SCIENTIFIC REPORT

EXPRESSION AND ACTIVITY OF INTRACELLULAR PATTERN RECOGNITION RECEPTORS IN DENDRITIC CELLS AND THEIR ROLE IN ANTI-VIRAL IMMUNITY

The ultimate goal of our studies was to discover novel mechanisms that regulate the functional activities and the collaboration of pattern recognition receptors (PRR) driving anti-viral responses. Detection of viral structures is mediated by a limited set of intracellular PRR among them Toll-like receptors (TLR) and retinoic acid induced gene-like (RLR) receptors. Activation of TLRs and RLRs by their specific ligands results in the activation of the nuclear factor-κB (NF-κB) and interferon regulatory factors 3 and 7 (IRF3/7) playing critical roles in antiviral immunity. This process is supported by the mitochondrial antiviral signaling protein (MAVS) that links the downstream signalling events to the NF-κB and IRF transcription factors. Stimulation of these receptors is known to be associated with the secretion of interferons (IFN) acting as potent soluble factors against viruses. Stimulator of IFN genes (STING) is a transmembrane adaptor protein involved in downstream activation of IRF3 via TANK-binding kinase-1 (TBK1) that mediates a potent defense mechanism against intracellular pathogens. The ETS-related transcripton factor ELF4 has recently been shown to interact with STING leading to TBK1-mediated activation and translocation of ELF4 to IFN promoters. The cooperative binding of STING, TBK1 and ELF4 increase the binding affinity of IRF3/7 to the newly identified EICE elements in the IFN promoters. It has also been shown that *elf4* gene expression could be increased by viral challange or IFNβ stimulation, while the overexpression of ELF4 protein results in the inhibition of viral replication confirming the essential role of ELF4 in establishing an antiviral state and facilitate anti-viral immune responses. Most importantly, ELF4 is involved in the orchestration of TLR4/7/9/ and also RLR-induced signal transduction confirming the regulatory potential of ELF4 acting by TBK1-mediated phosphorylation and translocation to the nucleus in a STING and MAVS dependent manner. Thus the EICE element in the *Ifnb1* promoter could be identified as a critical component that promotes the cooperative interaction of ELF4 via binding IRF3, IRF7 and p65 to IFN promoters, while the basal expression of ELF4 remains low indicating an induced regulatory circuit that can rapidly be mobilized to accelerate IFN responses **(1).**

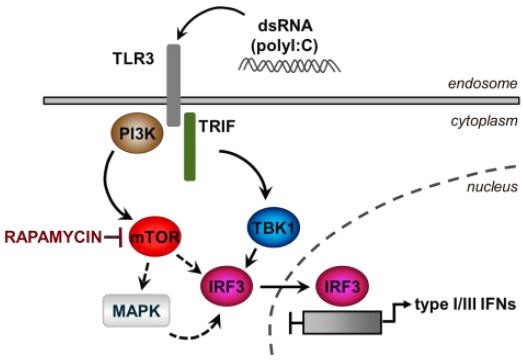
The cytosolic RIG-like receptor family members including RIG-I, MDA5 and LGP2 are involved in type I IFN production and antiviral immunity. We found that the expression of RLR genes and proteins and also the activity of the coupled signaling pathways are significantly higher in the human $CD1a^+$ monocyte-derived DC (moDC) subset than in its phenotypically and functionally distinct $CD1a^-$ counterpart. The specific activation of moDC with poly(I:C) or influenza virus (A/H1N1) induced IFNβ secretion via IRF3, whereas the induction of proinflammatory cytokine and chemokine (IL1β, IL-6, TNF-α, IL-8) responses were controlled predominantly by Toll-like receptor-3 (TLR3). The requirement of RLR-mediated signaling for priming autologous naïve $CD8^+$ T-lymphocytes in $CD1a^+$ moDC and inducing influenza virus-specific cellular immune responses was revealed by silencing RIG-I/MDA5 expression by siRNA in an *in vitro* infection protocol. These results demonstrated the moDC subset-specific activation of RLRs and the underlying mechanisms behind cytokine secretion was identified as a $CD1a^+$ inflammatory moDC subset with specialized functional activities. Furthermore, our immunohistochemical analyses provided evidence of the migratory potential of $CD1a^+$ confirmed by its presence in both human tonsil and reactive lymph nodes **(2).**

The question whether plasmacytoid DC (pDC) and conventional DC (cDC) characterized by different phenotypic, functional and migratory potential could collaborate in mediating

antiviral responses, the significance of pDC and cDC subtypes against different viruses was summarized in a review. In this article special emphasis was put on the the functional importance of TLR and RIG-I expression levels and the modulatory role of escape mechanisms involved in the recognition of different viral genomes **(3).**

Considering that certain viruses can induce malignant transformation and share defense strategies against viruses and tumors the involvement of TLR and MDA5 was investigated in human melanoma cells. The incidence of melanoma in the last three decades has increased worldwide and no effective treatment modalities have been developed sofar. All-trans retinoic acid (ATRA) and polyI:C are strong inducers of TLR3 and MDA5 expression, and polyI:C-induced TLR3 and MDA5 signaling causes cell death in melanoma cells *in vitro*. We found that the combined treatment of human melanoma cell lines with ATRA and polyI:C strongly increased the expression of TLR3 and MDA5 in both the WM35 and WM983A cells associated with significantly higher mRNA levels and secreted IFNβ, CXCL1, CXCL8/IL-8, CXCL9 and CXCL10 as compared to cells treated with either ATRA or polyI:C. The induced CXCL chemokines had a pivotal role in recruiting inflammatory and cytotoxic cells to the tumor microenvironment. Silencing of MDA5 by siRNA moderately affected IFNβ secretion, whereas TLR3 silencing interfered with both CXCL chemokine and IFNβ production. The supernatants of activated melanoma cells activated by ATRA+polyI:C increased the migratory potential of macrophages and $CD1a^+$ moDC significantly as compared to single treatment. Interestingly, this effect was TLR3 dependent and the consecutive treatment of melanoma cells with ATRA followed by polyI:C resulted in strong, TLR3 and MDA5 mediated chemokine and IFN responses. Thus cultured human melanoma cells can trigger professional antigen-presenting cells (APC) to develop inflammatory $CD1a^+$ cells. This novel mode of concomitant melanoma cell activation may represent a more efficient treatment option for future melanoma therapies **(4).**

Human DC subtypes express intracellular nucleic acid sensing TLRs, while the RLR family member RIG-I can recognize distinct types of RNAs in the cytoplasm. Both TLR3 and MDA5 sense the dsRNA analog polyI:C and elicits $CD8^+$ T-cell responses with anti-tumor activity. DC subtypes and subsets are specialized to recognize and internalize viruses, process viral proteins and present antigenic peptides to T-lymphocytes in a major histocompatibility complex (MHC) dependent manner. This process is accompanied by the activation of the NF-κB and MAPK pathways contributing to the induction of type I and/or type III IFNs (IFN-λ), which act as potent regulators of both innate and adaptive immune responses. The heterodimeric cytokine IL-27, produced by human $CD1c^+$ DC also exert antiviral activity upon stimulation by viral dsRNA through inhibiting viral replication and amplifying $CD8^+$ T-cell proliferation and cytotoxic activity.



Monitoring polyI:C-induced expression of type I (α β) and type III (λ1-3) interferons in different human DC subtypes revealed the secretion of type I and III IFN family members in both circulating $CD1c^+$ and moDC. The PI3K/mTOR pathway is also essential to elicit intact type I and III IFN responses in these DC subtypes. When the impact of rapamycin pretreated moDC on the effector functions of autologous naive $CD8^+$ T-cells was tested we found that the T-cell stimulatory capacity of polyI:C-activated moDC could dramatically be decreased by mTOR inhibition. IRF3 is considered as the master regulator of IFN production, but as a result of rapamycin pre-treatment IRF3 phosphorylation decreased after brief polyI:C

stimulation. The background of reduced type I and III IFN production induced by inhibited mTOR activity was also confirmed by blocking experiments using synthetic inhibitors interfering with PI3K activity and gene silencing targeting TBK1. These results suggested that mTOR exerts its modulatory effect through the classical PI3K/Akt/mTOR pathway and can regulate the induction of type I and III IFNs along with TRIF-mediated TBK1. Thus mTOR has a positive regulatory effect on type I/III IFN production via IRF3, which is under the simultaneous control of the PI3K/mTOR pathway and TBK1. These novel results provide with additional insight into the complexity of mTOR-mediated regulation of DC activities that could be relevant to improve the therapeutic potential of rapamycin in case of uncontrolled type I IFN production **(5)**.

Increasing body of evidence supports that mitochondrial MAVS proteins coordinate signals from cytosolic PRR to induce the expression of antiviral genes. The MAVS regulome and the cellular factors involved are the targets of intense research. To overcome competition in this field we focused to a different aspect of the involvement of mitochondria in innate immune responses and investigated oxidative stress characterized by elevated levels of damage-associated molecular patterns (DAMPS) released from injured or even living cells. One representative of DAMPS is extracellular mitochondrial DNA (mtDNA) containing evolutionary conserved unmethylated CpG repeats. Increased levels of reactive oxygen species (ROS) generated by recruited inflammatory cells modify mtDNA oxidatively resulting in the accumulation of 8-oxo-7,8-dihydroguanine (8-oxoG) lesions. We have examined the impact of native and oxidatively modified mtDNAs on the phenotypic and functional properties of plasmacytoid dendritic cells (pDC). Treatment of human primary pDC with native mtDNA up-regulated the expression of co-stimulatory and antigen-presenting molecules on the cell surface and increased TNF-α and IL-8 secretion from the cells. The effects were more apparent when pDC were exposed to oxidatively modified mtDNA. In a murine model, oxidized mtDNA was proved as a more potent activator of pDC compared to the native form, except for induction of IFNα production. Our results suggest that oxidized mtDNA derived from stressed, damaged or dying cells during inflammation exacerbates acute and chronic immune processes by eliciting the production of chemokines and pro-inflammatory cytokines from TLR9-expressing cells **(6).**

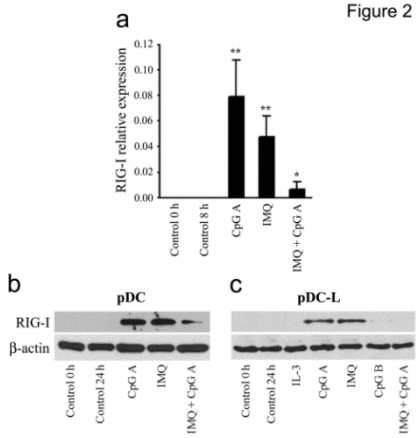
Initiation of allergic sensitization depends preferentially on the ability of inhaled allergens to generate danger signals that activate DC. Ragweed (*Ambrosia artemisiifolia*) pollen grains, which are considered too large to reach the lower respiratory tract, release subpollen particles (SPPs) of respirable size upon hydration. These SPPs contain allergenic proteins and functional NAD(P)H oxidases. We examined whether exposure to SPPs could initiate the activation of human moDC and found that treatment of moDC with freshly isolated ragweed SPPs increased the intracellular levels of ROS, up-regulated the cell surface expression of costimulatory and antigen-presenting molecules and increased the production of TNF-α, IL-6, IL-8, and IL-10. Co-culture of SPP-treated moDC with allogeneic $CD3^+$ pan-T cells resulted in increased secretion of IFNγ and IL-17 by T-cells of both allergic and non-allergic subjects, but induced the production of IL-4 exclusively from the T-cells of allergic individuals. We have also proved that these processes were mediated, at least partly, by the intrinsic NAD(P)H oxidase activity of SPPs. Our data suggest that inhaled ragweed SPPs are fully capable of activating DC in the airways and the NAD(P)H oxidase activity of SPPs is involved in the initiation of adaptive immune responses against innocuous pollen proteins **(7).**

Previous observations suggest that static magnetic field (SMF) exposure acts on living organisms partly through ROS reactions. In a study, the effects of SMF-exposure were studied in a murine model of allergic inflammation and also in human provoked skin allergy.

We found that even a single 30 min exposure of mice to SMF immediately following intranasal RWPE challenge significantly lowered the increase of total antioxidant capacity of the airways and decreased allergic inflammation. Under cell-free conditions SMF-exposure did not alter ROS production by RWPE, while diminished the RWPE induced increase in of ROS levels in cultured lung epithelial cells. Results of the human skin prick tests indicated that SMF exposure had no significant direct effect on provoked mast cell degranulation. The beneficial effects of SMF observed are likely due to the mobilization of cellular ROS-eliminating mechanisms rather than direct modulation of ROS production by pollen NAD(P)H oxidases **(8).**

Plasmacytoid dendritic cells (pDC) are professional type I IFN-producing cells that play essential role in antiviral immunity. The detection of intracellular pathogens is mostly dependent on endosomal PRRs. Since the possible interplay of these two systems has not yet been elucidated, we investigated the collaboration of endosomal TLRs and RIG-I in primary human pDC. We found that under steady-state conditions, pDC express RIG-I at very low level, but its expression gets rapidly and dramatically upregulated upon stimulation by the TLR7 ligand imiquimod (IMQ) or the TLR9 ligand type A CpG. We also demonstrated that pDC are able to sense and respond to the highly specific RIG-I ligand 5'-triphosphate double-stranded RNA (5'-ppp-dsRNA) only following activation by endosomal TLRs. Experiments on primary pDC with functionally blocked IFNα/β receptor 1 (IFNAR1) and on human pDC leukemia (pDC-L) cells defective in type I IFN secretion indicated that the upregulation of RIG-I expression in pDC upon stimulation by endosomal TLR occurs in a type I IFN-independent manner. Selective phosphorylation of signal transducer and activator of transcription 1 (STAT1) on tyrosine 701 was identified as an early signaling event in this process. These results showed that in contrast to many other cell types where RIG-I expression is induced by type I IFN, in pDC a disparate mechanism is responsible for the upregulation of RIG-I. Our findings also indicate that along with autophagy, an additional mechanism is operating in pDC to promote the detection of replicating viruses **(9).**

Figure 2



Protein antigens and subunit vaccines are poorly immunogenic, but their stimulatory potential can be enhanced by appropriate adjuvants. The primary targets of vaccine adjuvants are innate immune cells including monocytes, macrophages and DC subtypes considered as major regulators of immune responses. Upon stimulation with PRR ligands these cells can induce inflammatory reactions or mediate regulatory mechanisms. pDC and cDC subsets exhibit distinct tissue localization, migratory and functional activities and differ in their potantial to produce type I interferons. IC31® is a two-component adjuvant consisting of the artificial antimicrobial cationic peptide KLK that facilitates the uptake and delivery of ODN1 into TLR9 positive intracellular vesicular compartments and the TLR9 stimulatory oligodeoxynucleotide ODN1a. Due to its unique properties IC31® was implicated in TLR9 triggering induced by specific ligands. As TLR9 mediated stimulation is intimately linked to type I interferon production, and is also able to induce type I interferon production in a Stat1-dependent manner, we sought to identify the mode of action and the regulatory functions of the two-component adjuvant IC31®. The results revealed that IC31® was accumulated in the MHC class II positive compartments of peripheral blood macrophages and moDC that was accompanied by the inhibited secretion of TNF-α and IL-6 cytokines, while the production of

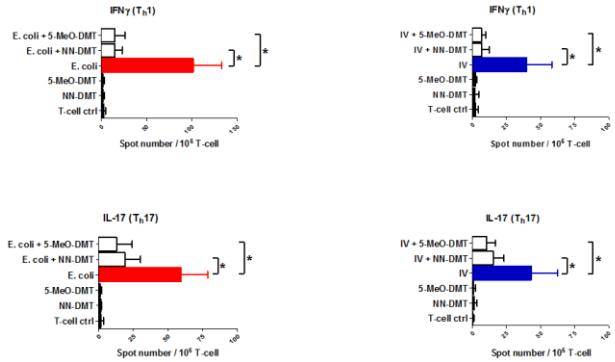
type I interferons was upregulated. Interaction with specific ligands induced signalling events through TLR3, TLR7 and TLR8 receptors, while TLR9 could be stimulated exclusively by the adjuvant. Phosphorylation of NF-κB gene and protein was inhibited in moDC, but was accompanied by increased type I interferon secretion indicating the operation of a unique signalling pathway. In conclusion, IC31® acting via the transcription factors NF-κB and IRF3 was validated as a potent adjuvant against intracellular pathogens and tumors. Furthermore, it acts in a pathway dependent manner via secreting IFNβ that supports the development of Th1-polarized immune responses relevant to protect against viral infections **(10).**

Proper stimulation of tissue moDC results in CCR7 chemokine receptor activation and moDC mobilization. This process is guided by matrix metalloproteinases (MMP) having endopeptidase activity and controlled by tissue inhibitor of metalloproteinases (TIMP) with MMP inhibitory potential. Our *in vitro* studies showed that MMP and TIMP proteins exhibited opposing activities in $CD1a^-$ and $CD1a^+$ moDC subsets. Under inflammatory conditions the synthetic inhibitor GM6001 interfered with the migration of moDC via inhibiting MMP activity, which may have therapeutic significance **(11)**.

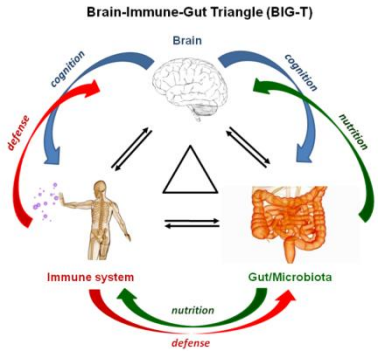
The functional importance of moDC regulation by MMP and TIMP proteins was further analyzed in another experimental setting, where the impact of mesenchymal like stromal (MSCl) cells on the functional activities of activated moDC was investigated. MSC play important roles in the maintenance of the bone marrow (BM) niche by inhibiting the differentiation of hematopoietic stem cells (HSCs) through direct cell-to-cell contacts and in concert with the released factors that support tissue repair through regulating cell differentiation and immunomodulation. Based on their wide spread physiological functions, we adressed the question how antiviral immune responses could be regulated by intracellular PPR in DC in the absence or presence of MSCl cells, followed by activation with specific ligands of the RIG-I helicase. We found that activated DC cocultured with MSCl cells exhibited reduced expression of CD1a and CD83 cell surface molecules serving as phenotypic indicators of DC differentiation and activation, respectively. Intrestingly, RIG-Imediated stimulation of DC through specific TLR ligands in the presence of MSCl cells resulted in significantly higher expression of the costimulatory molecules CD80 and CD86, than in the presence of bone marrow-derived MSC. In line with these results, the concentration of IL-6, IL-10 and CXCL8 cytokines increased in the supernatant of the DC-MSCl cocultures, while the secretion of TNF-α, CXCL10, IL-12 and IFNγ was reduced. Furthermore, the concerted action of mechanisms involved in the regulation of DC migration resulted in the blockade of cell trafficing indicating altered DC functionality mediated by MSCl cellderived signals and mechanisms resulting in a suppressive microenvironment **(12).**

The orphan receptor sigmar-1 is a transmembrane chaperone expressed in both the central nervous system and in immune cells. It has been shown to regulate neuronal differentiation and cell survival, and mediates anti-inflammatory responses and immunosuppression in murine in vivo models. It also has been shown to regulate neuronal differentiation and cell survival, and mediates anti-inflammatory responses and immunosuppression in murine in vivo models. Since the details of these findings have not been elucidated so far, we studied the effects of the endogenous sigmar-1 ligands N,N-dimethyltryptamine (NN-DMT), its derivative 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT) and the synthetic high affinity sigmar-1 agonist PRE-084 hydrochloride on human primary moDC activation provoked by LPS, polyI:C or pathogen-derived stimuli to induce inflammatory responses. Co-treatment of moDC with these activators and sigma-1 receptor ligands inhibited the production of pro-inflammatory cytokines IL-1β, IL-6, TNFα and the chemokine IL-8, while increased the secretion of the anti-inflammatory cytokine IL-10. The T-cell activating capacity of moDC was

also inhibited, and dimethyltryptamines used in combination with *E. coli* or influenza virus (A/H1N1) as stimulators decreased the differentiation of moDC-induced autologous Th1 and Th17 inflammatory effector T-cells in a sigmar-1 specific manner as confirmed by specific gene silencing. Thus we demonstrated for the first time the immunomodulatory potential of NN-DMT and 5-MeO-DMT on human moDC functions acrung via sigmar-1 thar could be harnessed for the pharmacological treatment of various autoimmune diseases and chronic inflammatory conditions of the CNS (Alzheimer's disease, Major Depression, Multiple Sclerosis) or peripheral tissues. Our findings also point out to a new biological role for dimethyltryptamines, which may act as systemic endogenous regulators of inflammation and immune homeostasis through the sigmar-1 receptor **(13).**



A review article has been prepared for summarizing the involvement of innate immunity in the context of Psychiatric and Neurological disorders. The role of cytokine feedback mechanisms between the immune and central nervous system (CNS) was discussedin this paper. Furthermore, we emphasized the topology of molecular pathways, which link together the gut-brain axis and intestinal immune cells. A particular focus was put on monocytes, macrophages(microglia) and dendritic cells, their collaboration with TLRs and RIG-I-like helicases and their involvement in inflammation and pathological conditions. We propose new perspectives for the pharmacological modification of innate immune cells and their response to inflammatory challenges in the brain. **(14).**



Besides protection against infections DC are essential in controlling self reactive immune responses that may cause autoimmune diseases and are important targets of therapeutic interventions in cancer. We provided evidence that secreted vesicles derived from cell culture supernatants of various origin are able to generate both Fas-dependent apoptotic and Fas-independent non apoptotic cell death. In contrast to classical RIP-independent Fas-induced cell death triggered by cross linked or membrane bound FasL, the vesicle-derived and secreted stimuli potentially could induce apoptotic processes that exhibited unique molecular and enzymatic characteristics. These pathways could partially be inhibited by blocking the enzymatic activity of cathepsin D and required the presence of RIP. Human blood-derived resting DC were found to be potent and promiscuous anti-cancer cytotoxic cells capable of inducing efficient and selective apoptotic cell death. Human blood circulation derived immature DC are capable of inducing efficient and selective apoptotic cell death and we could provide evidence that the supernatant of immature moDC stimulated with polyI:C or CL075 are capable of inducing RIP1-mediated cell death similarly to T- cells. Discovery of a DC-mediated non-classical RIP1-dependent cell death processes may play a fundamental role in the down regulation of activated immune cells by inducing immune tolerance and may open up new avenues for utilizing DC for killing apoptosis resistant tumor cells for cancer therapy We propose that this newly discovered cell death pathway could be critical when the classical apoptotic pathway is deregulated **(15)**.

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