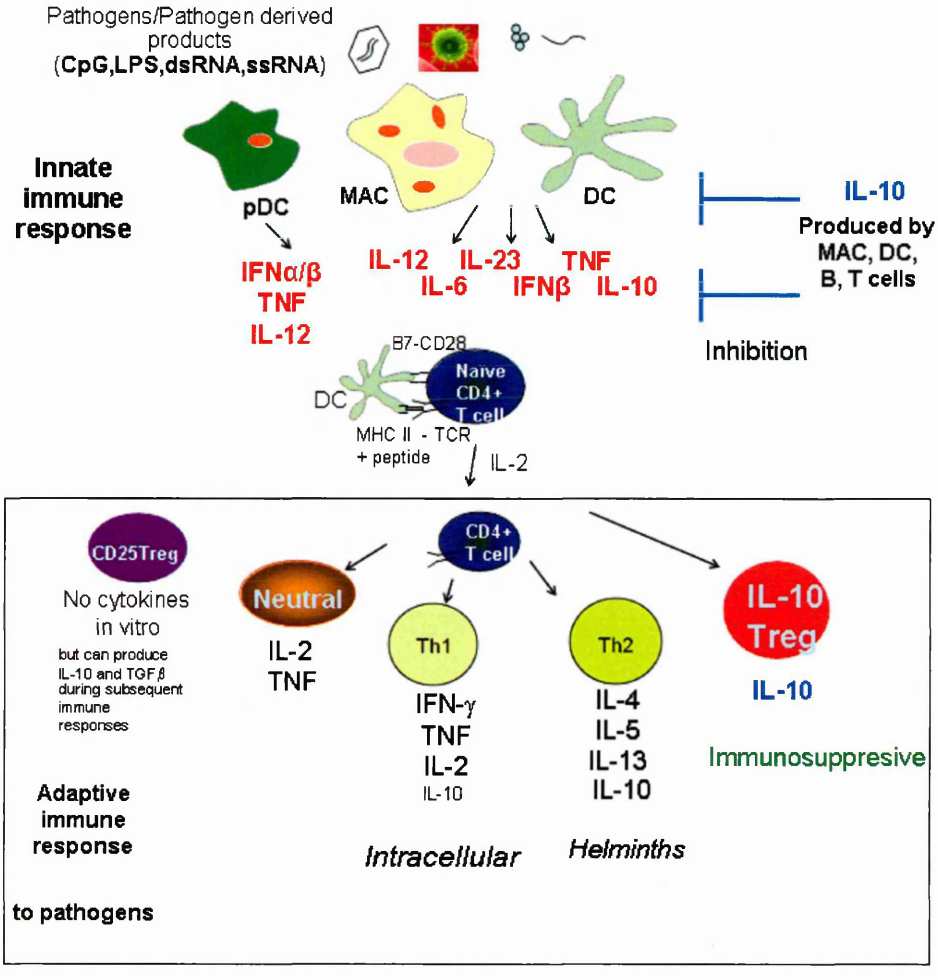



**Figure 1.5. Cells of the innate and adaptive immune system (used in our study) produce different patterns of cytokines upon stimulation and have different effector functions.** Plasmacytoid DC (pDC), Macrophages (MAC), and mDC are activated by pathogen-derived products to produce different sets of cytokines, important for initiation of the adaptive immune response. MHCII bound to peptide and co-stimulatory molecules, like B7, on the cell surface of DCs interacts with the TCR and CD28 on naïve CD4 T cells. Naïve CD4+ T cells producing IL-2 can differentiate into Th1 and Th2 populations distinguished by the cytokine profiles shown, and mediating protective responses against intracellular pathogens or helminth infections respectively. Neutral T cells producing IL-2 and TNF can also be derived *in vitro*. IL10-Tregs producing large amounts of the immunosuppressive cytokine IL-10 can also develop. The IL-10 cytokine is also produced by Th1, Th2, and macrophages, B cells and DC (but not pDC), and inhibits the differentiation and function of all these cell subsets. CD25 Tregs which also have inhibitory functions do not produce cytokines *in vitro* upon TCR stimulation.

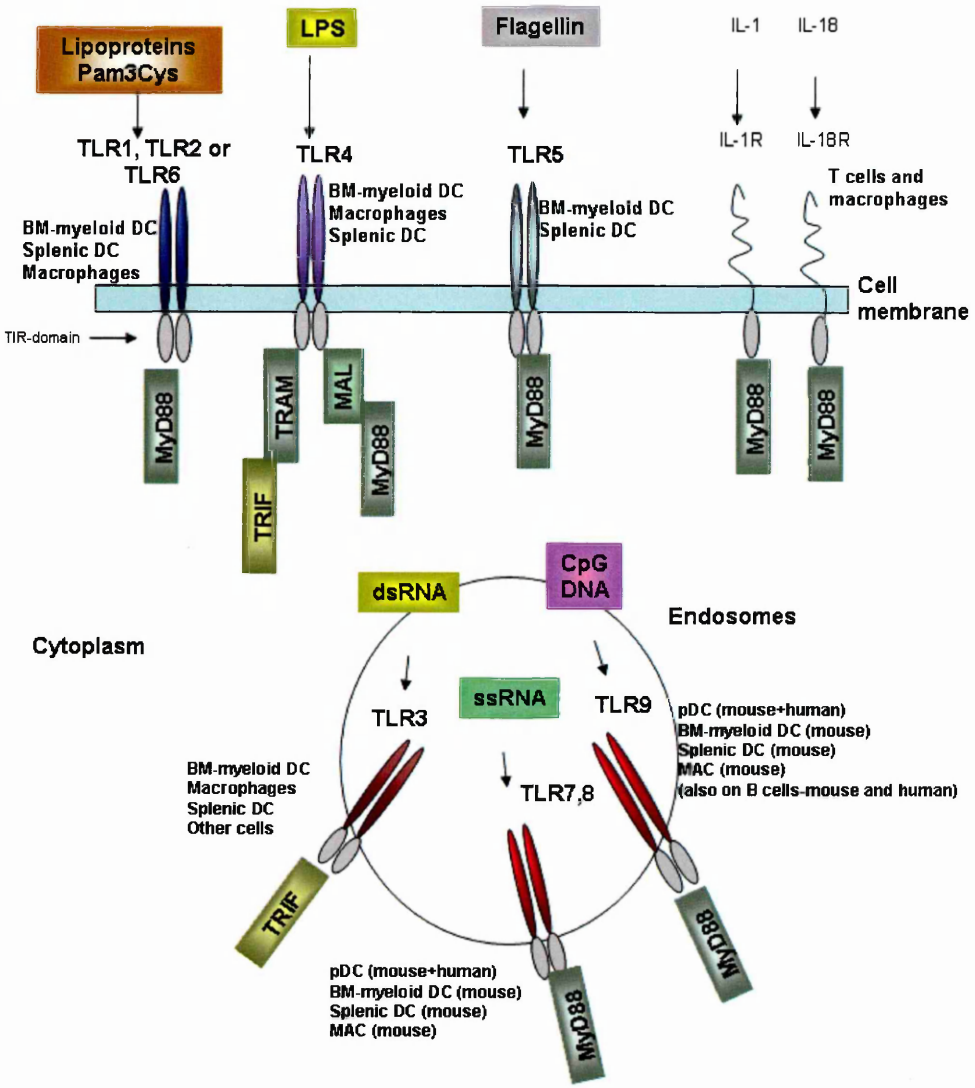



**Figure 1.6. Overview of the Toll like receptors (TLRs) and their differential expression in macrophages and DC subsets.**

Expression of the different TLRs on their specific cell subset and their TLR ligands are indicated. MyD88 (myeloid differentiation primary-response gene 88) is the key signalling adaptor for all TLRs, with the exception of TLR3 and TLR4. TLR3 signals instead through the TRIF (Toll/IL-1R [TIR]-domain-containing adaptor protein inducing IFNβ) adaptor molecule. TLR4 is the only TLR to signal through four adaptor proteins: MyD88, TRIF, MAL (MyD88 adaptor-like protein) and TRAM (TRIF-related adaptor molecule). IL-1R and IL-18R also signal through MyD88. TLR1,2,6 recognize lipopeptides from bacteria or the synthetic lipopeptide Pam3Cys. TLR4 binds lipopolysaccharides (LPS) from gram negative bacteria. TLR5 binds flagellin from bacterial flagella. TLR3 binds to double stranded RNA (dsRNA), TLR7,8 recognize single stranded RNA (ssRNA) from viruses. TLR9 binds unmethylated CpG DNA.TLR1,2,4,5,6 and the IL-1, IL-18 receptors (IL-1R, IL18-R) are expressed in on the cell membrane while TLR3,7,8,9 are expressed in endosomes.

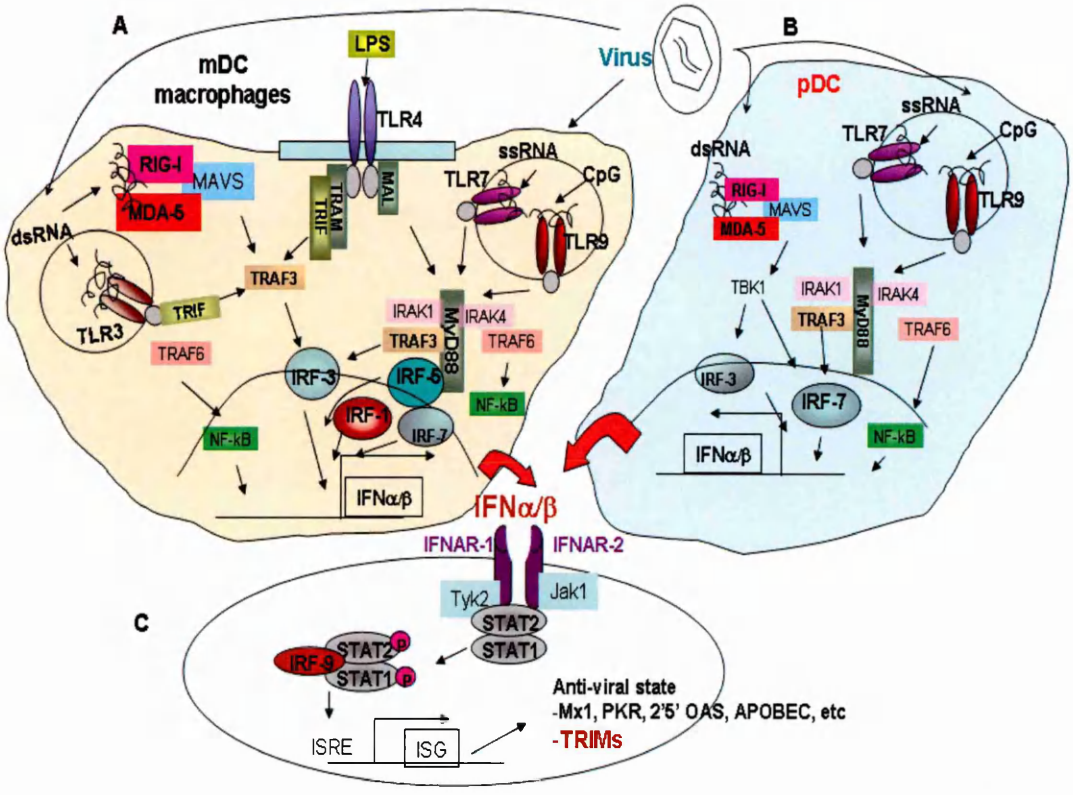



**Figure 1.7. Viral products activate cell-specific pathways to induce type-I IFNs and IFN-dependent expression of anti-viral proteins.**
**A)** Signalling pathways in macrophages and mDC to induce type-I IFNs and pro-inflammatory cytokines. **B)** Signalling pathways in pDC to induce type-I IFNs and pro-inflammatory cytokines. **C)** Type-I IFN signalling and establishment of an anti-viral state. Binding of type-I IFNs to their receptor (IFNAR1) leads to recruitment and activation of JAK1 and Tyk2 resulting in phosphorylation of STAT2 and STAT1 to form a STAT1–STAT2–IRF9 complex, which is known as ISGF3 (IFN-stimulated gene (ISG) factor 3) complex. These complexes translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA to initiate gene transcription. Importantly, this ISGF3 complex is the only complex that can bind specifically the ISREs in the promoters of certain IFN sensitive genes (ISG) to initiate their transcription. These genes can then induce an "anti-viral state" in cells exposed to IFNα/β by up-regulation the protein kinase R (PKR) which is activated by dsRNA and prevents transcription by the transcriptional initiation factor 2 (eIF2a), the 2'5'-oligoadenylate sinthetase (OAS) which degrade cellular and viral RNA, the Mx family of proteins which are GTPases that can act by recognizing nucleocapsid structures from some viruses, and potentially anti-viral TRIMs.

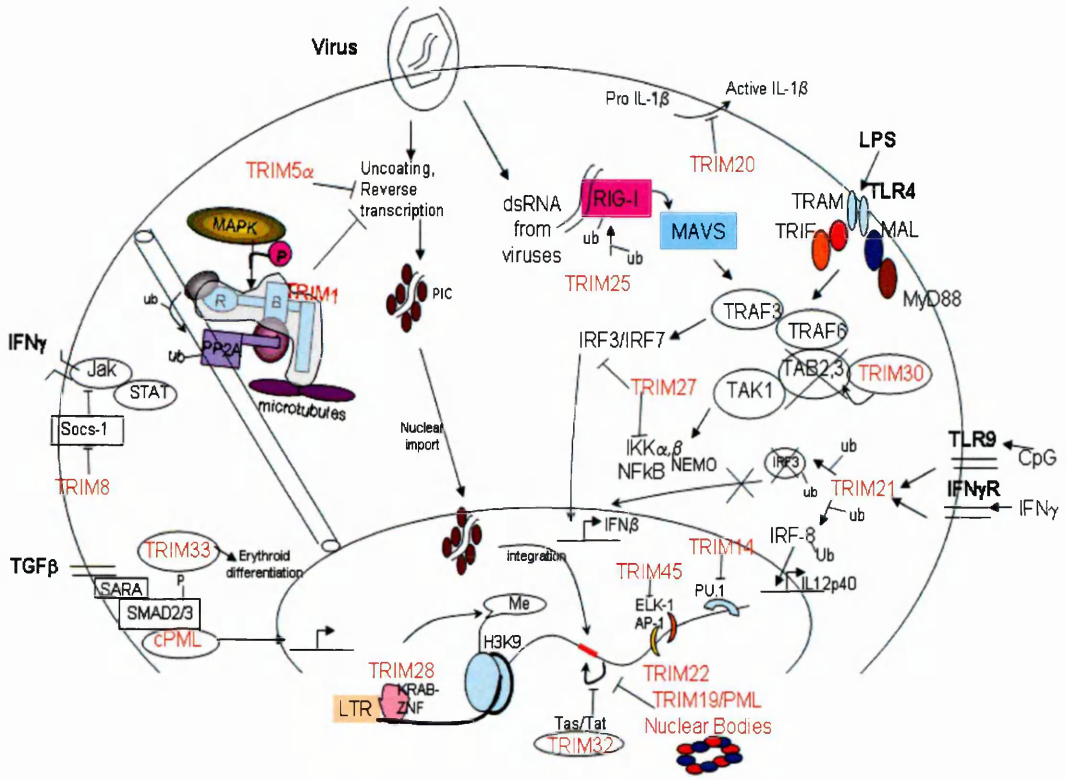



**Figure 1.8. An overview of the TRIM proteins involved in immune signalling and anti-viral functions.**

TRIMs in ubiquitination pathways: TRIM25 ubiquitinates RIG-I which is required for MAVS binding as well as the ability of RIG-I to induce IFNβ. TRIM21 involved in signalling to induce IL-12p40 production by non-proteolytic mechanism of ubiquitination of IRF-8 in macrophages stimulated with IFNγ and CpG. TRIM21 can act as a negative regulator of IFNβ by inducing IRF-3 ubiquitination and degradation. TRIM18 is a target of MAPK phosphorylation which leads to regulation of microtubule dynamics by association with the catalytic subunit of protein phosphatase 2A and targets it for ubiquitin-dependent degradation.. Non-ubiquitination pathways: TRIM27 which can be phosphorylated by IKKα,β,ε and TBK-1, inhibits NF-kB and/or ISRE activation mediated by these IKKs. Phosphorylation of TRIM27 leads to IRF-3 retention in the cytoplasm negatively regulating signalling. TRIM30 interacts with the TAB2-TAB3-TAK1 adaptor-kinase complex and degrades TAB2-TAB3 to inhibit production of IL-6 and TNF. TRIM8/GERP interacts with SOCS-1 and decreases its ability to inhibit IFNγ-induced JAK-STAT activation. In TGFβ signalling: TRIM33 interacts with phosphorylated Smad2/3 and results in induction of erythroid differentiation. Cytoplasmic TRIM19 (cPML) binds to Smad2/3 and SARA for signalling of TGFβ. TRIM20 modulates caspase-1 and IL-1β activation. TRIM45 inhibits transcriptional activity of AP-1 and ELK-1 probably used by some viruses for transcription of viral genes. TRIM14 inhibits the transcriptional activity of PU.1. Anti-viral TRIMs: Replication of MLV is restricted in embryonic stem cells where TRIM28 forms a complex with histone methyltransferases, histone deacetylase and HP1 family members to methylate histone H1 and promote chromatin condensation. TRIM32 interact with the transactivation of transcription (Tat) protein of HIV-1, HIV-2 and EIAV and may inhibit transcription of viral genes. TRIM22 may inhibit HIV-1 virus by downregulating HIV-1 Long terminal repeat (LTR)-directed transcription. TRIM5α blocks HIV-1 replication at the stage of early entry to the cells before reverse transcription, while TRIM1 blocks N-MLV possibly at the same stage.

# Chapter 2:

# Materials and Methods

## 2. Materials and Methods

### 2.1. Mice

129Sv/Ev WT, IFNα/β R knockout mice, BALB/c and C57BL/6, MyD88-deficient, and TRIF-deficient mice were used to provide macrophages, mDCs and/or pDC. BALB/c mice were used to obtain the CD4+T cells subsets for expression profiling. 129 Sv/Ev and IFNα/β R knockout mice were purchased from B&K Universal Ltd. All mice were bred at the National Institute for Medical Research (London, U.K.) and housed under specific pathogen-free conditions and following UK home office regulations. Female mice were used between 8 and 12 weeks of age. Mice were given irradiated food and water *ad libitum.*

### 2.2. Reagents

Culture medium (cRPMI) was RPMI 1640 (BioWhittaker) with 5% heat-inactivated fetal calf serum (FCS)(Labtech International), 0.05 mM 2-β-mercaptoethanol (Sigma), 10 mM HEPES (BioWhittaker), 100 U/ml penicillin (BioWhittaker), 100 μg/ml streptomycin (BioWhittaker), 2 mM L-glutamine (Sigma), and 1 mM sodium pyruvate. DC and macrophages were stimulated with *Salmonella minnesota* LPS (Alexis), poly (I:C) (Invivogen Life Technologies), phosphorothioate CpG DNA class B (CpG1018; 5'-TGACTGTGAACGTTCGAGA) (Invitrogen Life Technologies) or Influenza A virus strains A/Puerto Rico/8/34(H1N1) (PR8), and A/New Caledonia/20/99(H1N1) (CAL), grown at NIMR. Flt3 ligand was from Shangai Genomix (Shangai, China). Mouse GM-CSF

was obtained from Schering Plough. Monoclonal Antibodies (mAbs) used for isolation of DC subsets were anti-B220-FITC, anti-CD11c-PE, anti-CD11b-allophycocyanin (APC) (all BD Pharmingen or eBioscience). mAbs used in cultures for differentiation of T cell subsets were anti-IFNγ (XMG1.1) and anti-IL-4 (clone 11B11). Anti-mouse CD3 (clone 2C11) and CD28 (clone 37.51) mAbs used for T cell stimulation were purchased from BD PharMingen. mAbs used for T cell enrichment were anti-B220 (clone RA3-6A2), anti-CD8 (clone C291.2.43), anti-Class I-$A^d$/I-$E^d$ (clone 2G9), and for T cell isolation were anti-CD4-FITC, -PE or -CyChrome (clone RM4-5), anti-CD62L-PE (clone Mel-14), anti-CD45RB-FITC or -PE (clone C363.16A) and biotinylated anti-CD25 (clone 7D4) was followed by streptavidin (SA)-CyChrome or SA-APC, and isotype controls (all BD PharMingen). mAbs used for intracellular staining were anti-IL-2-FITC, -PE or APC (clone JES6-5H4) , anti-IL-4, PE (clone 11B1), anti-IL-5, PE (clone TRFK5), IFNγ, FITC (clone XMG1.1) and anti-IL-10-PE or –APC (clone JES5-16E3), anti-TNF-α-PE or APC (clone MP6-XT22) and isotype controls (all BD PharMingen).

### 2.3. Isolation of T cell subsets and generation of polarized T cells

$CD4^+$ T cells were enriched from total spleen cell suspensions and purified as $CD4^+CD62L^+CD45RB^{high}$ naive T cells (>98%), $CD4^+CD25^+$ Treg cells (>96%) using a MoFlo flow cytometer (DakoCytomation). Neutral (Th cells differentiated in the absence of polarizing cytokines and in the presence of anti-IL-4 and anti- IFNγ), Th1, Th2 and IL-10-producing Treg cells (IL-10- Treg) were derived *in vitro* in an APC-independent manner, as described [260]; [258]. Briefly, spleens from female

BALB/c mice were aseptically removed, pooled and mashed using a plunger from a 2ml syringe, through a 70-micron filter in the presence of cRPMI. The homogenized cells were treated with Ammonium Chloride, $NH_4Cl$ (0.83%) to remove red blood cells (RBC). The cells were washed and re-suspended in sort buffer (PBS, 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin). Cells were depleted of APCs and CD8 T cells (anti-B220, anti-CD8, anti-CD11b [Mac-1]) at a final concentration of 10μg/ml, by using BioMag goat anti-rat IgG magnetic beads (PolySciences, Inc.) and a magnetic strip. Following magnetic bead depletion, enriched CD4+ T cells were washed twice and incubated with a mix of the appropriate antibodies for purification of naïve CD4 T cells (CD4+CD62L+CD45RB+), or CD25 Tregs (CD4+, CD25+). Cells were incubated for 20 minutes at 4°C. Labelled cells were washed by centrifugation, passed through a 40-micron filter and purified by MoFlo cytometry. After purification, naïve CD4 T cells (CD4+CD62L+CD45RB+) or CD25 Tregs (CD4+CD25+), cells were plated in 24-well plates ($1x10^6$ cells per well) and stimulated in an APC-free environment with plate-bound anti-CD3 (α-CD3) (10 μg/ml) and soluble α-CD28 (2 μg/ml). For generation of neutral, Th1, Th2, and IL-10Treg, naïve cells (CD4+CD62L+CD45RB+) were also plated in 24-well plates ($1x10^6$ cells per well) and stimulated in an APC-free environment with plate-bound anti-CD3 (α-CD3) (10 μg/ml) and soluble α-CD28 (2 μg/ml) and their corresponding cytokine cocktail in cRPMI. Neutral cells were generated by addition of α-IL-4 (20 μg/ml), α-IFNγ (5 μg/ml). Th1 cells were generated in the presence of IL-12 (5 ng/ml) and α-IL-4 (20 μg/ml). Th2 cells were generated in the presence of IL-4 (10 ng/ml) α-IFNγ (5 μg/ml). IL-10-Treg were generated in the presence of vitamin D3 ($4x10^{-4}$ M), Dexamethasone ($4x10^{-6}$ M) (Vit/Dex). Cells were grown in an incubator (37°C, 5% CO2). On day 3, cells were split in a 1:3 dilution with their corresponding

cytokine cocktail into new 24-well plate in the absence of α-CD3 and α-CD28. On day 7, cells were collected and small fraction of the samples was stimulated for quality control with α-CD3 and α-CD28 for intracellular cytokine production by FACS, protein production by ELISA, and the rest of the cells were stimulated with α-CD3 and α-CD28 and samples collected at different time points for mRNA analysis by real time PCR. **Figure 2.1** shows a flow chart representation of the experimental approach used in our study.

### 2.4. GeneChip: Analysis of TRIMs

The microarray experiments were previously performed by a PhD student in the lab (John Shoemaker, 2006; [266]). Therefore the description of the microarray methodology is fully explained in his thesis [266].

The analysis of TRIM expression was done in my study using the previously available raw data of the different CD4 T cell populations by microarray. The computational algorithm used was GC-RMA and the absolute values were processed with GeneSpringv7.0 software (Silicon Genetics) for further normalizations. The values obtained were normalized to the 50% median (log2 =1) from each GeneChip and these values were further normalized by the median of all samples for each gene. For data presentation, normalized expression was transformed to log2 scale (log2 = 1.0, being the median). Importantly, the Affymetrix GeneChip contains 11 specific probes, that span the full length transcript for each gene measured, making it possible to detect some of the transcript variants described for TRIM genes.

### 2.5. Generation of Bone Marrow (BM)-derived macrophages

Bone marrow (BM)-derived macrophages were generated in the presence of L cell-conditioned medium containing M-CSF. BM cells were isolated by flushing femurs and tibia with culture medium (cRPMI containing 10% FCS). After centrifugation cells were RBC lysed as described above. Cells where then washed with cRPMI and plated at $0.5x10^6$ cells/ml in Petri dishes (60mm, Barloworld Scientific; volume 8 ml). Plates were placed in a tissue culture incubator at 37°C in 5% $CO_2$. At day 4, 10 ml of fresh L cell-conditioned medium were added and placed back in the incubator. At day 7, adherent cells were detached from plates by first removing the medium and adding ice-cold PBS. Plates were the placed in the fridge for 10-15 min. and cells harvested by gentle flushing. The purity was >95% macrophages as determined by staining cells for F4/80 by flow cytometry. The cells were then stimulated in 24-well plates as described in the *in vitro* stimulation of macrophages below.

### 2.6. Generation of Bone Marrow myeloid DC (mDC)

BM-derived CD11c+ myeloid DC (mDC) were generated in the presence of GM-CSF, as described previously [267]. BM cells were isolated by flushing femurs and tibia with cRPMI. Cells were centrifuged at 1300rpm for 5 minutes and RBC were lysed using 0.83% ammonium chloride ($0.5ml/1x10^7$cells). BM cells were plated at $10^6$ cells/ml in medium supplemented with 10 ng/ml GM-CSF in 6-well plates in a volume of 5 ml. At days 2 and 4, supernatant containing non-adherent cells was removed, the wells were washed gently, and fresh medium containing GM-

CSF (10 ng/ml) was added. At day 6, non-adherent cells were collected, centrifuged, re-suspended in fresh medium with GM-CSF (10 ng/ml), and cultured overnight in petri dishes (Nunc). The purity was >60-70%.

### 2.7. Generation of splenic pDC subsets

For the purification of splenic pDC, spleens were treated for 30 min at 37°C with 0.4 mg/ml Liberase Cl (Boehringer Mannheim), followed by RBC lysis as above. Cells were maintained throughout the procedure in cold PBS, 5% FCS, and 2.0 mM EDTA. Spleen cell suspensions were enriched for CD11c+ cells by AutoMACS using anti-CD11c microbeads and staining at the same time with CD11c-PE, CD8α-APC, and 120G8-Alexa488 for 20 min at 4C. Cells were then washed and enriched using an AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions to positively select the CD11c cells. The positively selected fraction was then spun down and re-suspended in FACS buffer at $20x10^6$ cells/ml. The enriched DC were purified using a MoFlo cytometer (DakoCytomation) as the CD11c+CD8α+, CD11c+CD8α–, and the $CD11c^{dull}20G8^+$ pDC. The purity was consistently <95%. **Figure 2.2A and 2.2B** show the isolation and purification sorting profiles.

### 2.8. Generation of Bone Marrow plasmacytoid DC (pDC)

BM derived Plasmacytoid DC (pDC) were generated by culturing BM cells in culture medium containing 100 ng/ml Flt3 ligand for 10 days. BM cells were isolated

from femurs and tibia as described above. Cells were RBC-lysed and cultured in medium containing 100 ng/ml Flt3 ligand for 10 days at $10^6$ cells/ml in 6-well plates in a volume of 5 ml. At day 5, 2.5 ml of medium was replaced by 2.5 ml of fresh medium containing Flt3 ligand. The resulting pDC were purified by flow cytometry as $CD11c^+CD11b^-B220^+$ using a MoFlo cytometer (DakoCytomation). In some cases the mDC population expressing $CD11c^+CD11b^+B220^-$ was also collected in a 2-way sort for comparison with the pDC population. The purity was always >96%. **Figure 2.2C and 2.2D** show the isolation and purification sorting profiles.

### 2.9. Flow cytometry analysis

Cell surface markers were analyzed to determine the purity of purified or enriched cell subsets. For macrophages and DC, cells were pre-treated for 10 min with anti-FCγII (anti-CD16/CD32, clone 2.4G2) to block FC receptors and minimize non-specific binding. Staining was performed in FACS buffer (PBS containing 1% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin) for 15 min on ice with the appropriate antibodies. Cells were then washed with FACS buffer and analyzed on a FACS Caliber flow cytometer (BD Bioscience). The Data was analyzed using FlowJo version 8 software.

### 2.10. *In vitro* stimulation of DC and macrophages, and quantitation of cytokine production

$0.25x10^6$ mDC were cultured in 500 µl medium in 48-well flat-bottom culture plates (Nunc) and $1x10^6$ macrophages were cultured in 1 ml medium in 24-well flat-bottom culture plates. mDC and macrophages were stimulated with medium alone, LPS (100 ng/ml), poly(I:C) (50 µg/ml), CpG1018 DNA (0.5 µM) or Influenza virus PR8 (A/Puerto Rico/8/34-H1N1) and New Caledonia (A/New Caledonia/20/99-H1N1) at 100 hemagglutinin (HA) U/ml. For pDC, $0.1x10^6$ cells were cultured in 200 µl medium in 96-well flat-bottom culture plates (Nunc) and stimulated with Influenza viruses and CpG as described above. After culture for 3, 6 and 24 hours, supernatants were collected, and the cytokine concentration was determined by immunoassay. Commercially available ELISA kits were used for the detection of IL-12p70, TNF, IL-10 (eBioscience; Ready-Set-Go) and IFNβ (PBL supplier). IFNα was measured by a sandwich ELISA with an anti-IFNα capture mAb (F18; Hycult), and a rabbit anti-IFNα polyclonal antibody (PBL supplier) followed by goat anti-rabbit HRP (Sigma-Aldrich).

### 2.11. Real-time quantitative PCR

Different cell types were stimulated with the indicated stimuli for 0, 3, 6 and 24 hours. RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany) and reverse-transcribed using oligo dT12-18 (GE Bioscience), random hexamer primers (Promega) and Superscript II RNaseH– reverse transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA was analyzed for TRIM expression by real-

time PCR assay in a 7900 Sequence Detection System using primer/probes sets (Applied Biosystems). The primer/probes were selected to detect all possible isoforms of TRIMs used in this study. In some cases (TRIM2,9,19,35) we tested primer/probes to detect different isoforms however no significant difference was observed in expression patterns of TRIMs between different isoforms. The primer/probes used in this study as well as their target exon for PCR are shown in Table 2.1. TRIM cDNA was amplified with TaqMan Universal PCR Master mix (Applied Biosystems) and expression values were normalized to HPRT1 (hypoxanthine phosphoribosyltransferase; Mm00446968_m1)(Applied Biosystems). TRIM mRNA expression for primer/probe pairs was normalized to the HPRT1 threshold value (CT value), or ubiquitin was used to normalize cytokine gene expression. Relative expression for all genes was calculated by the following empirical equation in Microsoft Excel:

Relative expression= POWER(1.8,((Ct, housekeeping gene)–(Ct, gene of interest))) * 10 000

### 2.12. Generation of heat maps for data presentation

The cycle threshold (CT) value of each TRIM was normalized by the CT value of HPRT as house-keeping gene to obtain the relative value for each condition. These values were then imported to GeneSpring GX 7.3.1 (Agilent Technologies) application. The values of each sample for individual TRIMs were normalized to the median of all samples for each gene. For data presentation, a value of 1.0 represents the median and is shown in yellow whereas high expression (relative to the median of each gene) is shown in red and low expression is shown in green. Further details in **Figure 3.7**.

### 2.13. Statistical analysis

Data from multiple experiments were analyzed by comparison to a defined control value using Dunnett's test. Analysis was performed using GraphPad Prism software (GraphPad). In defined cases, pair-wise comparison was by Student's paired *t* test. Values of $p < 0.05$ were considered significant.

### 2.14. Infectious units of Influenza virus in MDCK cells

Influenza virus PR8 (A/Puerto Rico/8/34-H1N1) and New Caledonia (A/New Caledonia/20/99-H1N1), were titrated in the susceptible Madin Darby canine kidney (MDCK) cells to determine infectious particles for each virus strain. This method is based on quantifying the cytopathic effects of the virus to distinguish viable from non-viable cells. Alamar blue provides a measurement of metabolic activity in the cell which results in the chemical reduction of Alamar blue from the oxidized state (blue, non-fluorescent) to the reduced state (red, fluorescent). Adherent MDCK cells were grown in a flask in IMDM medium containing 5% FCS. When cells reached a confluent state, then cells were washed with PBS and Typsin-EDTA was added and incubated at 37°C until detached. Cells were washed once with IMDM and re-suspended in a small volume for cell count. Cells were then adjusted to a density of $1x10^5$ cells/ml. Cells were plated (40µl=4000 cells) in a 96-well plate with 50 µl of IMDM and 10 µl of virus in 1:10 serial dilutions starting with undiluted sample. Cells were incubated for 3 days at 37°C and then the medium was removed by flicking the plate, washed once with 100 µl of IMDM followed by addition of 100 µl per well of IMDM containing 1x Alamar blue. The plates were transferred to an incubator at 37°C with 5% $CO_2$ for 1-2 hr. Alamar blue stained plates were loaded

into a Safire2 microplate reader (Tecan, Switzerland) and the fluorescence was measured with settings of 530 nm excitation and 590 nm emission wavelengths. The number of viral infectious units was expressed as tissue culture infectious dose ($TCID_{50}$) titre according to the method of Reed-Muench as described in [268]. The $TCID_{50}$ infectious unit were PR8 = $10^{4.8}$ /ml and New Caledonia = $10^{3.7}$/ml (see **Figure 2.3**). It is important to note that this protocol may not yield reliable values for virus titre. The protocol measured viable virus but probably only as a single cycle of infection and so the $TCID_{50}$ value could not be interpreted as typical infectious units of virus. Inclusion of trypsin into the medium would have allowed multiple cycles of replication and more typical units of infectivity.

### 2.15. Phylogenetic analysis of TRIM proteins

The amino acid sequences of all mouse TRIM proteins reported up to date were obtained from the Mouse Genome Informatics database (62 TRIM proteins available in this database)(http://www.informatics.jax.org). Amino acid alignments of the full length protein sequence of all TRIMs were obtained using the online ClustalW server (http://www.ebi.ac.uk/clustalw) [269]. Phylogenetic analysis and neighbour joining bootstrap analysis was performed on the amino acid alignment using the NJplot setting (from http://www.informatics.jax.org) and 500 replications. The same analysis was performed with all TRIMs not used in this study. Because a high number of TRIM proteins contain the B30.2 domain and it has been suggested that these domain may be a hotspot for evolutionary selection [28], [216] [270], we repeated the analysis described above using the protein sequence of the RBCC motif without the B30.2 domain or any other c-terminal sequence to the RBCC motif.

| Gene Title | assay ID chosen | PCR location(exon) | mouse chr |
|---|---|---|---|
| **Trim1/mid2** | *Mm00449285_m1* | *Exon 7-8* | X |
| **Trim2** | *Mm00453149_m1*<br>*Mm01219623_m1*<br>*Mm01219626_m1* | *Exon 1- 2*<br>*Exon 10-11*<br>*Exon 6-7* | 3 |
| **Trim3** | *Mm00803844_m1* | *Exon 8-9* | 7 |
| **TRIM6** | *Mm01273446_m1* | | 7 |
| **Trim8** | *Mm00474107_m1* | *Exon 3-4* | 19 |
| **Trim9** | *Mm01256267_m1*<br>*Mm01256265_m1* | *Exon 1-2*<br>*Exon12-13* | 12 |
| **Trim11** | *Mm01347817_m1* | *Exon 5-6* | 11 |
| **Trim12** | Mm00844231_s1 | Exon 2-2 | 7 |
| **Trim14** | *Mm01352552_m1* | *Exon 3-4* | 4 |
| **Trim16** | *Mm00459724_m1* | *Exon3-4* | 11 |
| **Trim18/mid1** | *Mm00839791_m1* | *Exon 6-7* | x |
| **Trim19/PML** | *Mm00476969 m1*<br>*Mm00476972_m1* | *Exon 1-2*<br>*Exon 4-5* | 9 |
| **Trim20/Mefv** | *Mm0040258_m1* | *Exon 1-2* | 16 |
| **Trim21/Ro52** | *Mm00447364_m1* | *Exon 5-6* | 7 |
| **Trim23** | *Mm00659668_m1* | *Exon 9-10* | 13 |
| **Trim24** | Mm01136963_m1 | Exon 17-18 | 6 |
| **Trim25/EFP** | Mm01304224_m1 | Exon 3-4 | 11 |
| **Trim26** | *Mm00499696_m1* | *Exon 5-6* | 17 |
| **Trim27** | Mm01136028_m1 | *Exon 7-8* | 13 |
| **Trim28** | *Mm00495594_m1* | *Exon 3-4* | 7 |
| **Trim30** | *Mm01274264_m1* | *Exon 5-6* | 7 |
| **Trim34** | *Mm00504218_m1* | *Exon1-2* | 7 |
| **Trim35** | *Mm00504120_m1*<br>*Mm01313806_m1* | *Exon1-2*<br>*Exon5-6* | 14 |
| **Trim37** | *Mm01307054_m1* | *Exon 21-22* | 11 |
| **Trim39** | *Mm00452564_m1* | *Exon 3-4* | 17 |
| **Trim44** | *Mm00522313_m1* | *Exon4-5* | 2 |
| **Trim45** | *Mm01304706_m1* | *Exon 3-4* | 3 |
| **Trim46** | *Mm01212757_m1* | *Exon 7-8* | 3 |
| **Trim59** | *Mm02527285_s1* | *Exon 3-3* | 3 |
| **Trim65** | *Mm01252954_m1* | *Exon 1-2* | 11 |
| **Trim68** | *Mm01165530_m1* | *Exon 1-2* | 7 |
| **HPRT1** | Mm00446968_m1 | | |

**Table 2.1. Primer-probes (Applied biosystems) and their PCR location used in this study**

Pre-design ABI primer-probes were chosen based on: 1) TRIMs known to be expressed in mice, 2) TRIM expression profile in T cells by Affymetrix, 3) TRIM reported in the literature to have some function in response to viral infections or immune response, 4) Chromosomal location, sequence homology and subfamilies.

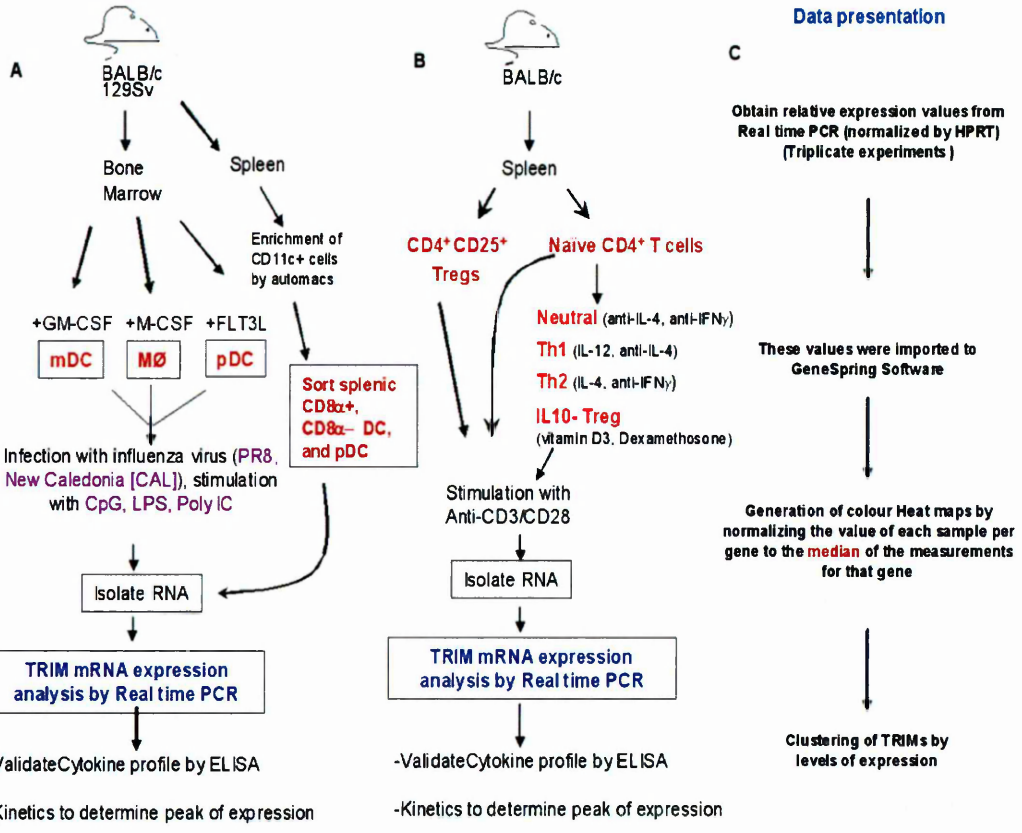



**Figure 2.1. Flow chart representation of the experimental approach used in this study.** **A)** Generation, stimulation and real time PCR analysis of macrophages and DC populations. **B)** Generation, stimulation and real time PCR analysis of CD4 T cell subsets. **C)** Strategy used to generate Heat maps from real time PCR values for data presentation.

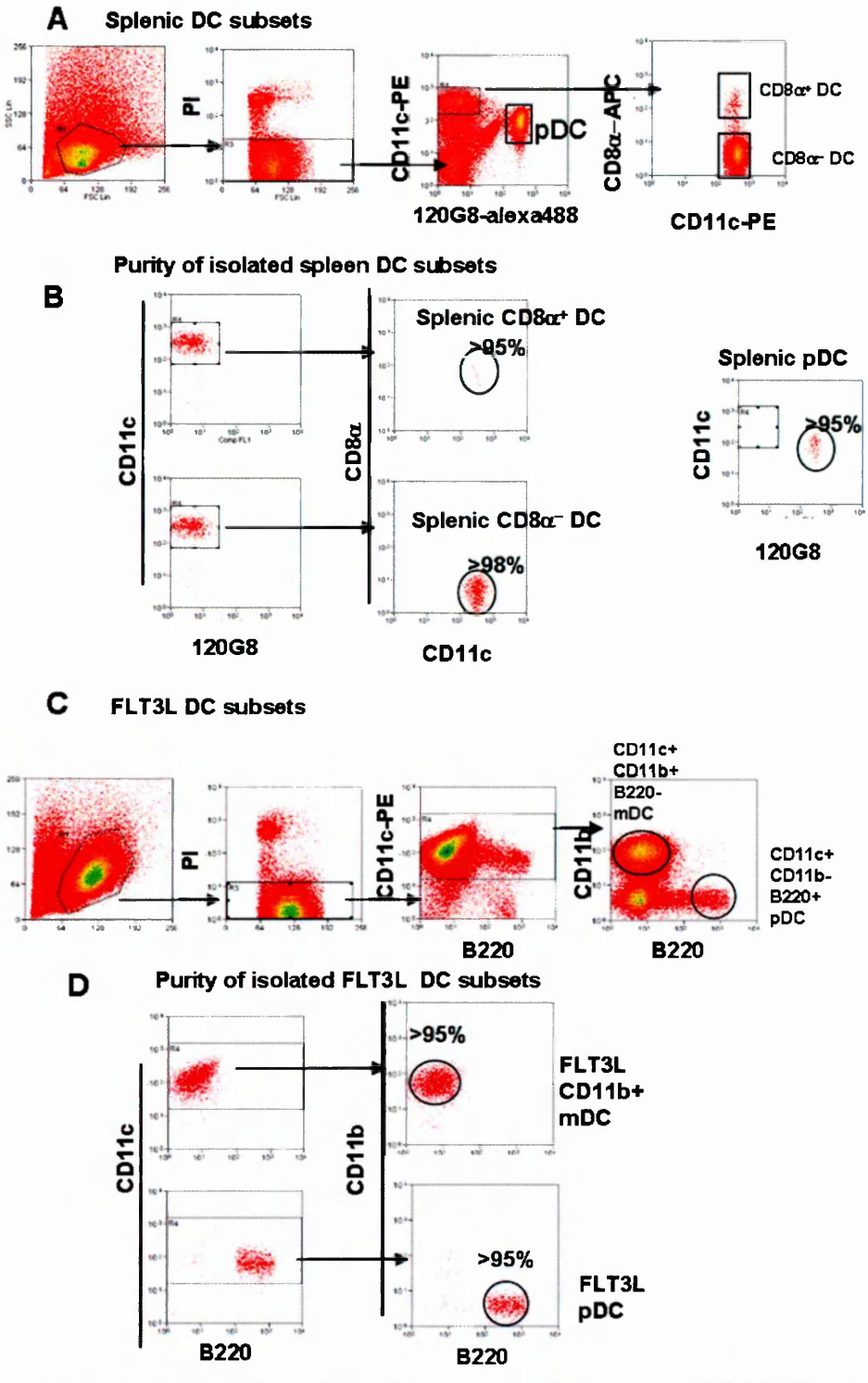



**Figure 2.2. Purification of splenic and plasmacytoid precursor DC (pDC) subsets by Flow cytometry and re-analysis**

**A)** Purification of splenic DC subsets by Flow cytometry. An example of FACS sort profile. The populations selected for purification are indicated in boxes. **B)** A typical post-sort re-analysis of the isolated CD8a+ DC, CD8a-DC and pDC populations. **C)** Purification of BM-FLT3L derived DC subsets by Flow cytometry. After 10 days cultured in FLT3L cells were purified by flow cytometry and an example of FACS sort profile of the selected populations is shown in boxes. **D)** A typical post-sort re-analysis of the isolated mDC (CD11b+) or pDC populations.

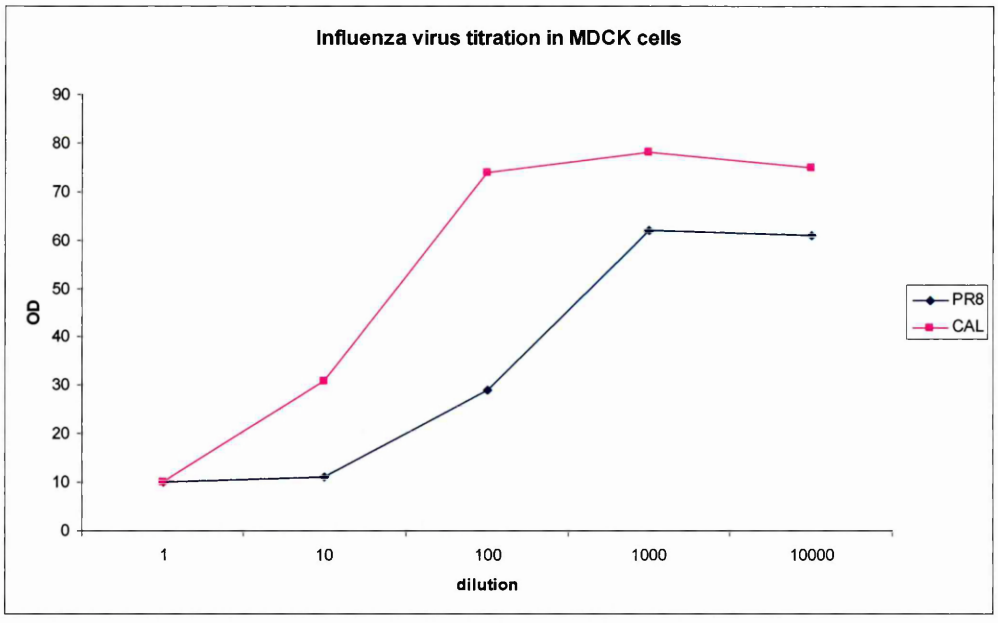



**Figure 2.3. Determination of Infectious units of Influenza virus in MDCK cells**
Influenza virus PR8 (A/Puerto Rico/8/34-H1N1) and New Caledonia (A/New Caledonia/20/99-H1N1), were titrated in the susceptible Madin Darby canine kidney (MDCK) cell line. The viruses were added in serial 1:10 dilutions starting with the equivalent to 100 HA units. The number of viral infectious unites was expressed as tissue culture infectious dose (TCID50) titre according to the method of Reed-Muench as described in [268]. The TCID50 infectious unit were PR8 = $10^{4.8}$ and Cal = $10^{3.7}$.

It is important to note that this method does not give an accurate measurement of the typical viral infectious units since trypsin was not added to the medium and therefore viable virus is detected only as single cycle of infection.

# Chapter 3:

# Results (I)

## Development of strategies for expression profiling of TRIM family members in primary cells of the immune system

## Chapter 3: Results (I)

### 3.1. Background: strategy for the analysis of TRIM expression in primary cells of the immune system.

The conserved features of the tripartite motif, and the large number of molecules containing this domain structure, strongly suggest that this motif has a distinct and essential basic function. TRIMs have been shown to be involved in very diverse functions including anti-viral functions and regulation of cytokine signalling. This has led to the suggestion that the basic common function of TRIMs may be related to the innate immune system [22]. This also begs the question as to whether more TRIMs remain to be discovered with functions related to immunity. To obtain information which would help to design studies or strategies to address this question, we set up a system to study whether TRIMs are differentially expressed in different primary immune cell subsets and whether their expression may be induced and/or regulated by specific cytokines that they produce. To date, there are no studies addressing systematically the expression or the role of TRIMs in the immune system. Thus, we have chosen to follow a global systematic approach to study the TRIM family of proteins to gain information as to their expression and potential function in immune cells. Broad expression studies using primary cells that can be purified to homogeneity are useful because they provide information in a biological system that represents close to physiological conditions. To do this it was necessary to first develop adequate systems for data analysis to be able to handle the large amount of information generated in such expression studies as presented in this thesis. The different cells of the innate and adaptive immune system chosen for our study have different effector functions during immune responses partially due to the cytokines they produce, thus knowledge of TRIM expression in these cells may help to give

information with respect to their regulation to subsequently study their potential functions.

### 3.1.1. TRIM expression by microarray analysis in CD4 T cell subsets

We studied six distinct primary $CD4^+$ T cell populations (naïve CD4+ T cells, neutral (cultured with anti-IL-4 and anti-IFNγ) , Th1, Th2, IL-10-Treg and CD25+Treg, which were described in section 1.5.2 and summarized in **Figure 1.5**) and produce different profiles of cytokines upon stimulation [266, 271]. Using previous data generated in our lab using an Affymetrix approach [266, 271], we now examined the expression profile of TRIMs in these six distinct primary $CD4^+$ T cell populations. As shown in **Figure 3.1**, we detected differential expression of TRIMs in T cell effector subsets after 6 hours stimulation with anti-CD3/CD28 (through T cell receptor signalling)(**Figure 3.1**). Some TRIMs were specifically expressed in unstimulated cells while others were induced upon stimulation. Conversely, some TRIMs were down-regulated upon stimulation (**Figure 3.1**).

### 3.1.2. TRIM expression by real time PCR analysis in CD4 T cell subsets

To gain additional information on TRIM expression in CD4 T cells, we used real time-reverse-transcription PCR (RT-PCR) to examine TRIM expression in these CD4+T cell subsets. To first ensure that analysis of TRIM expression would give comparable results to the data obtained by microarray, we obtained pre-designed primer-probes for RT-PCR, selected based on the data obtained by microarray analysis. Selected TRIMs that showed detectable differential expression in these cell subsets by microarray analysis are shown in **Figure 3.2A**. Expression analysis of these TRIMs by RT-PCR (**Figure 3.2B**) correlated for the most part with the

expression profile by microarray analysis, and confirmed that these TRIMs were selectively expressed in these cell subsets. TRIM21,14,26,39 showed high expression in unstimulated naïve CD4 T cells and CD25Tregs by microarray analysis (**Figure 3.2A**), and the expression of TRIM21,14,26 but not TRIM39 was down-regulated upon stimulation (**Figure 3.2A, B**), although down-regulation of TRIM39 was more clearly shown by RT-PCR analysis (**Figure 3.2B**). Expression of TRIM1 was selectively up-regulated only in Th2 and IL-10Treg upon stimulation as shown by microarray analysis and RT-PCR (**Figure 3.2A, B**). As it has been previously reported, Th2 cells produce high levels of the cytokines IL-4, IL-5, IL-10, and IL-13, but IL-10Treg only produce high levels of IL-10 [271], suggesting a possible correlation of IL-10 expression with TRIM1 expression in these cells. TRIM16 and TRIM46 were expressed in unstimulated Th2 and IL-10Treg and down-regulated upon stimulation as observed by both microarray and RT-PCR (**Figure 3.2A, B**). TRIM2, 9 were highly expressed in unstimulated IL-10Treg as observed by microarray and RT-PCR and upon stimulation their down-regulation was observed by RT-PCR but not as clearly by microarray (**Figure 3.2A, B**). These data obtained by two different methodologies confirmed differential expression of distinct TRIMs in CD4+T cell subsets. Although both methodologies gave similar results, it appeared that the RT-PCR based method may show greater sensitivity and a greater ability to discern differential expression.

### 3.1.3. TRIM transcript variants

The Affymetrix GeneChip is designed to contain 11 specific probes that span the full length of the transcript for each gene measured. In the microarray experiments we found that, in some cases, different probes for a specific TRIM

transcript appeared to give different results. This could be due to different sensitivities of the probe present in the GeneChip, or possibly due to differential expression of transcript variants in the same cell subset. Since some TRIM genes have been reported to encode transcript variants, we sought to examine if it is possible to detect differential expression of TRIM transcript variants in the T cell subsets by microarray and RT-PCR. Having established that RT-PCR provided consistent and comparable data to that obtained by microarray, with the additional advantage of higher sensitivity, we tested the possibility of differential expression of TRIM variants in these T cell subsets. For this, we selected some of the TRIMs whose expression was detected at different levels depending on the probe examined in the Affymetrix chip. For example, TRIM9 mRNA was detected at high levels by a probe in the Affymetrix chip for exon 10 of the TRIM9 gene in unstimulated IL-10Treg and in stimulated IL-10Treg and Th2 cells (**Figure 3.3A**). However, a probe spanning the exon 12-13 junction did not result in any detectable levels of TRIM9 in this unstimulated IL-10Treg subset and only low levels were observed upon stimulation in Th2 cells but not in IL-10Treg (**Figure 3.3A**), suggesting a possible differential expression of TRIM9 variants.

To test if this difference was due to real expression of different transcript variants, we obtained pre-designed primers-probes for RT-PCR specific for the same variants observed in the GeneChip. To achieve this, we first searched the Genebank database for reported sequences of mRNA variants for TRIM9, and found that the longest transcript variant is composed of 14 exons. This transcript variant can be detected with pre-design primers for RT-PCR specific for exon junctions 12-13, as well as with the same probe in the GeneChip (see **Figure 3.3B** for schematic representation). A shorter variant of TRIM9 containing only 10 exons has also been

reported in the GeneBank database. Thus, this variant could potentially be recognized with the GeneChip probe for exon 10 as well as with a pre-designed primers-probe for RT-PCR specific for exon junctions 1-2 (**Figure 3.3B**).

Expression of TRIM9 variant 1 by RT-PCR in the different CD4+T cells subsets (at 0 and 6 hours) showed high levels of expression in unstimulated IL-10Treg and a slight decrease upon stimulation, which correlated with the data obtained by microarray. Moreover, detectable levels of this variant were observed in Th2 cells upon stimulation, which also correlated with the expression pattern observed by microarray analysis (**Figure 3.3C**). However, high levels of TRIM9 variant 2 were detected in IL-10 Tregs by RT-PCR whereas we were unable to detect any expression of this variant by microarray analysis (compare **Figure 3.3A and C**). Therefore, we could not confirm differences in the expression of TRIM transcript variants using these techniques. This is most probably due to the higher sensitivity obtained by RT-PCR. This indicates that the analysis of gene expression by these two technologies may some times give different data although it suggests that the RT-PCR approach may be more robust. Therefore, it is important to pay close attention when analysis data by microarray analysis.

Of note, because of differences in the sequences of each primer-probes and genes, and the different GC content found in each of these sequences which can affect the sensitivity of detection, it is only possible to compare different samples for the same gene but it is not possible to compare expression levels between different genes (i.e. comparing different primer-probes). We ensured that in future experiments we used RT-PCR primer-probes that detect all possible variants for each of the TRIMs reported in the Genebank database. In conclusion, RT-PCR offers a

more reliable, sensitive technique although it is more demanding and time consuming.

### 3.1.4. Selection of primer-probes for TRIM analysis by real time PCR in immune cells

The data obtained by RT-PCR confirmed the differential expression of TRIMs in CD4+ T cell subsets (**Figure 3.2**), perhaps with greater sensitivity and precision, and revealed some TRIMs with interesting patterns of expression in these cell subsets. In addition, with the advantage of increased sensitivity and greater ability to discern differential expression (signal to noise ratio) by RT-PCR, we could obtain additional information with respect to TRIM gene expression that was not possible to observe by microarray analysis. We therefore expanded our study to include cells of the innate immune system, macrophages and DC, which also have different effector functions in part due to the cytokine profiles they produce. In addition we extended our study to examine additional TRIMs that may not have been detected by microarray analysis in T cells and selected additional TRIMs based on their reported putative functions in immunity and in anti-viral responses. We included here representative TRIMs from the different TRIM subfamilies and representatives located in clusters on different chromosomes (**Figure 3.4**). There are 62 mouse TRIMs described to date (mouse genome informatics website: http://www.informatics.jax.org) from which we chose 29 TRIMs that represent different subfamilies based on their domain composition and organization (shown with an asterisk on **Figure 3.4**, described in section 1.3 and shown in **Figure 1.2**; FN3, B30.2, PHD/BROMO, NHL, MATH, *ARF* domains) [22, 38]. Protein sequence homology and phylogenetic analysis of all mouse TRIMs revealed clusters or groups

of TRIMs sharing high degree of homology that can be found on chromosome (chr) 3, 7, 8, 11, and chr-17 (**Figure 3.4**). This is in agreement with previous phylogenetic studies on human and primate TRIM protein sequences [272] and supports previous suggestions that the TRIM motif may have evolved by gene duplications from a common ancestral gene [272, 273].

With this broad selection of representative TRIM proteins chosen for our study we set out to established time points of peak of expression by RT-PCR for each TRIM gene in different CD4+T cell subsets, and in addition in macrophages and DC, as outlined below.

### 3.1.5. Kinetics of TRIM expression in CD4+Tcells, macrophages and DC

To further gain insight into the expression patterns of TRIM proteins in different cells of the immune system we included in our studied macrophages, mDC and pDC, known to produce different patterns of cytokines upon different stimulation (explained in the introduction, section 1.5.1.2). As part of the innate immune system, macrophages and DC have different effector functions in part due to the cytokines that they produce. DCs detect pathogens, process and present antigens to T cells, thus initiating the adaptive immune response [113, 114]. On the other hand, macrophages are important in the early stages of the innate immune response by producing cytokines and molecules such as Nitric oxide (NO) to kill pathogens [111]. Therefore we hypothesized that a comparison of TRIM mRNA expression between macrophages, DC and the different CD4+ T cell subsets may reveal differential expression of TRIMs, and consequently, may provide information to help design studies to delineate their functions during immune responses.

We first performed kinetic experiments to determine the peak of TRIM expression by RT-PCR in the different cell subsets. For the differentiated CD4+ T cells described above and in the materials and methods, we collected samples at different time points after stimulations (0, 3, 6 and 24 hours) with anti-CD3/CD28 and analyzed their expression profile by RT-PCR. We first ensured that these cell subsets produced the expected cytokine profiles. It is well known that Th1 cells produce IFNγ [116, 117], whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13 [118, 119]. IL-10Tregs produce only high levels of IL-10, whereas naïve cells produce IL-2 and cells grown in neutral conditions produce IL-2 and TNF [271]. As a quality control for the respective cells, we show the expected Th1 hallmark cytokine IFNγ or the immunoregulatory IL-10 (shown in **Figure 3.5A**) and the full description is in [271]. As expected, IFNγ was exclusively produced by Th1 cells and only very low level of expression was observed in neutral T cells and IL-10Tregs. Conversely, IL-10 was highly expressed in Th2 and IL-10Tregs with only low levels in Th1 cells (**Figure 3.5A**) confirming the purity and the effector signature expected of these cell populations. In terms of TRIM expression in these CD4+T cell subsets, most of the TRIMs that were up-regulated reached the peak of expression at 6 hours (**Figure 3.5B**) and this data confirmed the results obtained with the microarray experiment.

We then analyzed TRIM expression in macrophages and mDC stimulated at different time points after stimulation with LPS and CpG (ligands for TLR4 and TLR9 respectively). Additionally, pDC were stimulated with CpG and inactivated influenza virus (ligands for TLR9 and TLR7 respectively). These TLR ligands also induce different patterns of cytokine expression. Macrophages are known to produce high levels of IL-10 and TNF upon stimulation with CpG or LPS, but not IL-12p70

[160]. Myeloid DC (mDC) also produce IL-10 and TNF upon CpG and LPS stimulation, and low levels of IL-12p70 [160]. pDC produce no detectable IL-10 upon stimulation with CpG, but are known to produce high levels of type-I IFNs (including IFNα and IFNβ), IL-12p70 and TNF [160]. We first verified their cytokine profiles, and as an example mRNA expression of TNF and IL-10 is shown in **Figure 3.5C**. Notably, IL-10 was not expressed in pDC upon CpG and virus stimulation whereas significant induction of IL-10 mRNA was observed in macrophages and to a lesser extent in mDC upon CpG and LPS stimulation. Conversely, TNF was induced in all cell types with the highest levels achieved by pDC stimulated with CpG (**Figure 3.5 C**). To confirm the viability and purity of the pDC subsets we also measured IFNα protein by ELISA. **Figure 3.5D** shows that pDC are able to produce IFNα upon influenza virus or CpG stimulation confirming the known phenotype of these cells [274], whereas no IFNα was detected in macrophages and mDC.

In terms of TRIM expression, we observed that a large number of TRIMs were up-regulated in macrophages and mDC and the expression of most of them reached a peak at 24 hours (**Figure 3.5E**). Similarly, most of the TRIMs that show up-regulation of expression in pDC also reached a peak of expression at 24 hours (**Figure 3.5F**), suggesting a possible autocrine effect by cytokine signalling. To compare the relative mRNA expression of the TRIMs we had selected in the different cell types, we chose the peak of mRNA expression for T cells (6 hours), macrophages and DC (24 hours).

### 3.1.6. Approach used for generation of colour heat maps of TRIM expression in T cells, macrophages and DC

Comparison of TRIM mRNA expression from RT-PCR analysed by standard techniques between the different cell types was complicated and difficult to interpret. **Figure 3.6** shows the histograms of TRIM expression in the different cell subsets by individual TRIM genes. Although some differences could be observed in the expression levels of some TRIMs between cell types, the large amount of data obtained made it difficult to observe correlations and also made assimilation of data almost impossible. Complex and large data sets unmanageable by standard techniques can be readily assimilated by generation of heat maps that represent levels of expression. To better observe TRIM expression patterns and correlate groups of TRIM expression with cytokine production in the different cell subsets in a way that could be more readily assimilated, we generated heat maps to represent TRIM mRNA expression by importing the values obtained from RT-PCR to the GeneSpring software. The values of individual TRIM gene expression were normalized to the median of all samples for each gene. The median is shown in yellow; high expression relative to the median is shown in red and low expression in green. As examples, TRIM9 and TRIM65 expression values are represented by histograms and also translated to heat maps in **Figure 3.7**. This shows the values of TRIM mRNA detected at the peak of expression (6 hours for T cells and 24 hours for macrophages/DC - determined in the previous kinetic experiments). Analysis of the differential expression of TRIMs using this approach of colour heat maps will be shown and discussed in the next chapter.

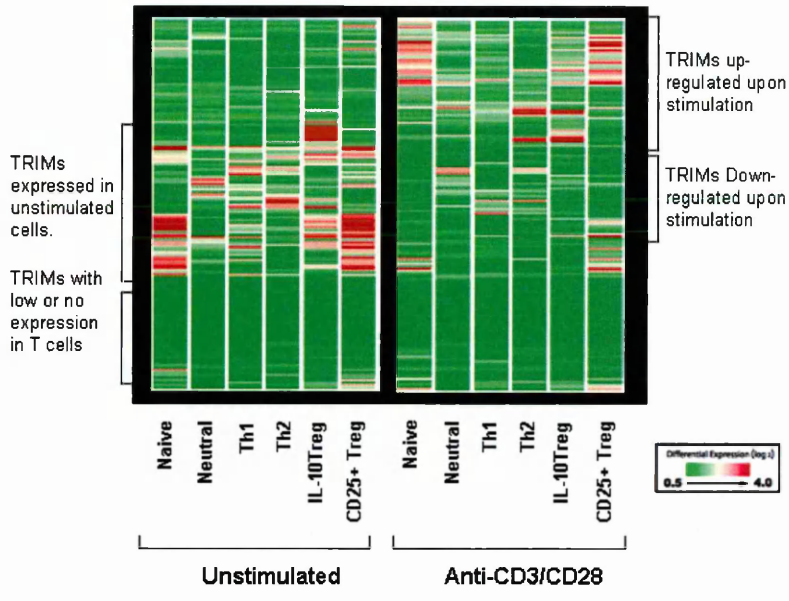
### 3.2. Discussion

Using microarray analysis we have shown differential expression of a number of TRIMs in six different CD4+T cell subsets that have different effector functions partly due to the different cytokine profiles they produce. We have also shown in this section that data of TRIM expression obtained by RT-PCR confirms the expression data obtained by microarray analysis, and this gives a level of confidence that the pre-designed primer probes chosen for PCR give reliable and reproducible results. In addition, using RT-PCR we were able to detect TRIM expression that was not observed by microarray analysis providing a more sensitive system for TRIM mRNA expression analysis and a greater ability to discern different levels of expression. This was highlighted by the fact that possible TRIM transcript variants were not detected by the use of specific probes in the GeneChip, while the same transcript was detected with the use of RT-PCR primer/probes (e.g. TRIM9, **Figure 3.3**). This is important, since many TRIM genes have been reported to express spliced transcript variants that may lead to TRIM isoforms. Moreover, some differences in isoform specific functions have been reported for some TRIMs [15]. Therefore, to obtain real information on gene expression and potential function of a gene we need to be able to account for all possible transcript variants. For our study we ensured that the primer-probes used for each TRIM gene recognize all possible transcript variants of a specific TRIM. Although this has the disadvantage of not being able to discriminate between transcript variants, it ensures detection of any possible variant for a gene and the study of TRIM variants was not an objective of this study. Another important aspect to take into account is the fact that primer-probes differ from one another in their sensitivity. Therefore PCR data obtained using different primers can not be

compared to each other since PCR amplification with different primers leads to different amplification efficiencies [275].

Having established an RT-PCR method to detect TRIM expression in immune cells we performed kinetics experiments of TRIM expression in the different T cell subsets, macrophages and DC and observed that, for the most part, expression of TRIMs that were up-regulated in T cells show an earlier peak of expression (6 hours) as compared to macrophages and DC (24 hours). Moreover, only a limited number of TRIMs showed up-regulation of expression in T cells and these TRIMs were different to the ones observed in macrophages/DC. This may suggest that the mechanism of induction of TRIM expression is different in T cells as compared to macrophages/DC, or could be explained by intrinsic differences between these cell types.

Using the method of RT-PCR for analysis of mRNA at the peak time of expression (6 hours for T cells; 24 hours for macrophages/DC) we obtained a large and complex set of data which was unmanageable by ordinary histograms. Thus we designed an approach to deal with this large amount of information by generation of heat maps of expression using the GeneSpring software. We now examined TRIM expression in all these cell types (six CD4+T cell subsets, macrophages, mDC and pDC) and upon different appropriate stimulations (CD3/CD28 for T cells; LPS and CpG for macrophages/mDC; CpG and influenza virus for pDC) to compare TRIM expression and determined whether there was any correlation of these data with cytokine production. This will allow us to observe patterns of TRIM expression as will be shown in the next chapter.




**-Microarray performed by John Shoemaker.**
**-TRIM Data analysis performed in this study (R. Rajsbaum).**

**Figure 3.1. Expression profile of TRIM proteins in CD4+ T cell populations by Microarray analysis.**
A GeneChip profile for TRIM expression in unstimulated CD4+ T cells or upon 6 hr stimulation with anti-CD3/CD28 (TCR pathway). The different cell populations were isolated or derived in culture as described in materials and methods. The GeneChip image was analyzed with GeneSpring software with the GC-RMA algorithm and values were normalized per chip to the median value of all chips and further normalized by the median of all genes (log2). Green represents genes under-expressed as compared to the median value. White represents the median value and red represents over-expression to the median value. All preparation of the microarray was performed by John Shoemaker [266] and the TRIM data was analyzed by myself in this study.

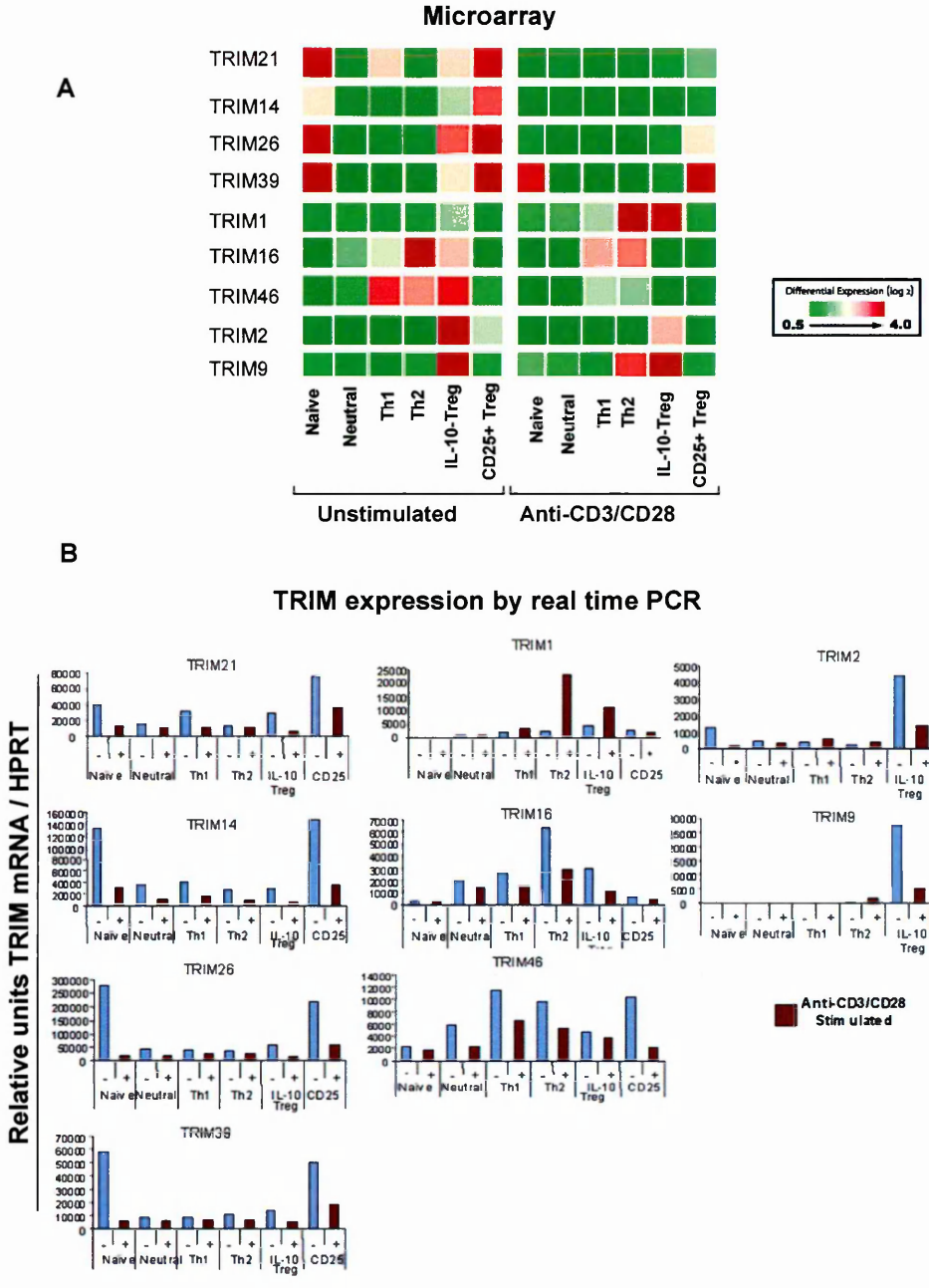



**Figure 3.2. Real time PCR data correlates with microarray analysis of TRIM expression.**

**A)** TRIM expression in different T cell subsets by microarray analysis. Selected TRIMs with high levels of expression are shown. **B)** Validation of TRIM mRNA expression in the different T cell subsets by reverse-transcription, real time PCR (RT-PCR). CD4+ T cells were either unstimulated (blue bars and - symbol) or stimulated for 6 hr with anti-CD3/CD28 (red bars and + symbol) and the Relative values (RU) of TRIM expression normalized to HPRT are shown.

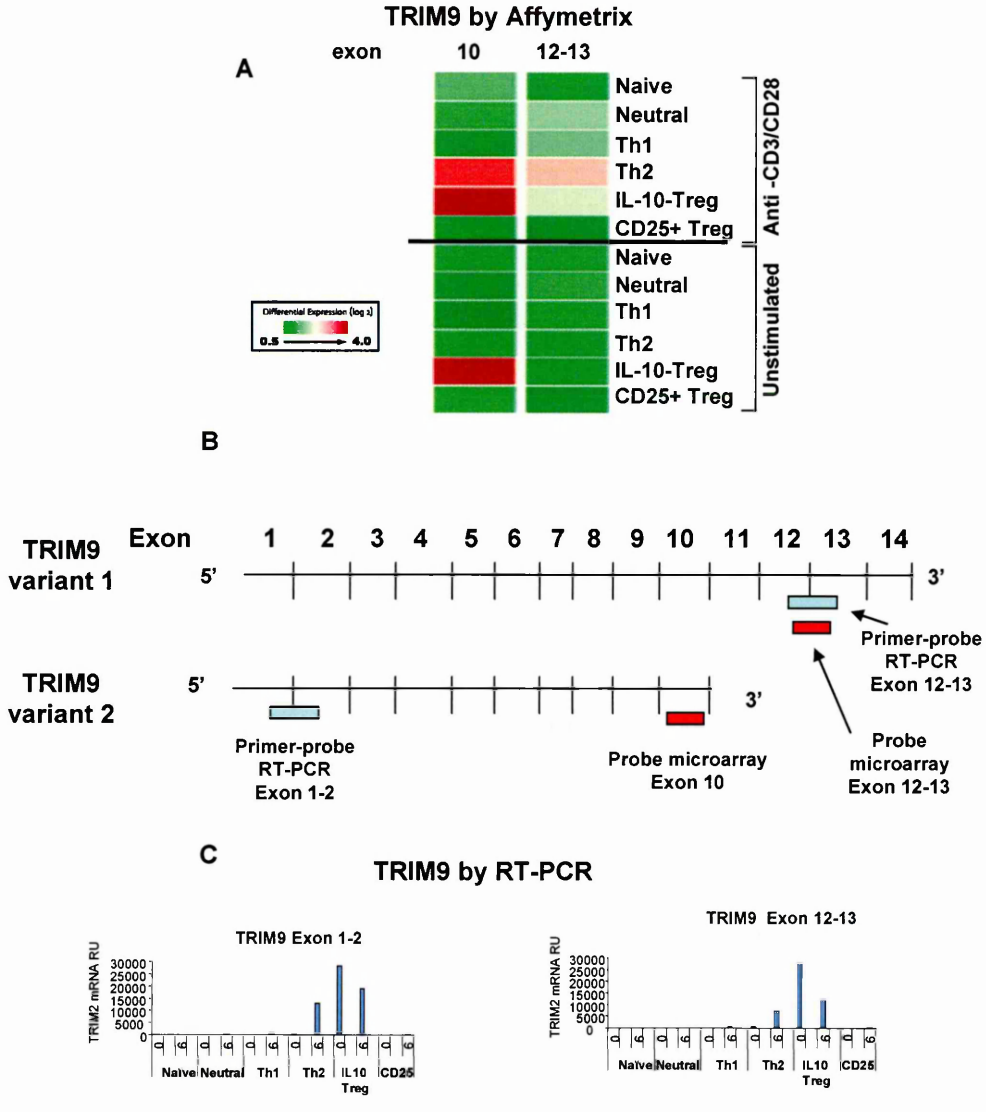



**Figure 3.3. TRIM transcript variants by microarray analysis and real time PCR.**
**A)** TRIM expression in different T cell subsets by microarray analysis. The Affymetrix chip contains probes that bind to specific exons of the TRIM transcripts giving possible information on expression of transcript variants. The different exons recognized by the probe in the chip are shown. **B)** Schematic representation of 2 different transcript variants for TRIM9 reported in the GeneBank. The full length variant 1 contains 14 exons, whereas the shorter variant 2 contains only the first 10 exons. The regions recognized by either the Affymetrix probes or the real time PCR primer-probes are indicated. The primer probe for exon 1-2 by real time PCR, recognizes the same transcript variant as exon10 by Affimetrix. The primer/probe for TRIM9 exon12-13 by RT-PCR recognizes the same variant as TRIM9 exon12-13 by microarray. **C)** TRIM mRNA expression of possible transcript variants expressed in the T cell subsets by real time PCR (RT-PCR). CD4+ T cells were either unstimulated (0) or stimulated for 6 hr with anti-CD3/CD28 (6) and the Relative values (RU) of TRIM expression normalized to HPRT are shown.

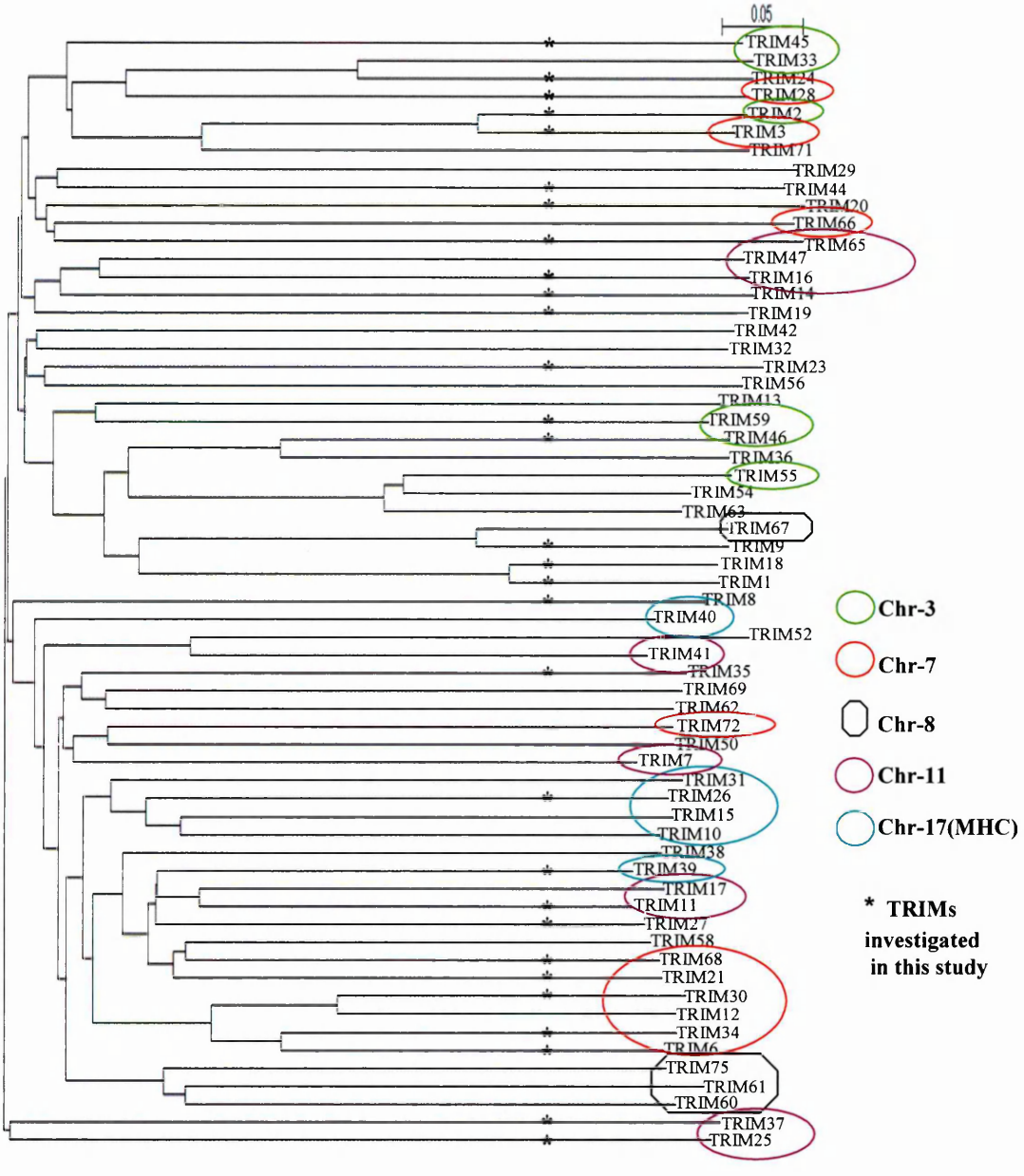



**Figure 3.4. Mouse TRIM family members by chromosomal location.**
A neighbour-joining tree based on the amino acid sequences of all full-length mouse TRIMs is shown. The scale represents 0.05 changes per site. The TRIMs located on different chromosomes (chr) are indicated in circles. TRIMs used in our study are indicated with a star. In addition to the TRIMs selected based on their expression in T cells by microarray analysis, we expanded our study and selected TRIMs that represent different subfamilies based on their domain composition and organization, protein homology, chromosomal location, and potential function as reported in the literature.

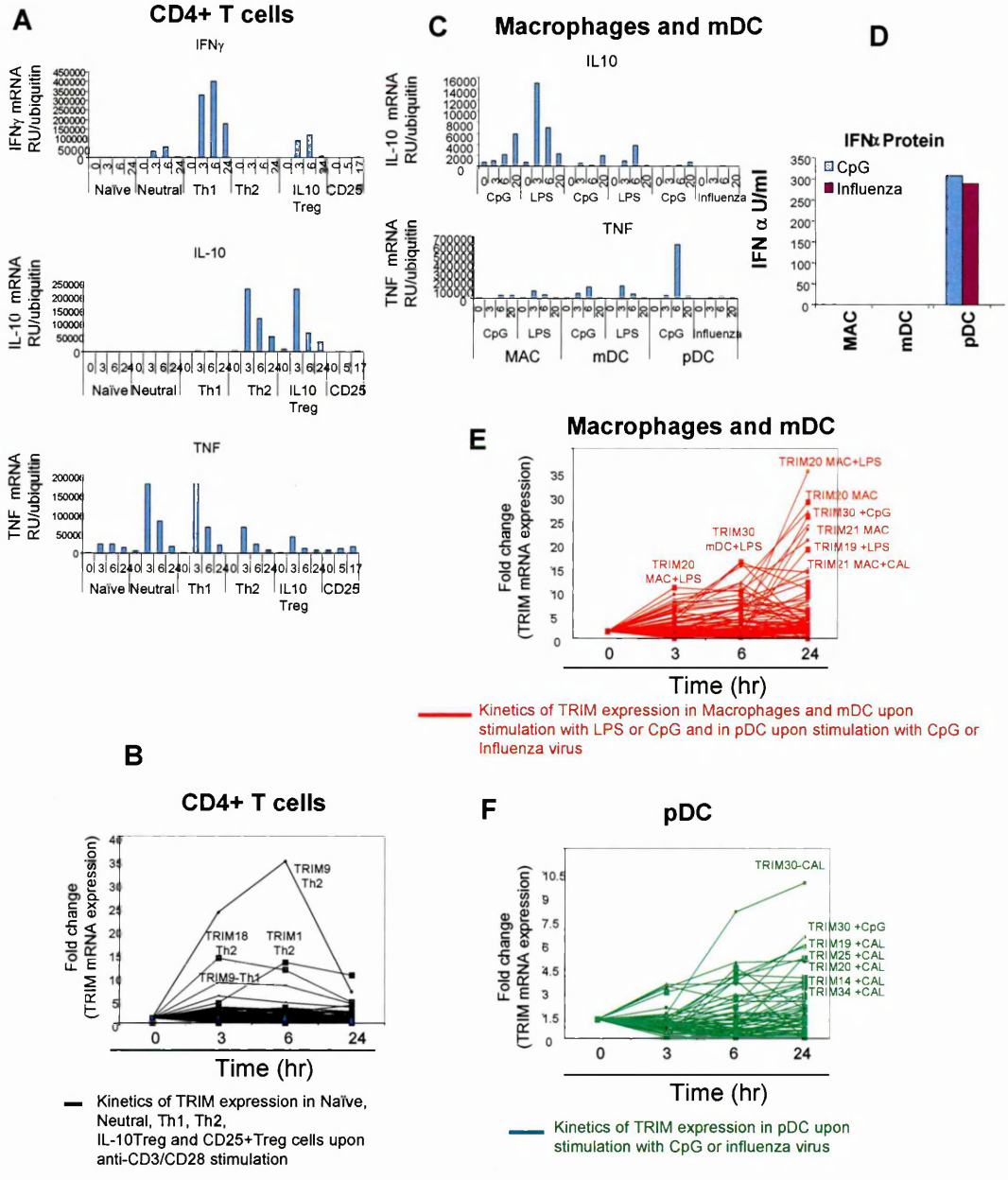



**Figure 3.5. Kinetics of cytokine and TRIM mRNA expression in CD4+T cells, macrophages and DC populations by Real time PCR analysis.**

**A)** CD4+ T cell populations were stimulated with anti-CD3/CD28 for the time indicated and mRNA expression of IFNγ, IL10 and TNF was determined by real-time PCR using SYBR green and ubiquitin as internal control (RU/ubiquitin). **B)** TRIM expression in CD4+Tcell was determined in the same samples by RT- PCR and normalized to HPRT as internal control. These values were then normalized to the unstimulated sample to give Fold change in TRIM expression. **C)** Macrophages (MAC) and mDC were treated with CpG or LPS and pDC were treated with CpG or inactivated influenza virus for the time indicated and mRNA expression of IL10 and TNF was determined by RT-PCR as described above. **D)** IFNα protein was determined by ELISA in the supernatants of pDC treated with CpG or inactivated influenza virus for the time indicated. **E)** TRIM expression in macrophages and mDC and **F)** pDC stimulated and analyzed by RT-PCR as described.

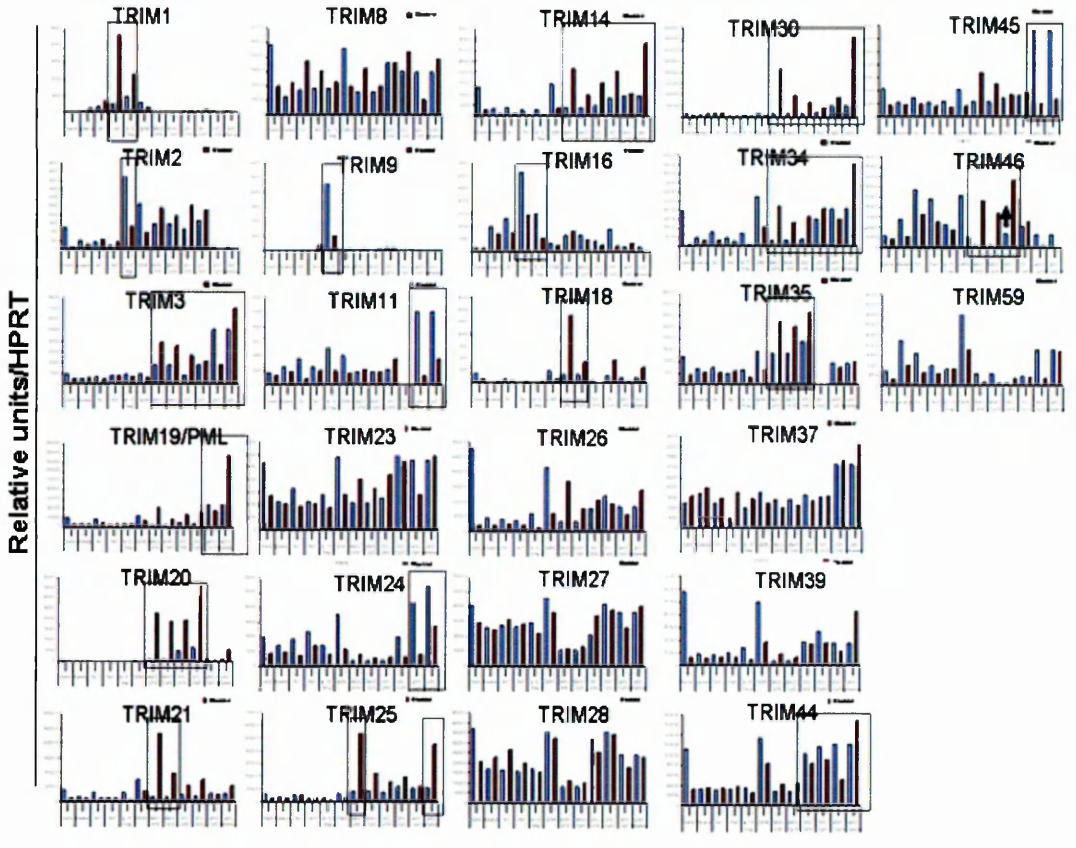



**Figure 3.6. Histograms of TRIM mRNA expression by real time PCR: difficult to interpret and observe correlations.**

Different cell types were either unstimulated (in blue) or stimulated (in red) with anti-CD3/CD28 for CD4+T cells or LPS and CpG for macrophages, mDC or CpG and inactive influenza virus for pDC. RNA was extracted and reverse-transcribed as described in material and methods. cDNA was analyzed for TRIM expression by real time PCR assay using primer/probes sets. TRIM expression values were normalized to HPRT to give relative units of expression (RU/HPRT). Samples with significantly higher levels of expression are shown in boxes. Difficult to observe correlations, therefore heat maps were generated (see Figures 3.7).

The reader is not expected to read the individual graphs. They are presented to show the difficulty of this type of graphical display.

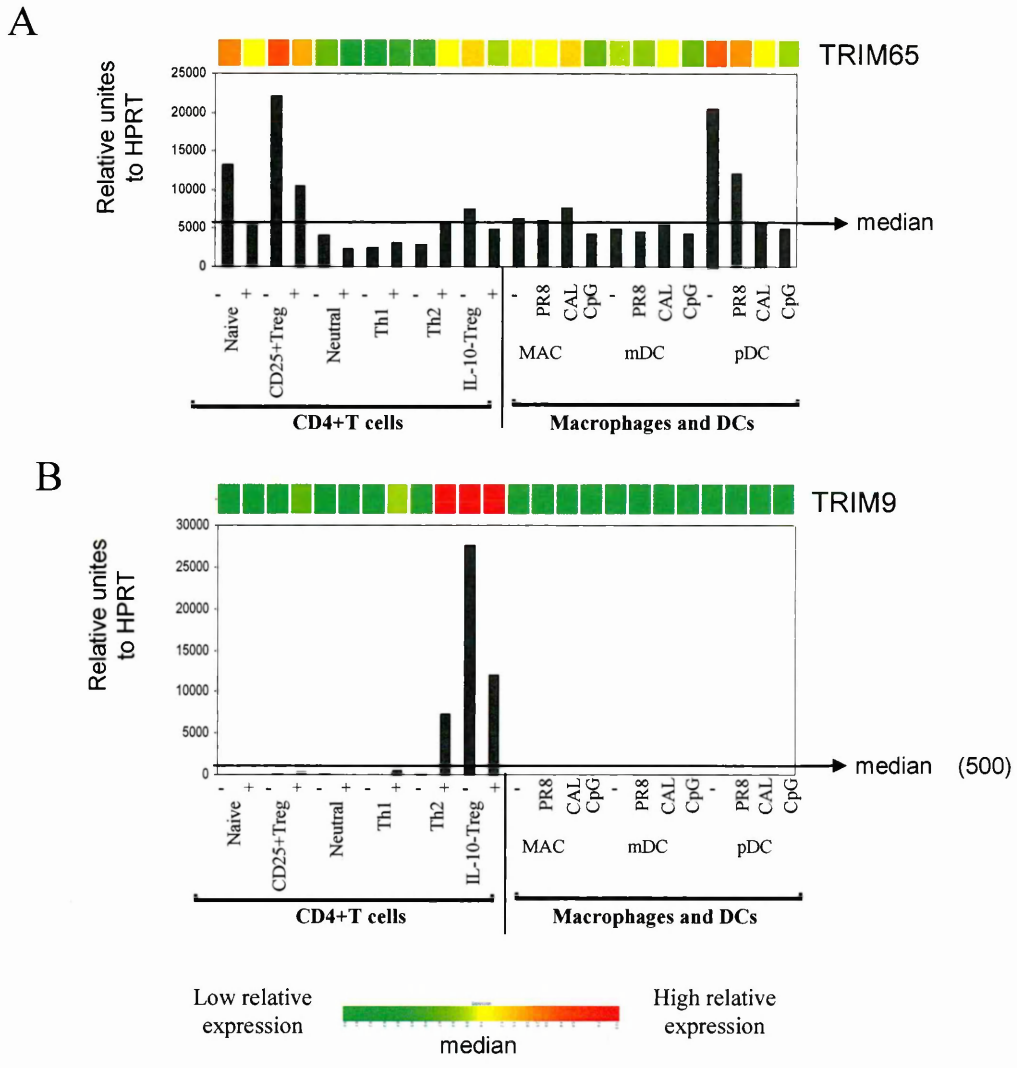



**Figure 3.7. Approach to convert data to a comprehensible format by translating histograms of real time PCR to colour heat maps.**
Different cell types were either unstimulated or stimulated with anti-CD3/CD28 for CD4+T cells or influenza virus PR/8, CAL and CpG for macrophages and DC. RNA was extracted and reverse-transcribed as described in material and methods. cDNA was analyzed for TRIM expression by real time PCR assay using primer/probes sets (Applied Biosystems-full list shown in materials Table). TRIM expression values were normalized to HPRT using the comparative method to obtain the relative value for each condition (RU/HPRT). Heat maps were generated by importing the relative values of TRIM expression to GeneSpring GX 7.3.1 software. The values of each sample for individual TRIMs were further normalized to the median of all samples for each gene. The median is shown in yellow whereas high expression (relative to the median of each gene) is shown in red and low expression is shown in green. A typical example of a histogram with low (but detectable) and high relative values represented as a colour heat map for TRIM65 is shown in panel **A**. In two exceptional cases (TRIM20 and TRIM9) (as an example TRIM9 is shown in Panel **B**) more than half of the samples did not yield detectable product for TRIM9; it was therefore not possible to obtain a median value. In these particular cases, an arbitrary number of 500 was chosen as a median to normalize to the rest of the conditions. This number was chosen based on the detection limit of the real time PCR (approximate PCR threshold value [CT] of 35 is equivalent to 500 relative units).

# Chapter 4

# Results (II):

# TRIMs are differentially expressed in CD4+T cells, macrophages and DC

## 4.1. TRIMs are differentially expressed in CD4+T cells, macrophages and DC

Having established conditions for analysis of TRIM expression by RT-PCR and complex data mining, we determined the expression of TRIMs in our panel of CD4+ T cells upon TCR activation (6 hr after stimulation) and compare this expression to the one obtained in macrophages and DC stimulated with TLR ligands (24 hr after stimulation). To investigate further the possible effect on TRIM expression during viral infections, we also expanded our analysis and included here infections of macrophages and DC with 2 different live influenza viruses A/Puerto Rico/8/34 (PR/8), and A/New Caledonia/20/99 (CAL) as well as stimulation with CpG.

Grouping of TRIMs by patterns of mRNA expression identified four different clusters of TRIMs (Fig. 4.1A, C-1 to C-4). TRIMs in cluster-1 (**Figure 4.1A, C-1**) (TRIM9, 1, 18, 46, 16) showed high expression in CD4+T cells with much lower to undetectable levels in macrophages and DC. TRIM9 showed a unique pattern of expression in resting IL-10Treg that was not further up-regulated after TCR triggering; however up-regulation was only observed in Th2 cells suggesting that its expression may not be attributable to cytokines produced by the different cells after activation. On the other hand, TRIM1 expression was up-regulated in IL-10 producing T cells upon stimulation (Th2 and IL-10 Treg) (Fig. 4.1, C-1) and less so in Th1 cells which produce lower amounts of IL-10 upon stimulation, suggesting a correlation with the IL-10 mRNA expression profile in these different effector T cell subsets (**Figure 4.1B and 4.1C**). However TRIM1 expression was also induced upon stimulation of CD25+Treg, which although they have the capacity to produce IL-10 during *in vivo* inflammatory conditions, do not produce IL-10 subsequent to *in vitro*

stimulation. Strikingly, the COS-FN3 domain was contained only in TRIMs in C-1 (Fig. 4.1A, TRIM9, 1, 18, 46) and not in TRIMs in clusters 2- 4, suggesting that the COS-FN3 domains may provide these TRIMs with specific characteristics which co-ordinately regulate functions in CD4 T cells. Although TRIM16 was also preferentially expressed in CD4+T cells, this TRIM does not possess a RING domain suggesting that its regulation of expression in T cells may differ from the COS-FN3 containing TRIMs.

A distinct group of TRIMs shown in clusters 2 and 3 (Fig. 4.1A, C-2 and C-3) was most highly expressed in macrophages and DC (TRIM2, 6, 3, 20, 35, 25, 14, 45, 19, 23, 21, 30, 26, 34, 8), and these TRIMs were further up-regulated in response to influenza virus infection. However, this group of TRIMs (C-2 and C-3) was further subdivided since a number of TRIMs were additionally expressed in naïve CD4+T cells and CD25+Tregs (Fig. 4.1A, C-3, TRIM14, 45, 19, 23, 21, 30, 26, 34, 8) but did not show an increase in expression in T cells upon stimulation. Of note, expression of TRIMs in C-2 and C-3, was especially high under conditions which induced the highest levels of type-I IFNs (further investigated in the next chapter). However, expression of TRIM 2 and 6 was restricted to macrophages and mDC and low to undetectable in pDC, even upon stimulation.

Cluster 4 comprised a group of TRIMs (TRIM24, 27, 28, 37, 39, 65, 68, 44, 59) which was expressed in pDC at high levels prior to stimulation, and for the most part their expression was either down-regulated or not affected upon stimulation. Only low levels of expression of these TRIMs were seen in macrophages and mDC (Fig. 4.1A, C-4) in the presence or absence of stimulation. Thus macrophages, mDC and pDC appear to have different intrinsic capacities to express particular TRIMs, regardless of the cytokines produced upon stimulation with microbes such as viruses

and their products. This cluster of TRIMs (Fig. 4.1A, C-4) was also expressed constitutively in naïve and CD25+Tregs.

B30.2 domains are present in members of the TRIM family, in addition to other molecules of diverse function and play a major role in protein-protein interactions (previously discussed in section 1.3.1, [26]. Furthermore, a group of TRIMs containing B30.2 domains have been suggested to have evolved to restrict viral infection [270]. We show here that TRIMs containing the B30.2 domain organization are not restricted to any particular cluster of expression, but are spread throughout C1 – C4 inclusively (Fig. 4.1A, solid circles). These findings support previous reports that B30.2 domains are responsible for a broad set of cellular functions in addition to anti-viral restriction [26].

In summary, TRIMs that possess a COS-FN3 motif are highly expressed in T cells with little to no expression in macrophages and DC (C-1). Another group of TRIMs were expressed in macrophages and DC and up-regulated by viral infection and CpG (C-2 and C-3). These TRIMs were further divided based on additional expression in CD4+ T cells (C-3). Finally, a distinct cluster of TRIMs (C-4) was expressed constitutively at a high level in pDC with very low expression in mDC and macrophages, and expression of these TRIMs was not further up-regulated by viruses.

## 4.2. Discussion

Using heat maps for analysis and presentation of a large set of data we have defined four clusters of TRIM molecules on the basis of their distinct expression in either CD4+ T cells or macrophages and DC, which have different innate and adaptive immune functions to an extent determined by their cytokine profile. A group

of TRIM (C-1) genes was preferentially expressed in CD4+ T cells and exclusively contained the COS-FN3 motif associated with protein-protein interactions. Additional clusters of TRIMs were defined on the basis of their up-regulation by influenza viruses in macrophages and DC. This group was subdivided (C-2 and C-3) since some TRIMs were also expressed in naïve T cells and CD25Treg (C-3). Conversely, a distinct group of TRIM genes was constitutively expressed in pDC (C-4). The fact that TRIMs could be grouped based on their levels of expression, suggests that there may be similar mechanisms of regulation of expression for these TRIMs. Moreover, the generation of heat maps and the definition of these clusters of expression will allow us to observe correlations with cytokine production (next chapter).

### 4.2.1. TRIM expression in T cells

Using two different expression profile methodologies (microarray analysis and real time PCR) and a computational approach to identify clusters of TRIM expression in primary immune cells we show for the first time a group of TRIMs (TRIM1, 9, 18, 46, 16) which are highly expressed in CD4+ T cells. TRIM1, TRIM9, TRIM18 and TRIM46 were highly expressed in CD4+ T cells, but less so or not at all in macrophages and DC (**Figure 4.1**), and exclusively contained the COS-FN3 motif which has been reported to bind microtubules [38]. Thus, our findings suggest that TRIM18, which has also been implicated in signalling pathways [276, 277], may be involved in immune function in addition to its previously reported role in microtubule dynamics in the context of the development of the ventral midline [106]. TRIM18 can form a large complex of proteins involved in the regulation of microtubules dynamics [106], and has been shown to be involved in the MAP kinase

p38- MEK1/2 signalling pathway [276, 277]. Similar to TRIM18, the function of TRIM9, since it is highly expressed in activated Th2 cells and in IL-10 Treg (**Figure 4.1A**), may not be restricted to its reported role in the central nervous system [278]. Based on our findings that the COS-FN3 domain was only found in TRIMs expressed in T cells, it is of interest to speculate that TRIM containing this motif may have similar signalling functions in T cells. Moreover, based on these findings it could be predicted that TRIM36, 67 (not done in this study) which also posses the RBCC-COS-FN3-B30.2 domain arrangement may be highly expressed in CD4+T cells. We also found TRIM16 (also known as the estrogen-responsive B box protein) to be highly expressed in CD4+Tcells and further up-regulated upon TCR stimulation in Th2 cells. However, TRIM16 does not contain the COS-FN3 domain organization suggesting that its role in T cells is different to the rest of TRIMs in C-1. Previous reports suggested that TRIM16 expression is important for keratinocyte differentiation [279]; however our data suggests that since TRIM16 is highly expressed in T cells its function may not be restricted to keratinocytes.

Human TRIM1 has been shown to inhibit N-tropic murine MLV [229]. Our findings that mouse TRIM1 is mainly expressed in CD4+ T cells and not macrophages and DC suggests that it may act as a restriction factor specifically in T cells, perhaps explaining the relative increased resistance of T cells to retroviral infection [280, 281]. Alternatively, our data may indicate that TRIM1 may have additional functions to anti-viral activities.

TRIMs in cluster C-3 were distinguished from those in C-2, since they were also expressed in certain CD4+ T cell subsets, albeit to a much lower extent (Fig. 4.1). In keeping with our findings TRIM8 and TRIM21, which we show here, fall in cluster C-3, have been previously shown to be expressed in T cells. Although it was

suggested that these TRIMs may be specifically involved in signalling pathways required for IFNγ and IL-2 production [200, 264, 282, 283], their high level expression in macrophages and DC, which do not produce IL-2 or IFNγ under these conditions, suggest that these TRIMs may have a broader function and may be important in innate immune responses.

### 4.2.2. TRIMs preferentially expressed in macrophages and DC

We additionally define two clusters of TRIM expression (cluster 2 and 3) based on their preferential expression in macrophages and DC upon infection with influenza virus. These TRIMs further showed more specific patterns of expression. TRIMs in C-2 showed very low to undetectable levels of expression in CD4+ T cells. However TRIM6 and TRIM2 were also not detected in pDC even upon stimulation, therefore these TRIMs may play specific roles in macrophages and mDC. Interestingly, TRIM2 and TRIM3 were both expressed in macrophages and mDC, in contrast with previous studies where TRIM2 and TRIM3 were predominantly expressed in brain tissues [284, 285] indicating that these TRIMs are not restricted to the brain and may play important roles in innate immunity. In agreement with previous reports we observed TRIM20, 35 to be preferentially expressed in macrophages and DC [100, 286]; however we found that this expression can be further up-regulated upon influenza virus infection.

Another TRIM worth mentioning here is TRIM19, which previous studies have shown to be important in terminal myeloid differentiation [66]. TRIM19 expression seems to vary depending on the cell type or the tissue studied [31], however the common feature of TRIM19 expression seems to be the capacity to be induced by type-I IFNs and IFNγ [225]. Consequently, it is not surprising that we

find TRIM19 expression highly increased in macrophages and DC upon stimulations that result in type-I IFN production. Moreover, the fact that pDC express such high levels of TRIM19 already in unstimulated conditions may be due to low constitutive production of type-I IFNs by these cells. Surprisingly, we found that TRIM19 is not significantly up-regulated in Th1 cells which produce large amounts of IFNγ, suggesting that the responsiveness to IFNs varies depending on the cell type studied or that it is the unique signalling through the type-I IFNR that results in its up-regulation. This highlights the importance of our study using different primary cell subsets.

Interestingly, we found a group of TRIMs that are constitutively expressed in pDC (C-4) and their expression is not augmented upon viral infection. Our observations that this cluster C-4 of TRIM genes is also expressed in T cells, may reflect the close relationship suggested between pDC and lymphoid cells from observations that pDC express a number of markers of the lymphoid lineage and the possibility of a common haemopoietic precursor [287].

We show that TRIM genes containing a B30.2 domain are not confined to a particular cluster defined by their expression and/or up-regulation by viral infection, in line with a broad function of B30.2 domains in protein-protein interactions. Indeed, the B30.2 domain can be found in proteins that belong to ten different families additional to the TRIM family, some of which have been shown to play a role in signalling in immune cells and proposed to have been selected as a component of immune defence [26].

Since the expression of a large number of TRIMs was induced upon influenza virus infection in macrophages and DC, this suggested a mechanism of co-regulation of gene expression. Having established an interpretable method for visualization with

heat maps, this may allow us to readily correlate TRIM expression with cytokine production and will be the focus of the next chapter.

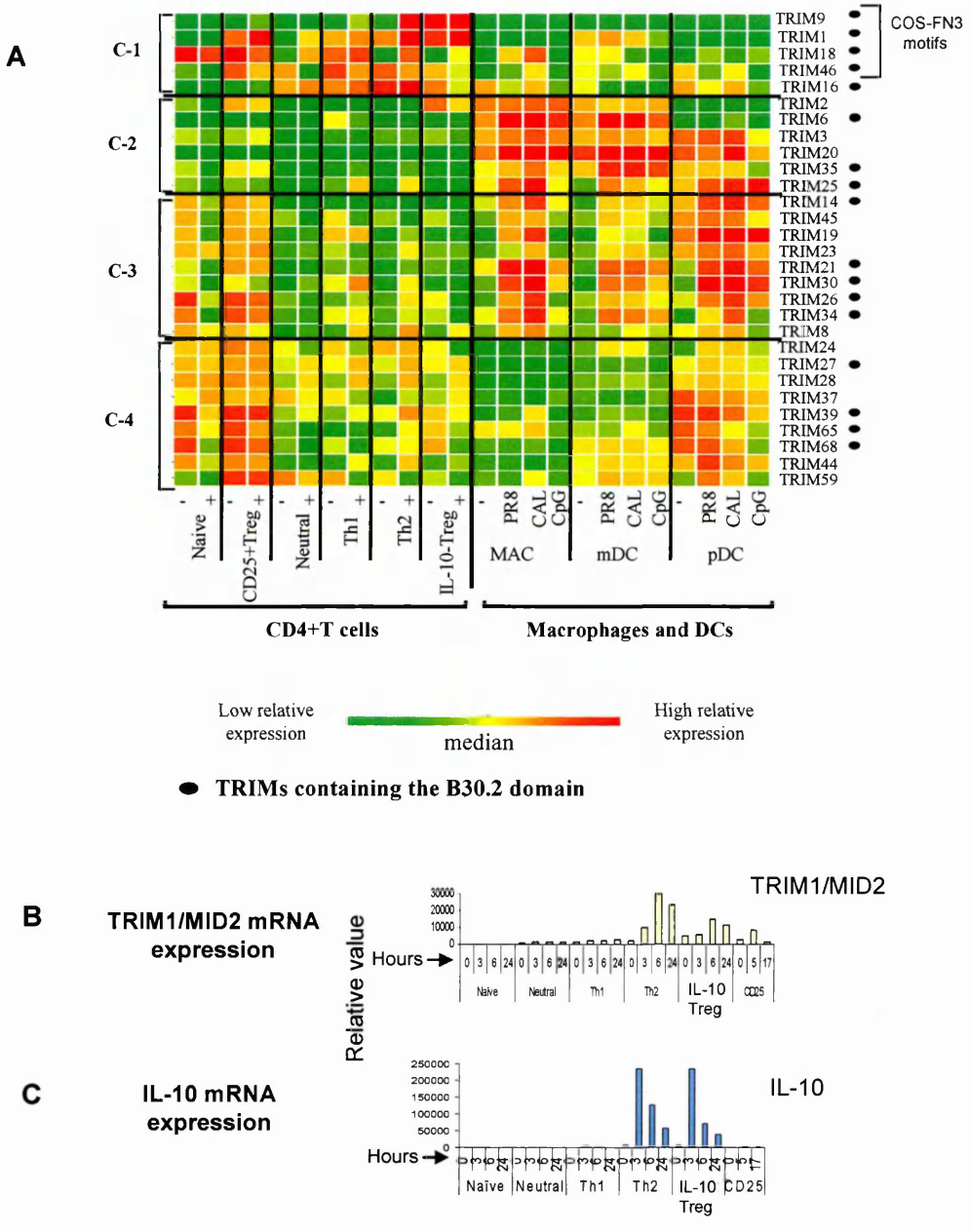



**Figure 4.1. TRIM are differentially expressed in resting and stimulated CD4+ T cells, macrophages and DC.**

**A)** TRIM mRNA expression was analyzed by real-time PCR in CD4+ T cells either unstimulated (–) or stimulated with anti-CD3/CD28 (+) for 6 h, and macrophages (MAC), mDC and pDC either unstimulated (–) or stimulated with influenza virus PR/8 or CAL and CpG for 24 h. A heat map was generated by normalizing the values of each sample to the median of all samples for each gene (as explained in Figure 3.7); green: low expression; yellow: median value; red: high expression. Genes were clustered by levels of expression: TRIM genes preferentially expressed in CD4+ T cells (C-1); TRIM genes preferentially expressed in macrophages and DC and up-regulated upon influenza infection (C-2 and C-3); and TRIM genes constitutively expressed at high levels in pDC (C-4). TRIM genes indicated with solid circles contain a B30.2 domain. **B)** Kinetics of TRIM1/MID2 mRNA expression in the different T cell subsets upon TCR stimulation, by RT-PCR. **C)** Kinetics of IL-10 mRNA expression in the different cell subsets upon TCR stimulation, by RT-PCR. Note that TRIM1 expression correlates with IL-10 expression in the different cell types.

# Chapter 5

# Results (III):

# Mechanism of regulation of TRIM expression

## 5.1. Mechanism of regulation of TRIM expression in DCs and macrophages.

In the previous chapter we showed the differential expression of TRIMs in primary cells subsets of the innate and adaptive immune system that produce different cytokine profiles. We showed 15 TRIMs (Clusters C-2 and C-3) that are preferentially expressed in macrophages and DCs and further up-regulated upon infection with influenza virus. This is of interest because only 7 of these TRIMs have been previously reported to be up-regulated by cytokines or viral infection and very few studies have addressed TRIM expression in primary cells. Those TRIMs that had been previously shown to be induced in epithelial, lymphoid or other cell lines when type-I or type-II IFNs were added to the cell culture include TRIM8/GERP, TRIM19/PML, TRIM20/MEFV, TRIM21/Ro52, TRIM25/EFP, TRIM30/RPT-1, TRIM34/IFP-1 [200, 218, 224, 282, 288-290]. However, a requirement for type-I IFNs or any other cytokine during viral infections has not been addressed. Moreover, the fact that expression of many TRIMs appeared to be co-regulated during influenza virus infection, makes it tempting to speculate that these TRIMs have common roles during immune responses to viruses and should be tested in future studies. Our experimental system of analysis of TRIM expression in primary cell subsets of the innate immune system which naturally produce different pattern of cytokines upon viral infection or TLR stimulation allowed us now to address the possible mechanism of regulation of TRIM expression. We started by searching for possible correlations of TRIM expression with cytokine production, as discussed in the next section.

### 5.1.1 Expression of a large number of TRIMs in macrophages and DCs correlates with the induction of type-I IFN in response to TLR ligation or infection with influenza virus

We showed that a large number of TRIMs (C-2 and C-3) are differentially up-regulated in macrophages and DC after exposure to viral and bacterial products which result in production of different cytokines. Therefore we asked if the presence of specific cytokines would correlate with TRIM expression. For this purpose, macrophages and mDCs were stimulated with CpG, LPS, dsRNA (poly I:C) and infected with influenza viruses PR8 and CAL while pDCs were infected with PR8 and CAL, and stimulated with CpG. We analyzed TRIM expression by RT-PCR and cytokine protein production after 24 hour stimulation. **Figure 5.1** shows TNF, IL-10, IL-12p70, and type-I IFNs profiles in macrophages and DC and a heat map of TRIM expression.

TNF was produced at intermediate levels by macrophages and mDC upon all treatments with no significant difference in the levels of expression. pDC expressed high levels of TNF upon CpG stimulation with much lower levels produced upon viral infection (**Figure 5.1A**). The TNF pattern of expression observed in the different cell types upon different stimulations did not appear to correlate with up-regulation of TRIM expression in C-2 and C-3 or C-4 (compare **Figure 5.1A with 5.1B**).

Macrophages produced relatively high levels of IL-10 when stimulated with PR8, CAL, LPS with lower levels produced upon CpG stimulation and significantly lower levels produce upon Poly I:C stimulation. mDC produce lower levels of IL-10 upon all treatments as compared to macrophages, whereas pDC did not produce any detectable levels of IL-10. This pattern of IL-10 production by all cell types and

treatments also did not appear to show any correlation with TRIM expression. In particular pDC do not produce IL-10 but expression of TRIMs in C-3 was strongly up-regulated upon viral infection and CpG. On the other hand, expression of TRIMs in C-2 was not detected in pDC, and this could possibly correlate with IL-10 production since expression of these TRIMs was highly induced in macrophages and DC. However, macrophages stimulated with Poly I:C produce little to no IL-10 and yet high levels of TRIMs in C-2 and C-3 were induced, suggesting that IL-10 is not involved in the up-regulation of TRIMs in C-2 and C-3.

Finally, IL-12p70 was not detected in macrophages and only low levels were observed in mDC upon all stimulations, whereas CAL virus induced the highest levels of IL-12p70 in pDC. Again, production of IL-12p70 did not seem to correlate with TRIM expression.

Of note, expression of TRIM genes in C-2 and C-3 was especially high under conditions which induced the highest levels of type I IFN (Fig. 5.1 A,B). Macrophages and mDC infected with 2 different strains of Influenza virus produced different levels of type-I IFNs. As compared to New Caledonia, PR8 infection produced lower levels of IFNβ in macrophages and mDC. A more significant difference was observed for IFNα produced by pDC. We observed that expression of TRIMs in cluster C-2 and C-3 was up-regulated in all cell subsets in response to viral infection and this expression showed the best correlation with IFNβ in macrophages and DC and IFNα in pDC (compare **Figure 5.1A and 5.1B**; [other type-I IFNs including IFNβ usually show similar patterns of expression as IFNα in pDC]).

CpG stimulation of macrophages and DC resulted in very low production of IFNβ as compared to infection with viruses (Fig. 5.1A). This correlated with lower induction of TRIM expression, suggesting a possible role of type I IFN in this up-

regulation. However, expression of TRIM2 and TRIM6 was restricted to macrophages and mDC and was low to undetectable in pDC, even upon stimulation. LPS stimulation also led to significant levels of IFNβ production in macrophages and mDC in correlation with up-regulation of TRIMs expression in C-2 and C-3. Notably, Poly I:C stimulation produced the highest levels of IFNβ in macrophages which correlated with the highest expression of TRIMs in C-2 and C-3. Moreover, Poly I:C induced very low levels of IFNβ in mDCs which also correlated with very low induction of TRIM expression (**Figure 5.1B**). The fact that influenza virus infection and dsRNA (Poly I:C) stimulations led to higher production of IFNβ in macrophages as compared to mDCs suggested intrinsic differences between these cell types, possibly related to a higher expression of TLR3 by macrophages.

TRIMs in C-4 were highly expressed in unstimulated pDC, and these TRIMs were not significantly affected upon stimulation, confirming our previous results and suggesting that macrophages, mDC and pDC have different intrinsic capacities to express this group of TRIMs.

### 5.1.2. Induction of TRIM19 upon TLR ligation is dependent on the TRIF adaptor molecule and correlates with IFNβ production

We have shown that expression of TRIMs in cluster C-2 and C-3 in macrophages is highly up-regulated upon Poly I:C stimulation, followed by influenza virus infection or LPS stimulation, and to a lesser extent if at all by CpG. This induction of TRIM expression seemed to correlate with production of IFNβ. Therefore we asked if this up-regulation of TRIM expression was due to a direct effect of the virus on TRIM expression, or an indirect effect as a result of the signalling cascade leading to the induction of cytokines upon infection. Influenza

virus infects macrophages and DC resulting in activation of TLR and non-TLR pathways to induce cytokine production (discussed in section 1.5.1). The TLR signalling pathways can rely on either the MyD88 or the TRIF adaptor molecules to recruit essential molecules required for induction of cytokine gene expression [154]. To further gain insight into the mechanism of regulation of TRIM expression we used BM macrophages derived from either MyD88 or TRIF deficient mice to investigate if induction of TRIM expression depends directly on TLR activation or downstream events resulting from specific signalling pathways. Expression of TRIM19/PML, a well known IFN-inducible gene [220], was not up-regulated upon stimulation of macrophages with CpG which only signals via the MyD88 pathway, and low to undetectable levels of IFNβ are induced (**Figure 5.2A**). In contrast, stimulation with LPS resulted in very significant up-regulation of TRIM19 expression and this was completely impaired in TRIF -/- macrophages (Fig 5.2A). LPS induction of IFNβ was completely dependent on the TRIF molecule since IFNβ production was completely impaired in TRIF -/- macrophages as it was also induction of TRIM19, indicating that TRIM19 may be induced directly by the TRIF pathway or indirectly by the induction of IFNβ production. In keeping with this, stimulation of TRIF -/- macrophages with Poly I:C resulted in only a partial reduction in the induction of TRIM19 expression as compared with wild type (WT) controls (Fig. 5.2A) which correlated with only a partial impairment of IFNβ induction in these TRIF-/- macrophages. This is consistent with previous reports that IFNβ can also be induced by dsRNA via the RIG-I/MDA5 pathway independently of the TRIF adaptor molecule [291].

These data suggest that induction of TRIM19 expression is due to an IFNβ autocrine loop and not directly dependent on the TRIF adaptor molecule. Moreover,

induction of TRIM19 expression and IFNβ production, were both not significantly affected in MyD88 -/- macrophages stimulated with LPS or Poly I:C (**Figure 5.2B**) indicating that TRIM19 expression as well as induction of IFNβ does not require the MyD88 adaptor molecule.

### 5.1.3. Induction of TRIMs in C-2 and C-3 upon TLR ligation is dependent on the TRIF adaptor molecule and correlates with IFNβ production

We now asked if the rest of the TRIMs in C-2 and C-3 followed the same pattern of expression as TRIM19 and if this correlates with type-I IFNs. We therefore focused our analysis of cytokine and TRIM expression in TRIF deficient mice as described above. **Figure 5.3A** shows IFNβ protein levels produced by WT and TRIF -/- macrophages upon TLR stimulations measured by ELISA. Similar to TRIM19 expression shown above, stimulation with CpG did not induce expression of TRIMs in C-2 and C-3 (with exception of TRIM2) (**Figure 5.3B**) nor did it induce much IFNβ production, suggesting that MyD88 is not involved in the regulation of TRIM expression in macrophages. Conversely, the expression of all TRIMs in C-2 and C-3 was up-regulated upon LPS stimulation and this up-regulation was completely impaired in TRIF -/- macrophages (**Figure 5.3B**) as was also the production of IFNβ (**Figure 5.3A**). Stimulation with Poly I:C led to even higher induction of all TRIMs in C-2 and C-3 in correlation with IFNβ production (**Figure 5.3 A, B**). This expression was only partially reduced in TRIF -/- macrophages which also correlated with only partial reduction of IFNβ production (**Figure 5.3 A, B**). In addition to these TLR stimulations we infected WT and TRIF -/- macrophages with CAL influenza virus and obtained similar results to the stimulations with Poly I:C (**Figure 5.3 A, B**) in that TRIMs in C2 and C-3 were up-regulated and their expression only partially

reduced in TRIF -/- as was seen for IFNβ production. The partial effect on IFNβ production confirms previous reports that influenza virus can induce type-I IFNs by TLR signalling or during viral replication by a TRIF independent mechanism involving RIG-I/MDA5 pathway [292]. Although at this point we can not completely rule out a possible role of the TRIF pathway or the non-TLR RIG-1/MDA5 pathways in regulation of TRIM expression, the summary of observations strongly suggest an effect of type-I IFNs on up-regulation of TRIM expression: 1) Stimulation of TRIF -/- macrophages with dsRNA-Poly I:C resulted in only a partial reduction of TRIM expression and not a complete impairment in TRIM expression. This indicates that there are other factors independent of TRIF signalling that may lead to induction of TRIM expression. 2) Another possibility is a direct effect of the RIG-I signalling pathway on TRIM expression, however, we have already shown that induction of TRIM expression in macrophages stimulated with LPS, which does not activate the RIG-I pathway, is completely impaired in TRIF -/- macrophages, correlating with IFNβ production. 3) We investigated the induction of TRIM19 expression, a known type-I IFN inducible gene [220], in macrophages derived from MyD88 -/- mice or TRIF -/-. We showed that TRIM19 expression was not affected in MyD88 -/- macrophages upon LPS or Poly I:C stimulations, correlating with IFNβ production. 4) We showed that expression of TRIM19, as well as the rest of TRIMs in C-2 and C-3 was not significantly up-regulated in macrophages upon CpG stimulation, in correlation with IFNβ low levels of induction.

### 5.1.4. Expression of TRIMs in C-2 and C-3 is dependent on type-I IFN signalling whereas those in C-4 are not.

Our data show that the expression of TRIM genes in clusters C-2 and C-3 is up-regulated in macrophages and DC upon viral infection (**Figure 4.1A and 5.1B**), and that the level of up-regulation appeared to correlate with the induction of type I IFN production (Fig. 5.1 and 5.3). In contrast, a distinct group of TRIM molecules comprising C-4 was expressed constitutively at high levels in pDC (Fig. 5.1B), and yet for the most part was not further up-regulated by viruses, although high levels of type I IFN were induced in these cells (Fig. 5.1A). To further investigate the possible involvement of type-I IFNs in the mechanisms of regulation of TRIM expression, macrophages, mDC and pDC were obtained from mice lacking the type I IFNα/β receptor (IFNα/βR–/–) and their TRIM expression was compared to equivalent WT cells, under the conditions of stimulation described earlier.

Expression of TRIMs within clusters C-2 and C-3, inducible by virus infection or CpG stimulation in macrophages and DC, was completely dependent on type I IFN production since their expression was not up-regulated in the IFNα/βR–/– cells (Fig. 5.4, C-2, C-3). Although expression of TRIM20 and TRIM35 was up-regulated upon stimulation, in contrast to the rest of the TRIM in C-2 and C-3, these increases were not completely dependent on type I IFN (Fig. 5.4, C-2, asterisks and black box). In keeping with our findings on the up-regulation of TRIM expression in C-2 and C-3 by virus and CpG, stimulation with LPS and dsRNA [poly(I:C)] (**Figure 5.5**), which signal via additional or different intracellular adaptor proteins downstream of TLR to produce type I IFN [292], also led to up-regulation of these TRIM in macrophages and mDC via a type I IFN-dependent mechanism, again with the exception of TRIM20 in macrophages/DC and TRIM35 only in mDC.

Collectively these data show that expression of the majority of TRIM molecules within clusters C-2 and C-3 is exclusively dependent on type I IFN signalling regardless of whether macrophages and DC are infected by different influenza virus strains or stimulated with TLR ligands. In contrast, the TRIM molecules contained within cluster C-4 were constitutively expressed at high levels in pDC, and were not significantly affected by a complete absence of signalling through the type I IFN receptor.

### 5.1.5. A region on mouse chr-7 contains TRIMs that are up-regulated in macrophages and DC by type-I IFNs.

We have defined clusters of TRIM (C-1 to C-4) based on their levels of expression in the different cell types (Fig. 4.1) and up-regulation in macrophages and DC in a type I IFN-dependent manner (Fig. 5.4 and 5.5). To determine whether TRIM genes may have co-evolved, we searched for co-regulation of expression of closely linked TRIM genes. Although TRIM proteins are spread across the human genome, previous studies have suggested that these proteins have evolved by gene duplication leading to groups of closely related TRIM on individual chr which may share functional similarities [20, 26, 293]. Similarly, mouse TRIM are found on almost all of the mouse chr and groups of closely related TRIM are observed on chr-7, in the MHC region on chr-17, and less closely related groups on chr-11 and chr-3 (**Figure 5.6A and 5.6B**) [270, 273]. TRIM genes mapping to mouse chr-3, chr-11 and chr-17 were expressed broadly in the different cell types with no distinct pattern of expression (**Figure 5.6C**). In contrast, the majority of TRIMs examined that map to mouse chr-7 (TRIM3, TRIM6, TRIM21, TRIM30 and TRIM34), syntenic to human chr-11, showed expression in macrophages and DC but not in T cells

following stimulation (Fig. 5.6D). Up-regulation of these TRIM in macrophages and DC by viruses was completely impaired in cells deficient in type I IFN signalling (Fig. 5.6E). TRIM6, TRIM21, TRIM30 and TRIM34 are the most closely related and group tightly in the F2 region of mouse chr-7 (Fig. 5.6B). The fact that these TRIM are phylogenetically related and are co-regulated by type I IFN suggests that they may have co-evolved to co-ordinate important anti-viral functions. In keeping with a role in anti-viral function, TRIM in this region (F2) of mouse chr-7 show high sequence similarity with TRIM in an equivalent region (p15.4) of the syntenic human chr-11, which have been demonstrated to have anti-viral activity (schematic representation shown in **Figure 5.6F**) [33, 210, 218, 272].

### 5.1.6. Microarray analysis of human macrophages revealed a similar pattern of TRIM expression to mouse TRIMs

So far our study on regulation of TRIM expression has only included mouse TRIMs, however many of the TRIMs shown to date to have anti-viral functions are human or primate TRIMs and some of them including TRIM5α and TRIM22 do not exist in mice. Therefore we wanted to examine if expression of the human TRIMs would correlate with those which we show in mice to be up-regulated upon viral infection or TLR stimulation in a type-I IFN dependent manner also correlate with type-I IFNs. For this purpose we searched the Microarray database (gene expression omnibus (GEO) website: www.ncbi.nlm.nih.gov/geo) for previous expression studies using human macrophages. Martinez et al. [294] have previously reported a microarray study on the transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization, and reported new molecules expressed during this differentiation process. However, they did not report any information

regarding to TRIM expression even though this microarray contained probes for most TRIM genes. Therefore we obtained the raw data freely available in the microarray database (accession number GSE5099) and analyzed these data using GeneSpring software. Strikingly, and consistent with our own findings in mice, up-regulation of the same TRIM genes in human macrophages was mainly observed under conditions which resulted in the induction of IFNβ (in this case LPS and IFNγ), as observed in this previously published microarray study [294](**Figure 5.7A**). In contrast, this was not observed in human macrophages stimulated with IL-4, which did not induce IFNβ production (Fig. 5.7A, indicated with an asterisk). TRIM genes, which we found to be constitutively expressed in mouse pDC (Fig. 4.1A, C-4), were not up-regulated in human macrophages under these conditions [294] (Fig. 5.7B). Strikingly, expression of TRIM3, TRIM5, TRIM6, TRIM21, TRIM22 and TRIM34, located on human chr-11, as previously discussed, and shown previously to have anti-viral activity [33, 210, 218, 272] are simultaneously up-regulated in human macrophages under conditions that led to induction of IFNβ [294] (Fig. 5.7A, C). This is in keeping with our data that the mouse TRIM genes located on the syntenic chr-7 are up-regulated via a type I IFN-dependent mechanism (Fig. 5.6E), supporting our hypothesis that these mouse and human TRIM genes located in the specific regions on chr-7 and chr-11, respectively, have co-evolved to combat viruses. The majority of the TRIM genes, which we showed in mouse macrophages to be up-regulated by virus in a type I IFN-dependent manner (Fig. 5.4), were also up regulated in human macrophages producing IFNβ (Fig. 5.7A, and Table 5.1). Additionally, human TRIM5, TRIM17, TRIM31, TRIM33, TRIM48 and TRIM62, which map to different chromosomal location, that are either non-existent in mouse or were not tested in our study, were up-regulated in human macrophages under these conditions. Taken

together, our study provides data which allows us to hypothesize that TRIMs expressed in the context of type I IFN signalling may be a broad predictor of anti-viral activity. It will be of interest to determine if all the TRIMs found in our study to be type-I IFN dependent indeed have anti-viral functions.

### 5.1.7. Type-I IFN dependent expression of TRIMs only partially correlates with the presence of a B30.2 domain

The B30.2/SPRY domain is a conserved region found in butyrophilin, a transmembrane protein belonging to the immunoglobulin super family [295]. The B30.2 domain is present in a large number of proteins that can be classified in 11 different families with diverse functions (discussed in section 1.3.1)[26]. A few TRIMs have been shown to restrict viral replication and a fraction of them interact with viral products through the B30.2 domain. Some studies have shown that the B30.2 domains of some TRIMs have been subjected to evolutionary pressure and possibly have been selected as an interacting domain with viruses. Not all TRIMs containing B30.2 domains have yet been found to restrict viral replication; however it is possible that they have not been tested for an appropriate panel of viruses. Moreover, as well as their potential involvement in direct restriction of viral replication, it is possible that TRIMs containing the B30.2 domain may be involved in innate immunity or other immune processes that may result in protection against viruses through indirect mechanisms. Interestingly, taken together our and previous studies have shown that TRIMs found to have anti-viral functions are also inducible by type-I IFNs (see table 5.2). In this study we further define these TRIMs not only to be type-I IFN inducible, but also to require type-I IFNs for up-regulation. Therefore we asked if the requirement for type-I IFN correlates with the presence of

the B30.2 domain suggested to have been selected to interact with viruses. The B30.2 domain is present in 32 out of 62 mouse TRIM proteins (52%) which have not all been shown as yet to restrict viral replication. Phylogenetic analysis showed that the B30.2 containing proteins investigated in our study grouped in a phylogenetic clade of related proteins (Fig. 5.8A), consistent with the fact that they all share a common C-terminal domain. We then examined if these TRIMs were expressed in macrophages and DCs in a type-I IFN dependent manner. **Figure 5.8B** shows that a large number of TRIMs containing the B30.2 domain fall in our clusters C-2 and C-3 which are dependent on type-I IFNs, suggesting they may have anti-viral functions. However, not all TRIMs containing a B30.2 domain were induced by type-I IFNs in keeping with the notion that B30.2 domains are also found in proteins involved in other functions [26]. Strikingly, many of the TRIMs that do not contain the B30.2 domain are also in our clusters C-2 and C-3 which are type-I IFNs inducible (**Figure 5.8C**). Some of these TRIMs including TRIM19 have been suggested to have anti-viral functions, thus type-I IFN dependency appears to correlate better with anti-viral activity than the presence of a B30.2 domain.

## 5.2. Discussion

We defined two clusters of TRIM genes (C-2 and C-3) based on their preferential induction in macrophages and DC upon influenza virus infection. Using cells from mice deficient in MyD88 and TRIF adaptor molecules we showed that this expression correlated with type I IFN production. Using macrophages and DC deficient in type-I IFN signalling we demonstrated that expression of TRIMs in C-2 and C-3, with exception of TRIM20 and TRIM35, was completely dependent on type-I IFNs.

We have shown that expression of all TRIMs in clusters C-2 and C-3 in macrophages was induced via a TLR mediated TRIF-dependent pathway which correlated with IFNβ production (**Figure 5.3**). We also showed that production of IFNβ and expression of TRIMs in C-2/C-3 in macrophages can be induced by a TRIF-independent pathway in response to influenza virus infection or dsRNA (Poly I:C) stimulation, consistent with the fact that viral infection and stimulation with dsRNA can activate the RIG-I pathway, independently of TLR activation [291]. The induction of TRIM expression in response to these stimulations could be explained either by a direct effect of the activation of TRIF pathway or indirectly by an autocrine effect of IFNβ. However, we demonstrated using macrophages, mDC and pDC derived from type-I IFN receptor deficient mice that expression of these TRIM depends on type-I IFN signalling. Although we did not test the role of the MyD88 signalling pathway on the expression of all TRIMs in C-2 and C-3, the fact that CpG does not induce significant levels of TRIM expression together with the fact that TRIM19 expression was independent of MyD88 signalling in macrophages, suggests that the rest of the TRIMs in C-2 and C-3 do not require the MyD88 pathway in macrophages.

We have also shown that infection with two different influenza viruses, PR8 and New Caledonia (CAL), which are both H1N1 strains, resulted in up-regulation of TRIMs in clusters C-2 and C-3 in a type-I IFN dependent manner. Notably, CAL virus induced higher levels of TRIM expression as compared to PR8 in all cell types, and the level of TRIM expression correlated with the levels of type-I IFN produced upon infection with these viruses. The fact that infection with CAL virus resulted in higher levels of IFNβ in macrophages and mDC, and higher levels of IFNα in pDC as compared to infection with PR8 could be due to changes in the sequence of the

non-structural protein (NS1) of these viruses, which is known to inhibit cytokine responses. NS1 protein can bind and sequester dsRNA before it activates IRF-3 or NF-κB [296, 297] via TLR3 or RIG-1 dependent pathways. However, using macrophages, mDC and pDC lacking functional type-I IFN receptor signalling we demonstrated that the induction of TRIMs in C-2 and C-3 (with exception of TRIM20, 35) was completely dependent on type-I IFN signalling and does not require viral replication. This is supported by the fact that up-regulation of these TRIMs in IFNRA -/- macrophages and DC is also completely impaired in response to LPS or Poly I:C, which are stimulations that do not contain virus or NS1 protein. These data could help in the understanding of the host response to viruses and consequently may have a great impact in the design of anti-viral strategies to protect people against influenza virus infections.

Unlike the majority of TRIM genes in C-2 and C-3, expression of TRIM20 and TRIM35 in macrophages and DC was not exclusively dependent on type I IFN, in keeping with previous reports that expression of these TRIMs can be up-regulated by TNF or IL-10, or M-CSF, respectively [100, 286]. However we found here that TRIM35 is expressed at higher levels in mDC derived with GMCSF as compared to macrophages derived with MCSF. Moreover, the expression of TRIM35 was further up-regulated upon stimulation in both macrophages and mDCs however this up-regulation was only completely dependent on type-I IFNs in macrophages but not in mDC suggesting the involvement of additional factors in the regulation of TRIM35 expression in mDC.

Expression of TRIM14, TRIM19, TRIM21, TRIM25, TRIM26 and TRIM34 (all in C-2 and C-3) has previously been shown to be up-regulated by influenza virus infection in a human epithelial cell line and it was inferred that this up-regulation was

caused by production of type I IFN [298]. Furthermore, expression of TRIM8, TRIM19, TRIM20, TRIM21, TRIM25, TRIM30 and TRIM34 (all in C-2 and C-3) was earlier shown to be up-regulated upon addition of either type I or type II IFN to a variety of cultured cells [200, 218, 224, 282, 288-290]. However, an exclusive requirement for type I IFN in the induction of TRIM expression has not been addressed. We now show that the expression of a large number of TRIM genes (C-2 and C-3) is up-regulated by influenza virus and TLR ligation in macrophages and DC, for the most part via a type I IFN-dependent mechanism (Fig. 5.4 and 5.5). Macrophages and DC are susceptible to influenza virus infection but are known to limit productive viral replication [299, 300], using a number of IFN-inducible anti-viral proteins including Mx, PKR and possibly TRIM19 [144, 301, 302]. We propose that the large group of TRIM that we defined as clusters C-2 and C-3 may all function to limit viral replication in macrophages and DC, possibly by different mechanisms. For example, TRIM25 by ubiquitinating RIG-I contributes to the signalling pathway required for IFNβ production [12]. Our findings that induction of TRIM25 by influenza virus infection is exclusively dependent on type I IFN in macrophages and DC indicates, however, that a tight autocrine loop is necessary for TRIM25 expression and IFNβ production in these cell subsets.

Our results suggest that macrophages, mDC and pDC may have different intrinsic capacities to express certain TRIM molecules regardless of their cytokine profile (Fig. 5.1, C-4). For example, those in C-4 were expressed constitutively in pDC at high levels as compared to macrophages and mDC and yet their expression was not up-regulated by viruses, nor dependent on signalling by type I IFNs (Fig. 5.4, C-4). This begs the question as to their function, particularly since pDC have been strongly implicated as dominant in the innate immune response to limit viral

infections as a result of their secretion of large amounts of type I IFN upon viral infections [187, 303]. Therefore constitutive expression of these TRIM genes in pDC may reflect distinct or additional anti-viral function of TRIM molecules in this specialized cell type without a requirement for type-I IFNs for expression. Our observations that this cluster C-4 of TRIM genes is also expressed in T cells may reflect the close relationship suggested between pDC and lymphoid cells from observations that pDC express a number of markers of the lymphoid lineage [287].

An other important implication of these different intrinsic capacities to express TRIMs is illustrated by the case of TRIM6, which we have shown is highly expressed in macrophages and mDC and is further induced by type-I IFNs. However, although pDC produce high levels of type-I IFNs we did not detect any expression of TRIM6 in this cell subset even upon stimulation. This may reflect specific functions of TRIMs in different cell subsets or involvement in different differentiation pathways [304].

The importance of our study in terms of this cell specific intrinsic capacity to express TRIM molecules may be related to the potential anti-viral function of TRIMs and intrinsic immunity [140]. In this respect, intrinsic immunity refers to viral restriction factors that are constitutively expressed in all cells of an organism. For example, it is normally assumed that macaque TRIM5α which restricts HIV-1 replication is expressed broadly in all cell types of this species. However a recent study has suggested that rhesus macaque DC are susceptible to HIV-1 infection due to dysfunctional expression of TRIM5α, whereas the anti-viral restriction activity is still functional in the macaque macrophages and T cells [305]. Again, this highlights the importance of our study using purified primary cells where the specific expression of potential anti-viral TRIMs can be observed.

Among the large number of TRIM dependent on type I IFN for their expression upon viral infection, was a group of homologous TRIM that mapped to the F2 region on mouse chr-7 (TRIM3, TRIM6, TRIM21, TRIM30, TRIM34) (Fig. 5.6B), syntenic to the region p15.4 of human chr-11, containing TRIM5, TRIM6, TRIM21, TRIM22 and TRIM34 [270], which have been reported to have anti-viral restriction activity (**Figure 5.6F**) [33, 210, 218, 272]. Furthermore, mouse TRIM6, TRIM30 and TRIM34 are phylogenetically related to human TRIM5, TRIM6, TRIM22 and TRIM34 [270]. With the exception of mouse TRIM30, none of these mouse TRIM proteins have so far been shown to have anti-viral activity. Taken together our findings suggest that mouse TRIM3, TRIM6, TRIM21, TRIM30 and TRIM34 located on chr-7 may have evolved similar mechanisms for viral restriction as the human TRIM genes located on chr-11. The human TRIM molecules located on human chr-11 have been suggested to exert their anti-viral restriction by B30.2 domain-dependent interactions [272]. In keeping with this, four out of five TRIM genes (exception TRIM3) located on mouse chr-7 contain a B30.2 domain, suggesting that as in human these TRIM genes have co-evolved to restrict viruses. However, TRIM genes without the B30.2 domain, like TRIM19, can also be involved in anti-viral functions [22]. Therefore, our classification of a large number of TRIM defined on the basis of their induction by viruses via a type I IFN-dependent mechanism in macrophages and DC may be an alternative predictor of anti-viral activity to the presence of a B30.2 domain or their chromosomal location, although this remains to be tested.

We show that TRIM genes containing a B30.2 domain are not confined to a particular cluster defined by their expression and/or up-regulation by viral infection via type I IFN, in line with a broad function of B30.2 domains in protein-protein

interactions. Indeed, the B30.2 domain can be found in proteins that belong to ten different families additional to the TRIM family, some of which have been shown to play a role in signalling in immune cells and proposed to have been selected as a component of immune defence [26].

Using microarray data deposited in the gene expression omnibus (GEO) database analyzed a previous microarray experiment of human monocyte derived macrophages differentiated in the presence of MCSF and polarized with either LPS and IFNγ or alternatively IL-4 [294]. We were able to show that the human TRIM orthologs to mouse TRIMs are also induced in conditions where type-I IFNs are present (**Figure 5.7**). Although this does not prove that all the human TRIMs are actually type-I IFN inducible, it is a good correlation implying that the expression of these human and mouse TRIM genes are regulated in similar ways. This is important because it indicates that not only the TRIM proteins have been conserved during evolution, but also their mechanisms of regulation of gene expression remained conserved through evolution. Therefore these genes must play essential roles within the IFN system, and although not necessarily in direct interaction with viruses, it suggests they are involved in these innate immune processes. In this context, it is interesting to speculate that since TRIM proteins probably evolved from a common ancestral gene [272, 273], they may have carried with them their promoter regulatory sequences containing ISREs necessary for IFN responsiveness [147]. However since not all TRIMs are type-I IFN inducible, this begs the question as when, during evolution, was this capacity to respond to IFNs gained or lost. It remains to be seen if TRIM gene promoters may also share high degree of similarities that may answer this question and if this promoter sequence similarity may also predict anti-viral functions.

In this study we have shown groups of mouse TRIM genes that are expressed either in CD4+ T cells, or alternatively in macrophages and DC. Clusters of TRIM expression were further subdivided on the basis of their up-regulation by influenza viruses or TLR ligands via a type I IFN-dependent mechanism in macrophages and DC, or in contrast their constitutive expression in pDC independently of type I IFN production. This grouping of TRIM genes based on their expression and regulation may provide leads to delineate the potential functions of this diverse family of proteins

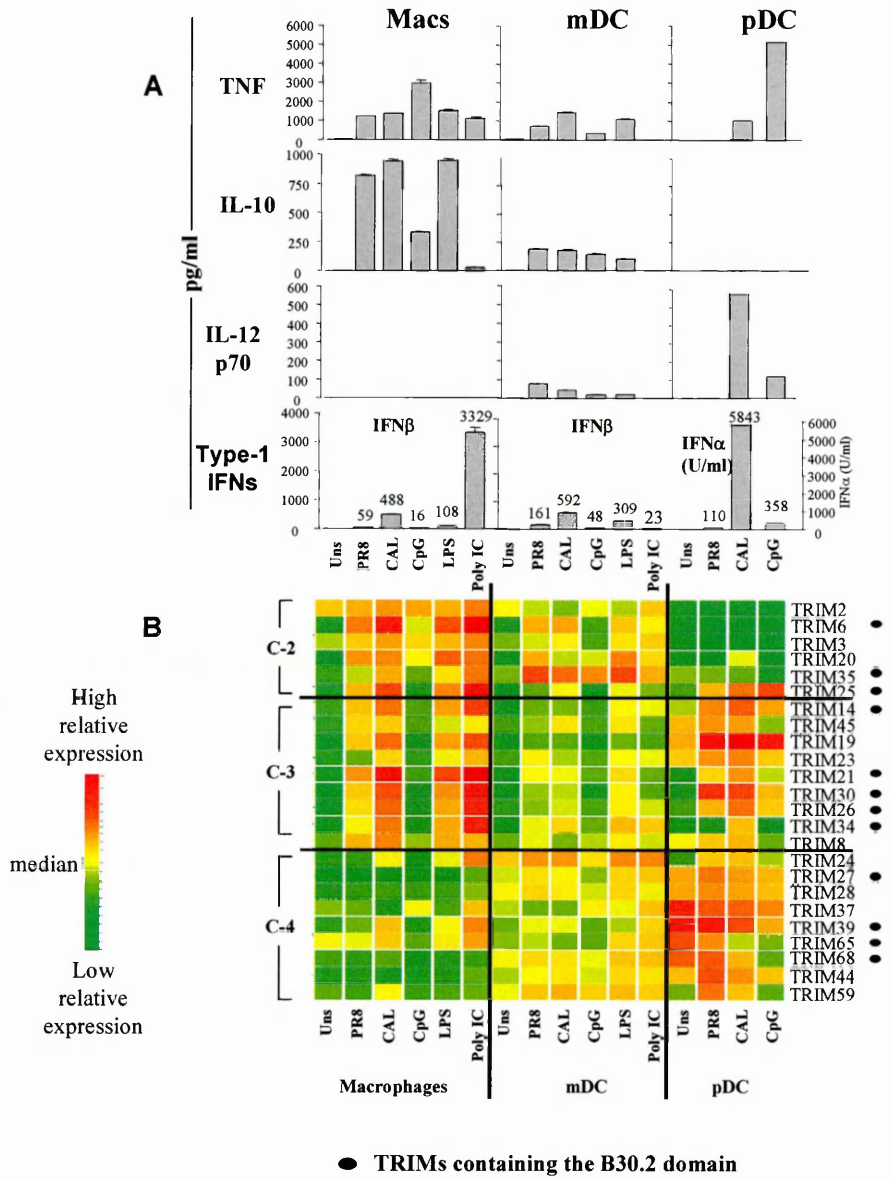



**Figure 5.1. Induction of TRIM expression in response to viral infection or TLR stimulation in macrophages and DC correlates with type-I IFN production.**
**A)** Macrophages (MAC) and mDC were unstimulated (Uns) or treated with influenza virus strains PR/8 and CAL, or TLR ligands CpG, LPS or dsRNA (Poly I:C). pDC were treated with influenza virus PR8, CAL or CpG. After 24 h, cytokine protein was measured by ELISA. **B)** TRIM mRNA expression was analyzed by real-time PCR in macrophages (MAC), mDC and pDC described in panel (A). A heat map was generated by normalizing the values of each sample to the median of all samples for each gene (further details in Figure 3.7); green: low expression; yellow: median value; red: high expression. Genes were clustered by levels of expression: The TRIMs shown in the previous section to be preferentially expressed in CD4+ T cells (C-1) were omitted here; TRIM genes preferentially expressed in macrophages and DC and up-regulated upon influenza infection (C-2 and C-3); and TRIM genes constitutively expressed at high levels in pDC (C-4).

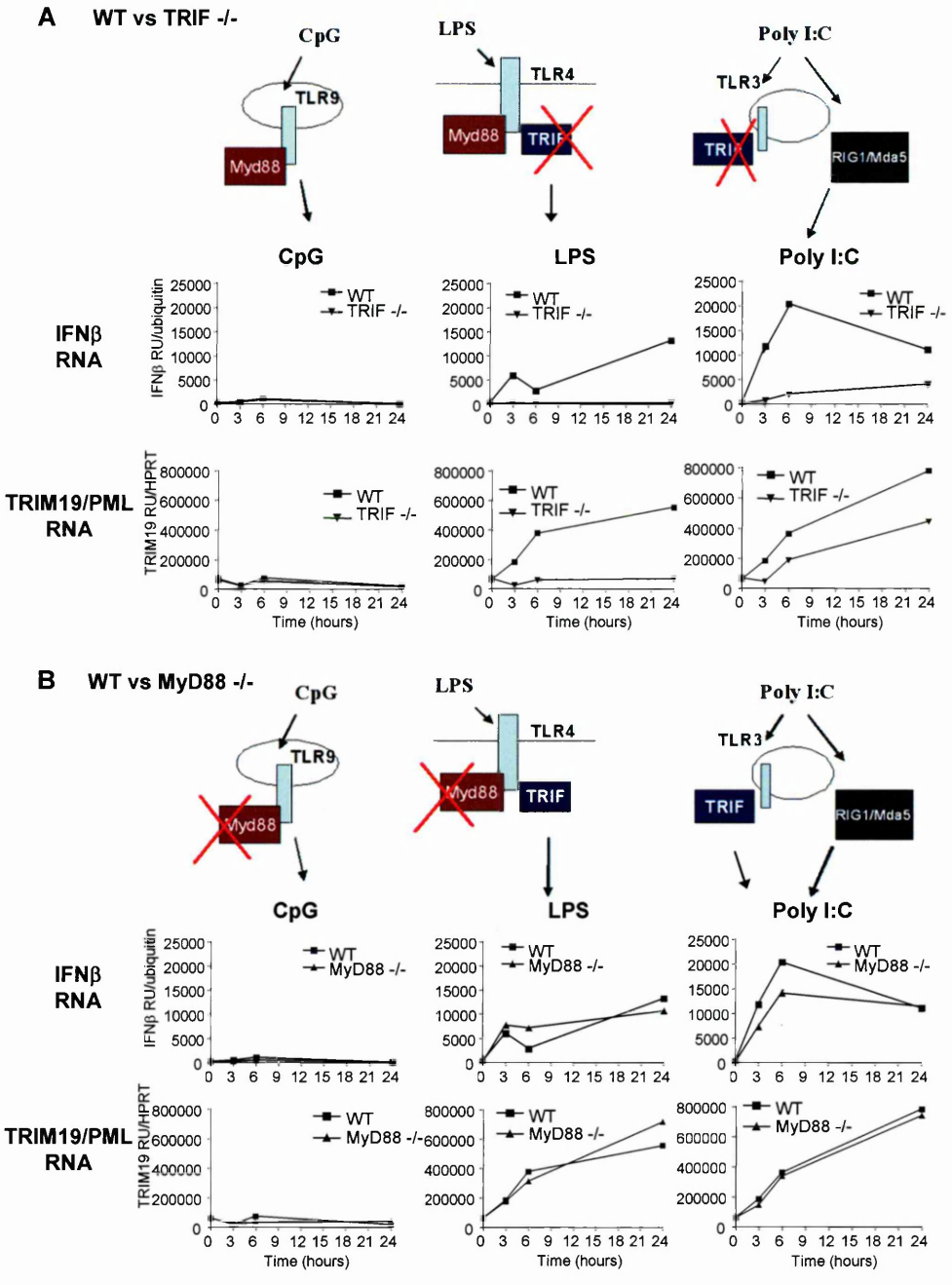



**Figure 5.2. Induction of TRIM19/PML expression upon TLR stimulation is dependent on the TRIF adaptor molecule and correlates with IFNβ production.**
BM derived macrophages from wild type (WT) C57BL/6, MyD88-deficient **(A),** and TRIF-deficient mice **(B)** were stimulated with CpG, LPS, or Poly I:C and samples were collected at different time points. IFNβ mRNA expression was determined by RT-PCR using SYBRgreen and TRIM19/PML expression was determined by RT-PCR using primer-probes.

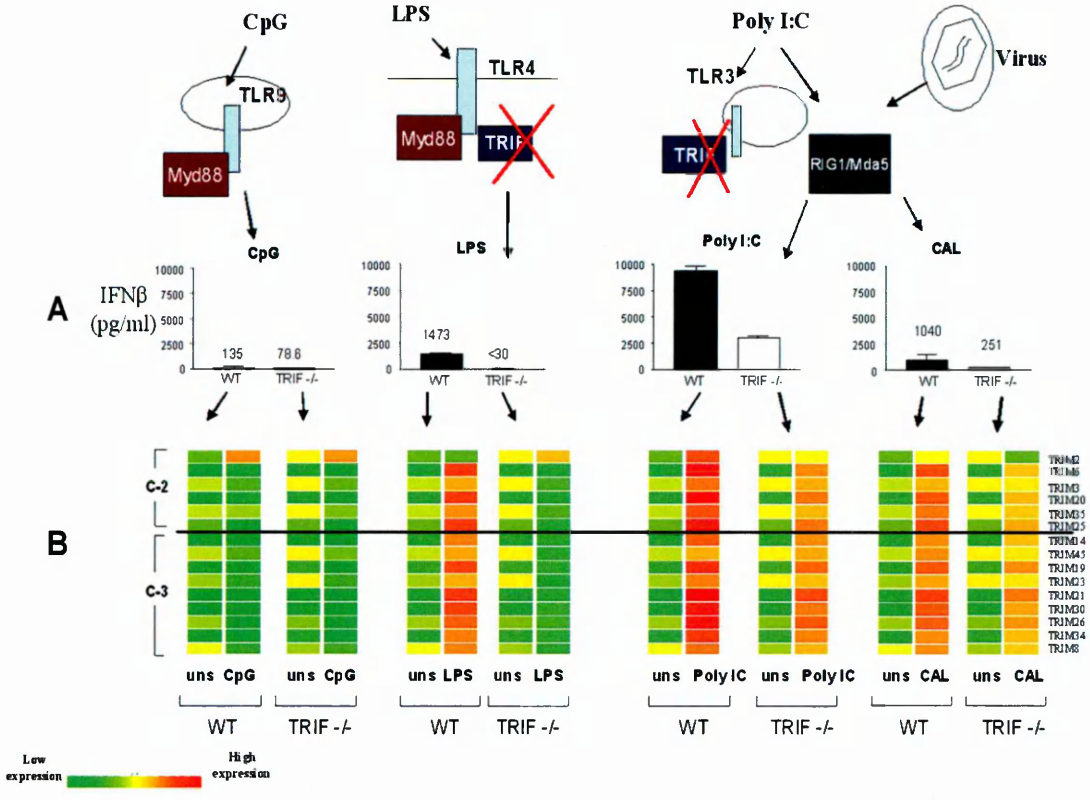



**Figure 5.3. Induction of TRIMs in C-2 and C-3, and IFNβ production are completely impaired in TRIF -/- macrophages stimulated with LPS but not Poly I:C or influenza virus.**

**A)** BM derived macrophages from wild type (WT) C57BL/6 and TRIF-deficient mice were unstimulated (Uns) or treated with Live influenza virus CAL, CpG, LPS or Poly I:C. After 24 h, IFNβ protein was measured by ELISA and **B)** cells were collected for RNA extraction and analysis of TRIM mRNA expression by real-time PCR. A heat map was generated by normalizing the values of each sample to the median of all samples for each gene (as in Figure 5.1); green: low expression; yellow: median value; red: high expression. Only the TRIMs in clusters C-2 and C-3, which expression is significantly up-regulated in WT macrophages upon any of these stimulations, is shown. Data are representative of three independent experiments. A model representing the possible mechanism of IFNβ production by these TLR ligands is shown on top of the figure. CpG activates TLR9 and results in very low levels of IFNβ production by a MyD88-depend, TRIF- independent pathway. LPS activates TLR4 and results in significant production of IFNβ only by a TRIF-dependent pathway. Poly I:C stimulation or infection with influenza virus can activate both TLR3 or the non-TLR, RIG-I/MDA5 resulting in significant levels of IFNβ production by both TRIF dependent and independent mechanisms. For more details see the text. Data are representative of three experiments.

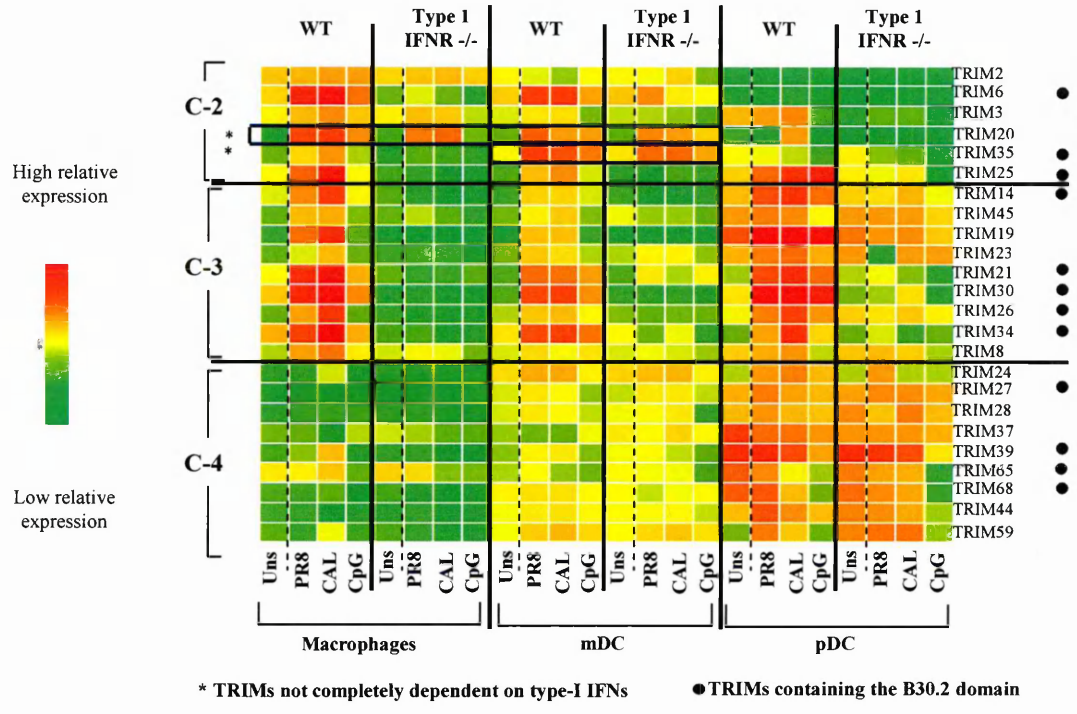



**Figure 5.4. Type-I IFN dependent and independent expression of TRIMs upon Influenza virus infection or CpG stimulation in macrophages and DC.**
Expression of many but not all TRIM induced by CpG or influenza virus is exclusively dependent on type I IFN in macrophages and DC. Macrophages, mDC and pDC from WT and IFNα/βR–/– mice (Type-I IFNR –/–) were infected with influenza virus PR/8 or CAL, or stimulated with CpG for 24 h. TRIM expression was determined by real-time PCR. The heat map was generated as in Fig. 4.1. TRIM expression in C-2 and C-3 was completely dependent on type I IFN. *TRIM20 (macrophages/mDC) and TRIM35 (mDC) expression was not completely inhibited in the IFNR –/– cells. TRIM in C-4 were expressed constitutively in pDC at high levels independently of type I IFN. Data are representative of three experiments for macrophages/mDC and two experiments for pDC. TRIM indicated with solid circles contain a B30.2 domain. Expression of TRIM6, TRIM14, TRIM19, TRIM21, TRIM25, TRIM30, TRIM26 and TRIM34 was highly up-regulated upon viral infection (six to 24 times up-regulation, $p<0.001$), and to lower levels TRIM3, TRIM45, TRIM23 and TRIM8 expression was up-regulated upon stimulation (three to six times up-regulation, $p<0.05$), all in a type I IFN-dependent manner.

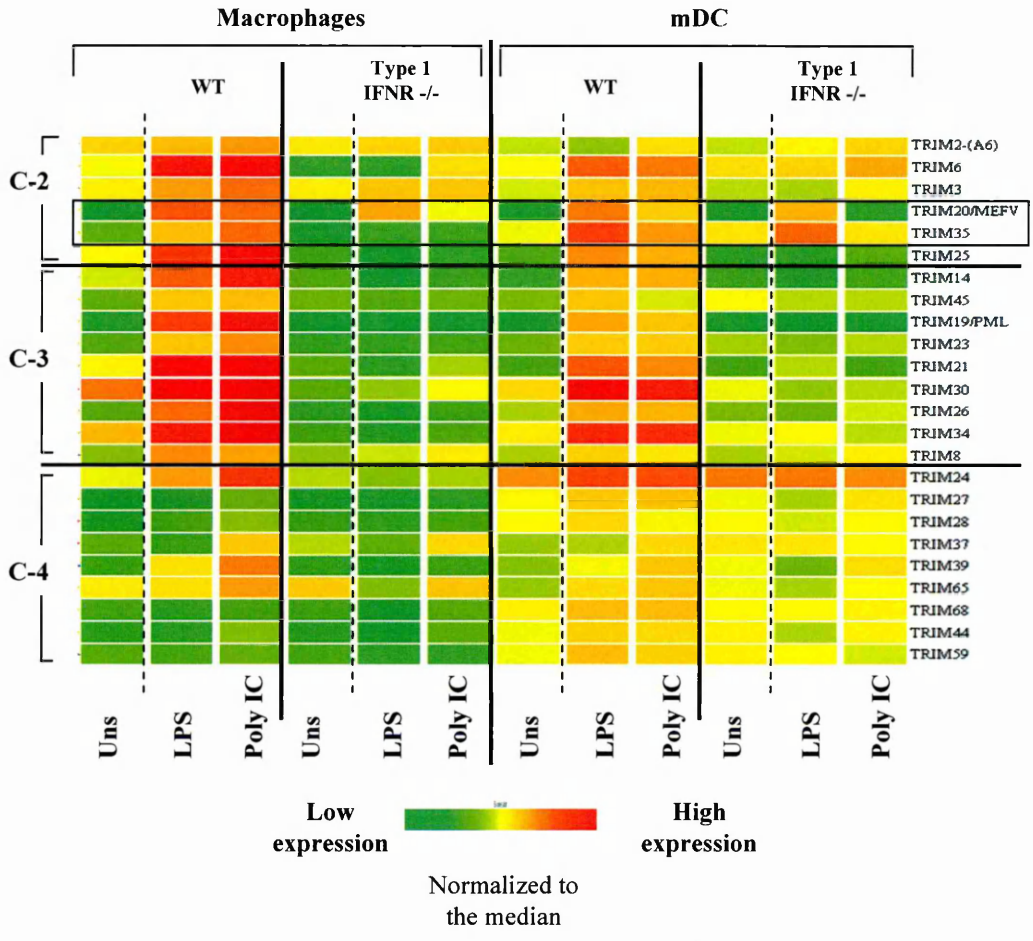



**Figure 5.5. Most TRIMs in C-2 and C-3 are induced by type-I IFNs in macrophages and mDC upon stimulation with LPS and Poly I:C.**

Macrophages and mDC from WT and IFNα/βR–/– mice (Type-I IFNR –/–) were stimulated with LPS or dsRNA (Poly I:C) for 24 h and TRIM expression was determined by real-time PCR. The heat map was generated as in Fig. 3.8. TRIM expression in C-2 and C-3 was completely dependent on type I IFN. *TRIM20 (macrophages/mDC) and TRIM35 (mDC) expression was not completely inhibited in the IFNR –/– cells. Data are representative of three experiments for macrophages/mDC. Expression of TRIM6, TRIM14, TRIM19, TRIM21, TRIM25, TRIM30, TRIM26 and TRIM34 was highly up-regulated upon viral infection (six to 30 times up-regulation, $p<0.001$), and to lower levels TRIM3, TRIM45, TRIM23 and TRIM8 expression was up-regulated upon stimulation (three to six times up-regulation, $p<0.05$), all in a type I IFN-dependent manner. Note that TRIM24 in macrophages is also significantly up-regulated but only statistically significant with Poly I:C, and also in a type-I IFN dependent manner.

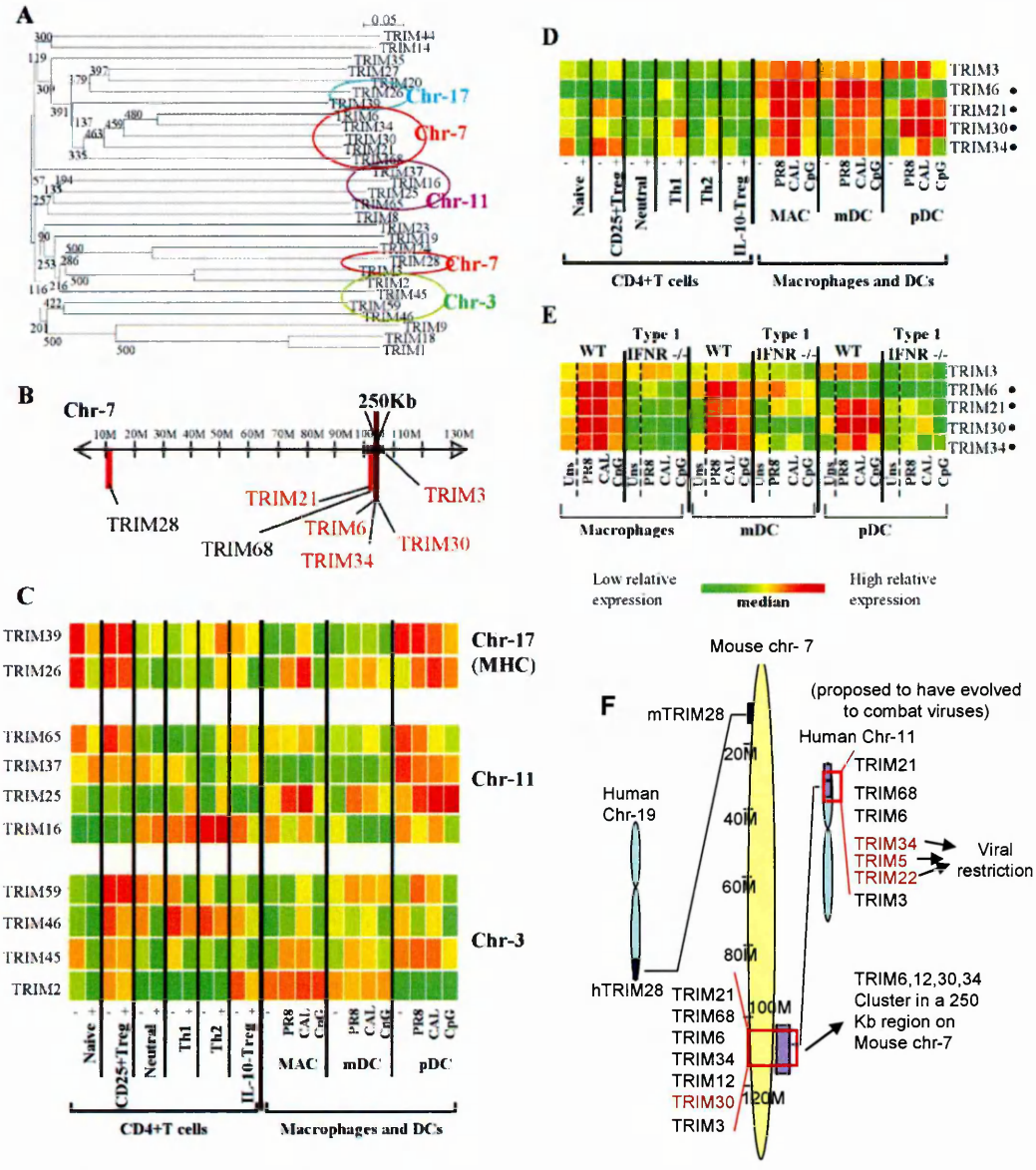



**Figure 5.6. A group of TRIM on chr-7 is preferentially expressed in macrophages and DC and up-regulated exclusively by type I IFN.**

**A)** Phylogenetic analysis of mouse TRIM proteins used in our study. A neighbour-joining tree based on the amino acid sequences of TRIM without their C-terminal domain is shown. Numbers indicate bootstrap proportions after 500 replications. The scale represents 0.05 changes per site. **B)** Representation of TRIM located on mouse chr-7. TRIM6, TRIM30 and TRIM34 are grouped within a 250-kb region on chr-7. TRIM3, TRIM6, TRIM21, TRIM30, TRIM34 (in red) and TRIM68 are located in the F2 region of mouse chr-7. The scale shown indicates megabases (M) from the centromere. **C)** Heat map representation of TRIM mRNA expression in CD4+ T cells, macrophages (MAC) and DC grouped by chromosomal location. Expression of these TRIM does not show any clear correlation with their homology or chromosomal location (selected from Figure 4.1). **D)** Heat map representation of TRIM3, TRIM6, TRIM21, TRIM30 and TRIM34, showing their preferential expression in macrophages and DC (selected from Figure 4.1). **E)** Heat map representation of TRIM3, TRIM6, TRIM21, TRIM30 and TRIM34 mRNA expression in macrophages, mDC and pDC from WT and IFNR1-deficient mice as described in Fig.5.4. Expression of all these TRIM genes, which are located on chr-7, is dependent on type I IFN. TRIM indicated with solid circles contain a B30.2 domain. Values were normalized as described. **F)** Schematic representation of the mouse TRIMs that map to mouse chr-7 and the syntenic human TRIMs which some have been described to have anti-viral activity.

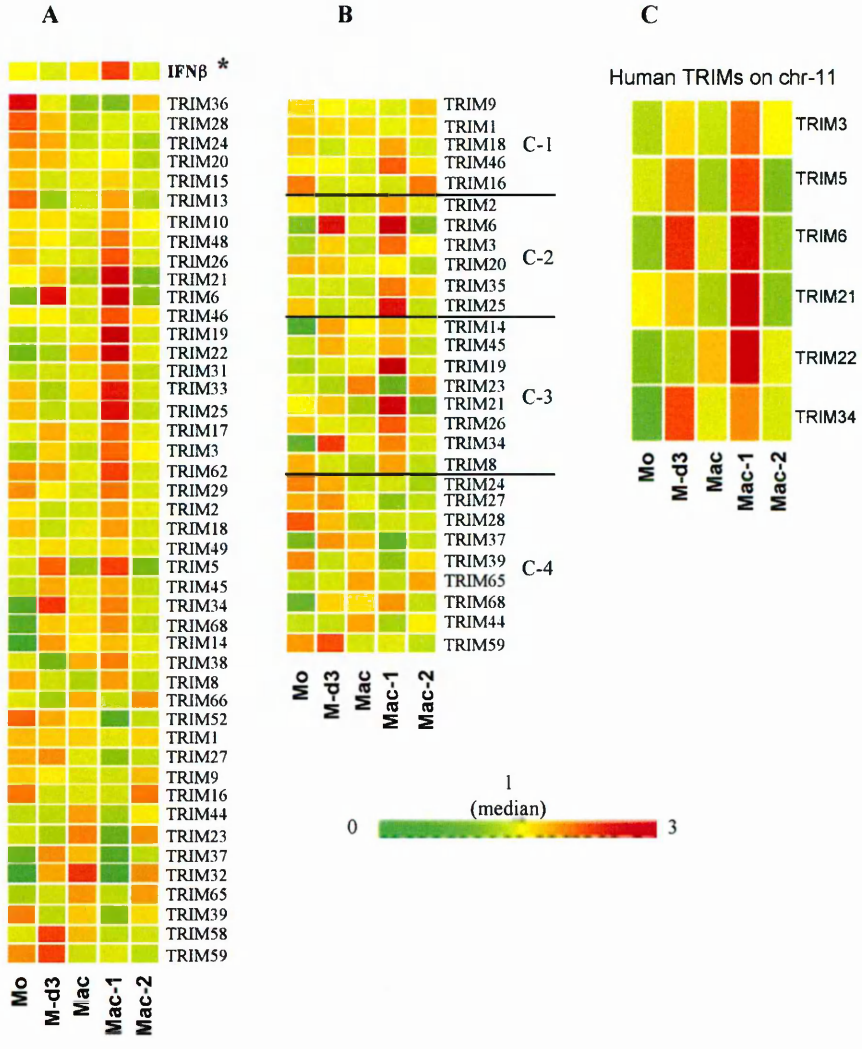



**Figure 5.7. Expression of TRIMs in human monocytes and macrophages in different stages of differentiation and activation, from Martinez et al. [294].**
**A)** Microarray study performed by Martinez et al [294] on human monocytes (Mo) differentiated with MCSF for 3 days (Mo-d3) or 7 days to generate macrophages either untreated (Mac) or treated with LPS/IFNγ (Mac-1) or IL-4 (Mac-2). The microarray data was obtain from the gene expression omnibus (GEO) website (www.ncbi.nlm.nih.gov/geo), accession number GSE5099 and was analyzed by GeneSpring. Expression of all TRIMs present in the affymetrix array HG-U133 is shown. Data was normalized to the median (yellow) of all samples for each gene. In the colour scale, red indicates expression over the median value (up to 3 times) and green represents expression under the median value. A large number of TRIMs are up-regulated upon LPS/IFNγ treatment, correlating with induction of IFNβ (indicated with a star). Hierarchical clustering by Pearson correlation is shown. **B)** Expression of Human TRIMs from Martinez et al [294] organized into the clusters defined in our study (C-1 to C-4, only the TRIMs used for our study in mouse cells are shown). Most of the human TRIMs in clusters C-2 and C-3 are up-regulated in human macrophages treated with LPS and IFNγ which produce IFNβ, supporting our data in mouse on their induction by type-I IFNs. **C)** Expression of human TRIMs from Martinez et al, that map to human chr-11 syntenic to the mouse chr-7. All human TRIMs that map to the region p15.4 of human chr-11 are up-regulated upon LPS and IFNγ treatment which induce IFNβ similarly to our data with TRIMs located on the F2 region of mouse chr-7.

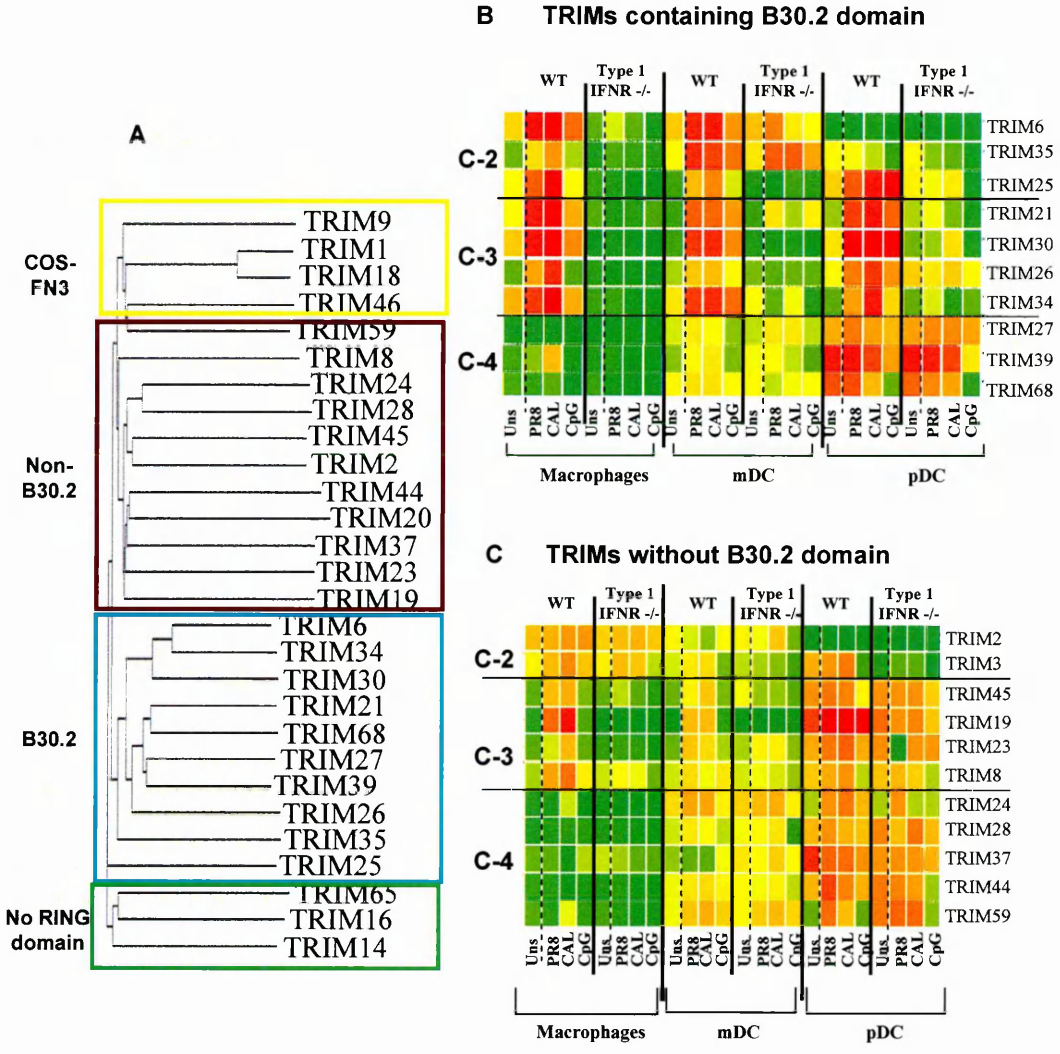



**Figure 5.8. Type-I IFN dependent expression of TRIMs and the B30.2 domain.**
**A)** Phylogenetic analysis of mouse TRIM proteins used in our study. A neighbour-joining tree based on the full length amino acid sequences of TRIM with their C-terminal domain is shown. Numbers indicate bootstrap proportions after 500 replications. TRIMs containing the COS-FN3 domains are in the yellow box. TRIMs without B30.2 domain are in the red box. TRIMs with a B30.2 domain are in the blue box. TRIMs with no RING domain are in the green box. B) Heat map representation of TRIM mRNA expression in macrophages (MAC) and DC upon influenza virus infection or stimulation with CpG, grouped by the presence **(B)** or absence **(C)** of B30.2 domains (these data was selected from Figure 5.4). Although the majority of TRIMs containing the B30.2 domain are type-I IFN dependent, many of the TRIMs without B30.2 domain are also type-I IFN dependent. Furthermore, TRIM19, which does not have a B30.2 domain, has also been shown to have anti-viral functions, therefore these data suggest that type-I IFN may be a better predictor of anti-viral activity than the B30.2 domain. TRIMs in C-1 were excluded from this figure since they are preferentially expressed in CD4+ T cells. TRIM65,16,14, are also not included here because they lack a RING domain. Mouse TRIM20 does not have a B30.2 domain.

| TRIM | Expression in human macrophages by microarray (Martinez etal) | Expression in mouse macrophages by RT-PCR, our study |
|---|---|---|
| TRIM1 | N.A. | N.A. |
| TRIM2 | Low U.R. | Low U.R. |
| TRIM3 | U.R. | U.R. |
| TRIM5 | U.R. | N.E. |
| TRIM6 | U.R. | U.R. |
| TRIM8 | D.R. | U.R. |
| TRIM9 | N.A. | N.A. |
| TRIM10 | U.R. | N.S. |
| TRIM13 | U.R. | N.S. |
| TRIM14 | Low U.R. | U.R. |
| TRIM15 | N.A. | N.S. |
| TRIM16 | D.R. | D.R. |
| TRIM17 | U.R. | N.S. |
| TRIM18 | Low U.R. | Low U.R. |
| TRIM19 | U.R. | U.R. |
| TRIM20 | N.A. | U.R. |
| TRIM21 | U.R. | U.R. |
| TRIM22 | U.R. | N.E. |
| TRIM23 | D.R. | U.R. |
| TRIM24 | N.A. | N.A. |
| TRIM25 | U.R. | U.R. |
| TRIM26 | U.R. | U.R. |
| TRIM27 | D.R. | N.A. |
| TRIM28 | N.A. | N.A. |
| TRIM29 | U.R. | N.S. |
| TRIM30 | N.E. | U.R. |
| TRIM31 | U.R. | N.S. |
| TRIM32 | D.R. | N.S. |
| TRIM33 | U.R. | N.S. |
| TRIM34 | U.R. | U.R. |
| TRIM35 | U.R. | U.R. |
| TRIM36 | N.A. | N.S. |
| TRIM37 | N.A. | N.A. |
| TRIM38 | Low U.R. | N.S. |
| TRIM39 | N.A. | N.A. |
| TRIM44 | D.R. | N.A. |
| TRIM45 | Low U.R. | U.R. |
| TRIM46 | U.R. | U.R. |
| TRIM48 | U.R. | N.S. |
| TRIM49 | N.A. | N.S. |
| TRIM52 | D.R. | N.S. |
| TRIM58 | N.A. | N.S. |
| TRIM59 | N.A. | N.A. |
| TRIM62 | U.R. | N.S. |
| TRIM66 | D.R. | N.S. |
| TRIM68 | Low U.R. | N.A. |

**Table 5.1. Comparison of TRIM expression in mouse macrophages infected with Influenza virus (our study, Real time PCR) and human macrophages stimulated with IFNγ and LPS (from Martinez et al. [294]; by microarray analysis).**
Note that these stimulations in both mouse and human macrophages respectively lead to expression of IFNβ. Not affected, N.A.; Genes up-regulated, U.R.; Genes down-regulated, D.R.; Genes not studied, N.S.; Not exist, N.E

| Cluster | TRIM | Shown when IFNs added | Have anti-viral activity | In MØ Require type1-IFN | In mDC Require type1-IFN | In pDC Require type-1IFN | C-terminal domain |
|---|---|---|---|---|---|---|---|
| C-2 | TRIM2 | | | | | | FIL/Ig/NHL |
| | TRIM6 | | | √ | √ | | B30.2 |
| | TRIM3 | | | √ | √ | √ | FIL/Ig/NHL |
| | TRIM20 | IFNα/γ | | partially | partially | √ | - |
| | TRIM35 | | | √ | partially | | B30.2 |
| C-3 | TRIM25 | IFNα | √ | √ | √ | √ | B30.2 |
| | TRIM14 | | √ | √ | √ | √ | B30.2 |
| | TRIM45 | | Potential | √ | √ | √ | FIL/Ig |
| | TRIM19 | IFNα/β/γ | √ | √ | √ | √ | - |
| | TRIM23 | | | √ | √ | √ | ARF |
| | TRIM21 | IFNγ | | √ | √ | √ | B30.2 |
| | TRIM30 | IFNα/β/γ | √ | √ | √ | √ | B30.2 |
| | TRIM26 | | | √ | √ | √ | B30.2 |
| | TRIM34 | IFNα/γ | √ | √ | √ | √ | B30.2 |
| | TRIM8 | IFNγ | √ | √ | √ | √ | - |

**Table 5.2. Induction of TRIM expression by type-I IFNs may suggest anti-viral activity.** Summary of the TRIMs found in our study to be dependent on type-I IFNs in macrophages, mDC and pDC. Our data confirmed previous studies that showed some TRIMs to be inducible by IFNs when added to the cell culture (shown in red). With exception of TRIM1 which expression is not up-regulated in macrophages and DC, the expression of all the TRIMs found to have anti-viral activity are also regulated by type-I IFNs. This may suggest that type-I IFNs may predict anti-viral function of TRIMs. The data for anti-viral activity was compiled from different reports: [12, 22, 210, 231].

# Chapter 6:

# Future Perspectives

## 6.I. Summary of the Thesis

In this project we have performed a comprehensive analysis of expression of TRIM molecules in different cells of the innate and adaptive immune system including macrophages, mDC and pDC, and a panel of CD4+ T cells, which are all known to have different patterns of cytokine production. The large amount of information generated in this study and the complex set of data obtained here demanded an efficient method of data analysis to be able to observe any correlations between TRIM expression and cytokine production. By generating heat maps for real time PCR data presentation we defined clusters of co-regulation of TRIM gene expression that provide a broad picture which can undoubtedly help to point at strategies for defining TRIM functions.

We have defined four clusters of TRIM molecules on the basis of their distinct expression in either CD4+ T cells or macrophages and DC, which have different innate and adaptive immune functions to an extent determined by their cytokine profile. A group of TRIM genes was preferentially expressed in CD4+ T cells and exclusively contained the COS-FN3 motif associated with protein-protein interactions (Cluster 1). Additional clusters of TRIM were defined on the basis of their up-regulation by influenza viruses and TLR ligands via a type-I IFN-dependent mechanism in macrophages and DC (Clusters 2 and 3), suggesting that this large group of TRIM may play a role in anti-viral responses. Conversely, a distinct group of TRIM genes was constitutively expressed in pDC independently of type-I IFN production (Cluster 4). The classification of TRIMs in this study according to their expression in cells of the innate and adaptive immune system and their dependency on type-I IFNs, provided us with important information that can be used in future

studies to define function of specific TRIMs that may be involved in either innate or adaptive immune responses.

## 6.2. Future studies based on the classification of TRIMs by expression in cells of the immune system and type-I IFN dependency

Taken together, our study opens new avenues for defining the function of TRIM proteins. Based on our classification of TRIM proteins by their levels of expression we can propose to study TRIM function in the context of their clusters of expression in the respective primary cell subsets where they are found to be specifically expressed. However, future studies to complement our findings should also include the analysis of TRIMs protein to test if the changes in mRNA expression indeed translate into changes in protein levels. In addition, future studies should also take in consideration the fact that many TRIMs may have post-translational modifications that may affect their function, as well as the possible presence of TRIM isoforms.

The following are some of the proposed studies to delineate TRIM function, which come to mind, based on our classification of TRIM molecules.

### 6.2.1. Future studies on TRIMs in Cluster-1

An important outcome of our study is the fact that TRIMs in C-1 showed a strong correlation of structural homology with preferential expression in CD4+ T cells. TRIM1, 9, 18, 46 in C-1 possess the COS-FN3 domains and this raises the possibility that other TRIMs containing this domain organization not tested in our study may also be preferentially expressed in T cells. Therefore, our study could be expanded to test if the other COS-FN3 containing TRIMs (TRIM36 and TRIM67),

would follow the same pattern of expression as the TRIMs in C-1. Another important aspect of these findings is that TRIMs in C-1 (TRIM1, 9, 18, 46) have been previously reported to bind microtubules via this COS-FN3 domain [38]. Microtubule organization has been shown to be important during immune function since microtubule dynamics is crucial for T cell activation and can regulate the establishment of cell polarity, cell migration, and direct secretion of cytokines and cytolytic granules [306]. Therefore, generation of conditional knockouts of these C-1 TRIMs in T cells may reveal specific effects on function *in vitro* and *in vivo*. In addition, since all of these TRIMs share high degree of homology and TRIM18 has been previously implicated in MAP kinase signalling pathways [276, 277], it is possible that other TRIMs in C-1 may also be involved in signalling pathways. To test this possibility, future studies could include searching for post-translational modifications in TRIM proteins which are important in signal transduction pathways (e.g. phosphorylation or ubiquitination). Over-expression studies of these TRIMs to test if they are involved in pathways to induce cytokine production could be complemented by the generation of conditional knockout mice where CD4+ T cells are generated to lack functional forms of these TRIMs. Other experimental approaches could include knockdown assays in CD4+ T cells using small interfering RNA (SiRNA) of these TRIMs either individually or in combination to explore their possible contribution in common functions in T cells.

### 6.2.2 Future studies on TRIMs in C-2 and C-3

The large number of TRIMs classified in C-2 and C-3 that we found highly expressed in cells of the innate immune system supports a role of these TRIMs in innate immune responses. Since many of the TRIM proteins that have been

previously shown to have anti-viral activity are also inducible by type-I IFNs, it could be hypothesized that induction of TRIM expression in a type-I IFN dependent manner may predict anti-viral function. To test this, future studies could focus on all of the type-I IFN dependent TRIMs (TRIMs in C-2 and C-3) to test their potential anti-viral activity against a broad panel of viruses, not only retroviruses, and should include *in vitro* and *in vivo* experimental systems. Viral replication could be tested by using GFP labelled viruses and Flow cytometry to obtained information at the single cell level and the effects on the innate immune system (e.g. cytokine production) or direct anti-viral activity. Testing the anti-viral effects of each TRIM could also be done by knocking down TRIM expression using SiRNA or knockout mice, or conversely over-expressing specific TRIMs against the panel of viruses. If anti-viral activity is detected, indirect mechanisms via induction of innate signalling pathways (e.g. cytokines) could be tested by using cells lacking functional forms of different cytokine receptors, for example TNF receptor knockouts or the type-I IFNRA1 -/-.

Our study also opens a new opportunity to expand the investigation to the rest of the TRIMs in this family to address their possible regulation by type-I IFNs. This could be done by repeating our experiment using cells from the type-I IFNRA -/- versus wild type controls including all the rest of the TRIM family members and analyze their expression by using new, low cost high sensitivity and high throughput microarray Chips (Illumina Sentrix).

#### 6.2.2.1. Inducible expression of TRIMs by type-I IFNs versus type-II IFNs (IFNγ).

Signalling by Type-I (e.g. IFNα/β) and Type-II (IFNγ) IFNs may lead, in some cases, to induction of common genes. However, some distinct differences in these

pathways may result in induction of specific genes for each pathway. IFNγ signalling results in the formation of STAT1 homodimers which bind GAS elements, a distinct sequence from the ISRE sequence in type-I IFN inducible genes [147, 181](For more details see section 1.5.1.6.). Although it is possible that TRIMs in C-2 and C-3 are specifically induced by type-I IFNs but not IFNγ, in our study we can not rule out the possibility that IFNγ may induce TRIM expression in macrophages and DC. Also, we cannot rule out the possibility that IFNγ may induce the production of IFNβ and thus subsequent up-regulation of TRIM expression. To test the possible role of IFNγ in induction of TRIM expression, IFNγ could be added to macrophages and DC in the presence or absence of stimulation with different TLR ligands. Alternatively, TRIM expression could be tested in cells from IFNγ receptor knockout mice upon different stimulations of whole organ cell suspensions (e.g. mouse spleen or human PBMC). The IFNγ effects could also be blocked using specific antibodies or performing the experiments in presence or absence of SiRNA for IFNγ. If these experiments provide data that can distinguish TRIMs induced by type-I IFNs from TRIMs induced by IFNγ, then the up-regulation of these type-I IFN specific TRIMs could be used in the future as an indicator of the presence of type-I IFNs during *in vivo* infections where the cytokine protein may be more difficult to detect due to sensitivity or kinetic constrains.

#### 6.2.2.2. Induction of TRIM expression in CD4+ T cells by type-I or type-II IFNs

We have shown that TRIMs in C-2 and C-3 are highly induced in macrophages and DC in a type-I IFN dependent manner, but for the most part, the expression of

these TRIMs was not significantly up-regulated by IFNγ in CD4+ T cells. This is judged by the fact that TCR stimulation of Th1 cells which produce high levels of IFNγ, did not result in a significant increase of TRIM expression. This discrepancy could be explained by intrinsic differences between macrophages/DC and CD4+ T cells to express specific TRIMs, or alternatively TRIM genes promoters could contain only ISRE elements and not GAS elements and therefore would be responsive only to type-I IFNs and not IFNγ, as explained above. However, in our study we did not measure type-I IFNs produced by CD4+ T cells, consequently we can not rule out that TRIMs are not induced in T cells by type-I IFNs. Since most cells are capable of producing IFNβ upon viral infection and also express the type-I IFN receptor, it is thus possible that the TRIMs found in our study in C-2 and C-3 may also be induced in CD4+ T cells in conditions where type-I IFNs are present. This could be tested using CD4+ T cells derived from the type-I IFN receptor deficient mice and infections with viruses that may target CD4+T cells and may result in production of IFNβ. On the other hand, it is also possible that expression of TRIMs in C-2 and C-3 may be induced by type-I IFNs in a paracrine manner when TLR or virus stimulated DC are present. This could be tested firstly by adding exogenous type-I IFN cytokine to the T cell culture, in the presence or absence of TCR activation. If up-regulation of TRIM expression is detected, then the possible type-I IFN dependent effect could be then tested in CD4+ T cells derived from IFNRA -/- mice. These experiments could indicate if CD4+ T cells, macrophages and DC have different intrinsic capacities to express this group of TRIMs, or if it is only the lack of type-I IFNs that leads to no up-regulation of these TRIMs in T cells.

### 6.2.3. Future studies on TRIMs in C-4

An interesting outcome of our study is the finding of highly expressed TRIMs in pDC (TRIMs in C-4). These cells are highly specialized and are extremely important in secreting high levels of type-I IFNs during viral infections [303]. This raises the question as to whether TRIMs in C-4 may be related to type-I IFN production, differentiation pathways, or anti-viral functions. Although this group of TRIMs is also expressed in high levels in CD4+T cells suggesting a potential relationship between these cell subsets as previously suggested [287], the generation of mice lacking functional forms of these TRIMs in either pDC or CD4+ T cells conditionally, may reveal answers to these questions.

## 6.3. Future studies on individual TRIMs: TRIM19/PML

Our study demonstrated a large number of TRIMs that show interesting patterns of expression and could be chosen to be studied individually. We have identified the expression of 15 TRIMs that are exclusively dependent on type-I IFNs in primary DC and macrophages. Within this group, TRIM19/PML showed an interesting pattern of expression since it was also highly expressed in pDC even without stimulation. In addition, TRIM19/PML is also attractive for further investigation because it has been previously shown to be involved in a variety of cellular functions including the TGFβ signalling pathway [204], IFNγ signalling pathway [48], chromatin remodelling, transcriptional repression and activation as part of the nuclear bodies [31], however its predominant role is still unclear. Induction of TRIM19 expression by type-I IFNs may have an effect in the regulation of a wide variety of signalling and differentiation pathways and it is possible that TRIM19 may thus function at a broader level with respect to immune responses to pathogens.

**Figure 6.1** shows a model of the mechanism of regulation of TRIM19 expression and its effects on other cellular functions. *In vivo* studies are very limited and have failed to provide any mechanism on TRIM19/PML function during *in vivo* infections with VSV and LCMV [226]. Although a few studies have been reported on the possible effects of TRIM molecules on influenza infection in epithelial cell lines *in vitro*, little has been done on the potential role of TRIM19 in *in vivo* during influenza virus infection.

To test the possible anti-viral role of TRIM19/PML *in vivo*, we propose to infect TRIM19/PML -/- mice with influenza virus infection and analyze for:

-Cytokine production (Type-I IFNs, IFNγ, IL-12, IL-23 TNF, IL-10, TGFβ)

-Viral load

- Weight loss

- Antibody titres

We have performed some preliminary experiments to test the possible role of TRIM19/PML in regulation of cytokine production in response to TLR stimulation. Using *ex-vivo* DC from wild type and PML -/- mice stimulated with CpG we have observed that IL-12p40 levels are significantly higher in myeloid $CD11b^{+}$, $CD8\alpha^{-}$ DC from PML -/- mice as compared to equivalent cells from littermate controls (data not shown). This could have an important effect during clearance of bacterial infections and intracellular pathogens that require Th1 responses, since it has been shown that IL-12 from infected macrophages or DC is essential to sustain effector Th1 cells generated *in vivo* to mediate long-term protection to intracellular pathogens [307-309]. Thus far although bacteria can induce IFNγ or type-I IFNs, nothing is known as to whether TRIMs are induced during bacterial infections or whether they

have any anti-bacterial effect. To address this point we will perform *in vivo* experiments in TRIM19/PML -/- mice infected with *Listeria monocytogenes*. These studies will include:

- Testing for bacterial load and time for development of pathogenicity
- Cytokines in serum and cell suspensions from infected organs
- CD4 and CD8 responses and macrophages/DC *ex-vivo*

These studies will have a great impact in our understanding of TRIM function in immunity and may lead to develop better strategies to control viral and bacterial infections.

In addition, it will be of interest to investigate the possible role of TRIM19 in CD4+ T cells. Given that TRIM19 may be involved in TGFβ and IFNγ signalling, it is of interest to test whether TRIM19 plays a role in the TCR dependent differentiation of effector CD4+ T cells including Th1, Th2, Th17 and IL-10Treg.

The data that we present in this thesis points to a major role of the TRIM family of proteins in the innate immune responses to viruses however, our study also opens new possibilities to study TRIM proteins as they may also be involved in broader responses to pathogens and may also be involved in adaptive immune responses.

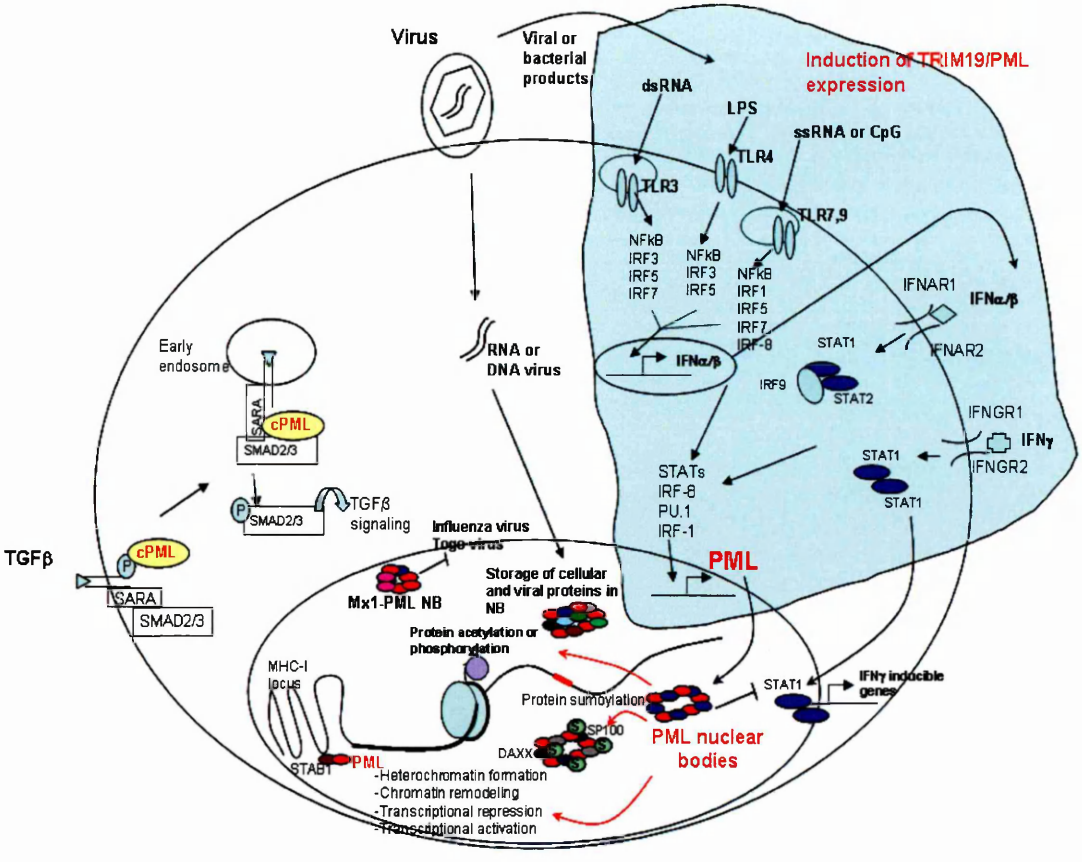



**Figure 6.1. Different functions of TRIM19/PML upon induction by viruses via type-I IFNs.**

Schematic representation of the mechanism of TRIM19/PML up-regulation of expression upon viral infection via type-II FNs and the many cellular functions attributed to TRIM19 (PML). Upon viral infection, viral products (ssRNA, dsRNA, CpG) stimulate TLRs to induce type-I IFNs production by activating a set of transcription factors that may differ between cell types (NF-kB, IRF-3, IRF-5, IRF-7, IRF-1, IRF-8, discussed in Section 1.5.1.5). Type-I IFNs (and also IFNγ), then bind to their receptors to induce expression of TRIM19 by activating STATs, IRF-8, IRF-9, IRF-1. TRIM19/PML then can exert its functions which include: interactions with other proteins like SP100 and DAXX to form nuclear bodies (NB) which are important in regulation of transcription, storage of proteins, sumoylation and anti-viral functions. TRIM19/PML can regulate chromatin remodelling of the MHC-1 locus, or inhibit replication of viruses like Influenza, Thogoto virus and others. TRIM19 can also negatively regulate IFNγ signalling by inhibiting STAT1 transcriptional activity. The cytoplasmic isoform of TRIM19 (cPML) binds to the TGFβ receptor and serves as an adaptor molecule to recruit SARA and SMAD2/3 and initiate TGFβ signalling.

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