Discussion and future directions

Tridib Das

In this thesis we addressed the role of A20/Tnfaip3, a negative regulator of NF-κB signaling in dendritic cells (DCs) in immune regulation that is essential to prevent autoimmune disease. We examined a novel DNGR1-cre mediated targeted deletion of the *Tnfaip3* gene in mice, which primarily affects cDC1s and studied their activation in the context of a spontaneous chronic autoimmune liver disease (**Chapter 3**). In a house dust mite (HDM)-induced neutrophilic airway inflammation, based on LysM-cre-mediated deletion of A20/*Tnfaip3* from myeloid cells, we examined the role of IL-17RA-signaling (**Chapter 4**). We surprisingly found that IL-17RA-signaling played no detectable role in HDM-induced neutrophilic airway inflammation. In aged *Tnfaip3*$^{CD11c\text{-}KO}$ mice, which lack A20/Tnfaip3 expression essentially in all DCs, we noticed that splenic B cell numbers were significantly reduced. We therefore examined the developmental stages of B cells in these mice in detail in **Chapter 5** and found that B cell development in the bone marrow was hampered at the immature B cell stage in 6-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice and at the pre-B cell stage in 24-week-old Tnfaip3$^{CD11c\text{-}KO}$ mice. It had been previously demonstrated that A20/Tnfaip3-deficient bone marrow-derived (BM)-DCs could directly activate B cells *in vitro* independent of T cell help. Using this model crossed onto a *Cd40lg*^{KO} background, we examined whether this also occurred *in vivo* (**Chapter 6**). Despite the crucial role of CD40L in T-B cell communication in acquiring germinal center (GC) B cells and IgG1 class-switched plasma cells, we still observed glomerular membrane thickening in *Tnfaip3*$^{CD11c\text{-}KO}$*Cd40lg*^{KO} mice. We concluded that in these mice the kidney pathology was most likely mediated by autoreactive, T cell independent IgA, because IgA was deposited and serum anti-dsDNA IgM and IgA levels were enhanced. Finally, we aimed to investigate how IL-23 signaling affected Th17 and B cell populations in *Tnfaip3*$^{CD11c\text{-}KO}$ mice (**Chapter 7**). We observed that the autoimmune phenotype in mice with dendritic cell-specific deletion of Tnfaip3/A20 was independent of the IL-23/IL-17 axis.

In this chapter, we discuss the role of A20/Tnfaip3, primarily in DCs, but also other immune cells, to keep the immune system in balance and prevent autoimmunity. We translate our findings to patients and discuss the outlook of A20 research in autoimmunity.

A STEP INTO THE PAST, EQUALS A STEP INTO THE FUTURE: UNDERSTANDING DC ONTOGENY

Nobel laureate Ralph Steinman's discovery of DCs in the 1970's boosted our knowledge of immune homeostasis[1]. DCs are orchestrators of the immune system from tolerance to immunity[2]. This delicate balance is maintained by the expression of tolerogenic or immunogenic co-stimulators and cytokines[3, 4]. Over the years various types of DCs were

discovered and this led to a confusing nomenclature of DC subsets in different organs and different species, and challenged the comparison of individual experimental studies. At one point in time, questions were asked whether DCs are truly any different from monocytes at all[5]. In 2014, when experiments described in this thesis were ongoing, a consensus was proposed on the basis of DC ontogeny, which corresponded well across different species[6]. Deciphering various transcription factors or cell-specific expression markers to isolate DC subsets became a 'hot topic', either by targeted deletion of those transcription factors[7] or by genetic tracing using fluorescent reporters in mice[8, 9]. Promising results were found for cDC1s and pDCs, but to date, cDC2s have no single transcription factor with exclusive control over their development. This is due to (i) key transcription factors such as IRF4, which are also used by other myeloid or adaptive immune cells[10] and (ii) the finding that cDC2s can also be derived from lymphoid progenitors during certain conditions[11]. An overview of the main pDC and cDC transcription factors is depicted in **Figure 1**. Very recent work identified a subdivision of cDC2s into cDC2A and cDC2B based on T-bet or RORγt expression, respectively, and divides them into anti-inflammatory and pro-inflammatory cDC2s[12]. Our work did not take these ongoing divisions of cDC subsets into account.

Our interest settled on loxP-Cre-mediated gene deletion, whereby the DNGR1-cre (Clec9a-cre) was shown to mostly affect cDC1s, and a smaller proportion cDC2s and moDCs in steady state[8]. By crossing our *Tnfaip3*^{DNGR1} mice with ROSA-EYFP mice we demonstrated, in parallel to the work of Schraml *et al.* in the kidney[8], that primarily cDC1s (~95%) were affected in the liver (**Chapter 3**). A proportion cDC2s and moDCs (~30-40%) also showed deletion, as evidenced by YFP expression, in steady state. Interestingly, during inflammation these proportions were reduced for cDC1s from ~95% YFP-positivity to ~60% YFP-positivity. While Schraml *et al* did not report the exact percentages of YFP^{+} cells in DC subsets during *L. monocytogenes* inflammation, their CD11c^{+}GR1^{-} population had ~55% YFP-positivity and likely encompassed most DC subsets. Thus, during inflammation, there appears to be a positive selection for the YFP-negative (reflecting the Tnfaip3-sufficient) population of cDC1s in our study. What the origin of these cells are is yet unknown, but they could be derived from monocytes as they can express cDC markers in tumors[13]. In the time that the experiments described were ongoing, several novel cDC1 specific conditional cre transgenic mouse models became available such as XCR1-cre or Karma-cre[14] that are superior to the DNGR1-cre mouse. Even during viral infection, these models remain specific for cDC1[14]. The utilization of these models would have given cleaner results in comparison to the DNGR1-cre mice when analyzing the systemic effects of activated cDC1s, as described in **chapter 3**. Nevertheless, in *Tnfaip3*$^{DNGR1\text{-}KO}$ mice cDC and moDC activation was associated with T cell activation, elevated IFN-γ production and plasma cell differentiation (**Figure 2**). Specifically, plasma cells were present that produced autoreactive IgA recognizing periportal

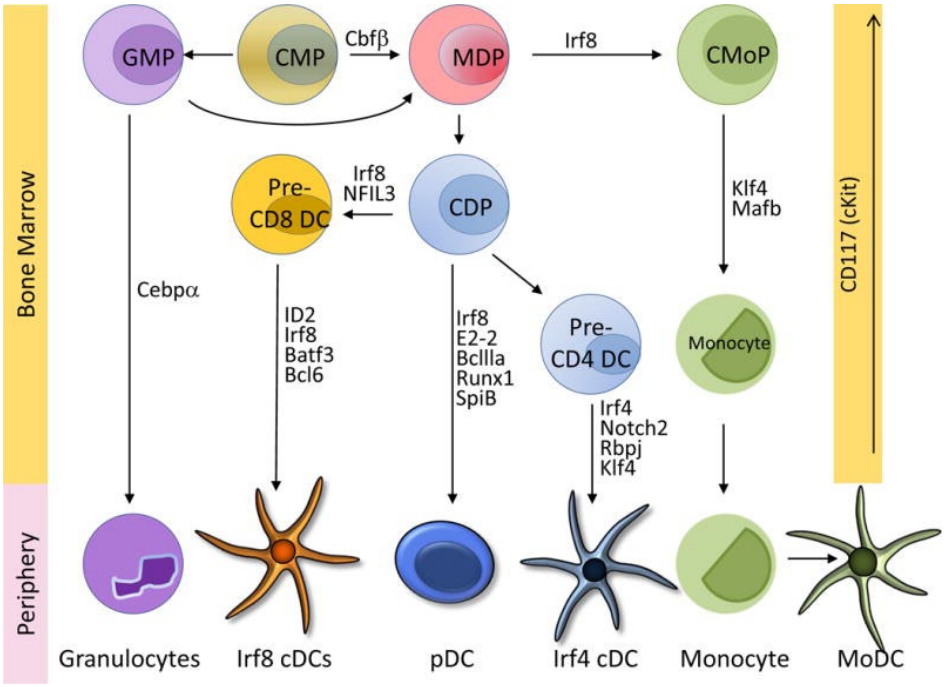

Figure 1: Differential expression of transcription factors regulating DC differentiation
From the common myeloid progenitor (CMP), stems off a granulocyte-monocyte progenitor (GMP) and macrophage-dendritic cell progenitor (MDP). The latter splits off into a common monocyte progenitor (CMoP) that may develop into monocytes or into the common DC progenitor (CDP). The CDP may give rise to pDCs or a precursor DC (pre-DC) for $CD4^+$ or $CD8^+$ DCs. ID2, inhibitor of DNA binding 2; BATF3, basic leucine zipper transcription factor ATF-like 3; IRF, interferon-regulatory factor; Bcl6 or Bcl11a, B cell lymphoma 6 or 11a; Runx1, Runt related transcription factor; Rbpj, Recombination signal binding protein for immunoglobulin Kappa J Region; Klf4, Krueppel-like Factor; Mafb, MAF BZIP transcription factor B. From Murphy et al, Annu Rev Immunol, 2016[7].

cytoplasmic liver antigens, possibly leading to inflammatory infiltrates in those areas. By administering anti-IL-6 antibodies *in vivo*, we blocked IL-6 for multiple weeks and found a simultaneous absence of Th17 cells (Thomas Koudstaal et al., unpublished data). Thus, lack of IL-6 and Th17 did not reduce the liver inflammation. We were unable to demonstrate whether IFN-γ was responsible for the inflammation, which likely contributes to the pathology as IFN-γ transgenic mice on a liver specific promoter (serum amyloid P component) resembled our liver phenotype[15]. Evidence was obtained for an indispensable role for cDC1s in a *Batf3*$^{-/-}$ mice in a primary biliary cirrhosis model (Reuveni/Zigmund, pers. commun.), which would support our findings of liver pathology in the *Tnfaip3*^{DNGR1} model of activated cDC1s. Liver inflammation was previously demonstrated in the context of multiorgan inflammation by Kool *et al* in the SLE-like phenotype[16] and by Xuan et al with CD11c-cre mediated deletion of *Tnfaip3*[17]. Another group indepen-

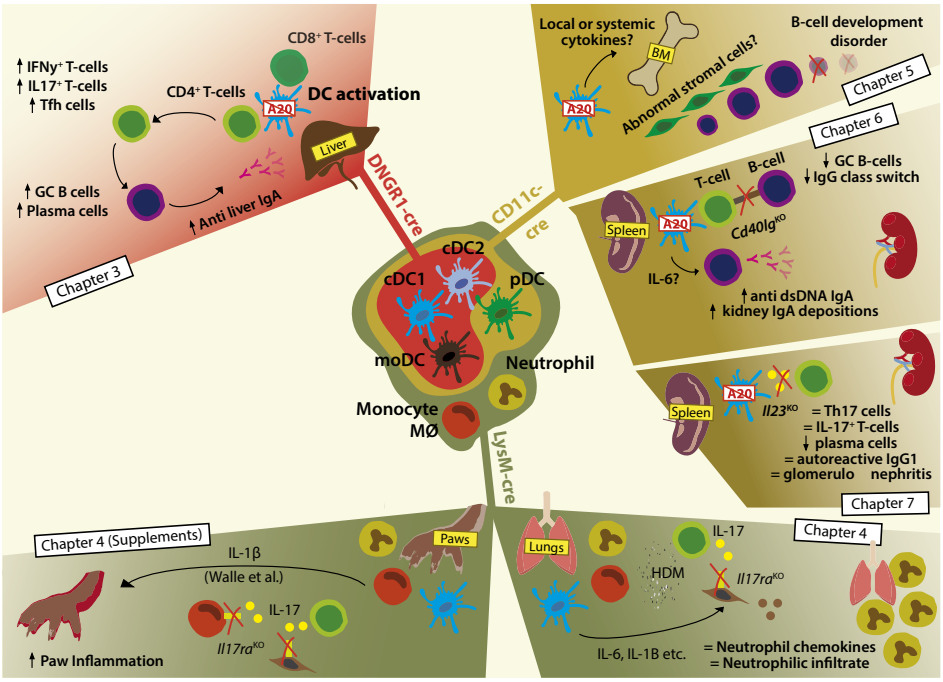

Figure 2: A summary of the Tnfaip3$^{fl/fl}$ and Cre-LoxP models (DNGR1-cre (red), CD11c-cre (brown) and LysM-cre (Green) applied in this thesis, as well as the studied organs. Additional crosses onto other backgrounds (*Cd40lg*^{KO}, *Il23*^{KO} and *Il17ra*^{KO}), which are described in the specific chapter blocks are indicated. "↑" means a significant increase, "↓" means a significant decrease and "=" means no significant differences.

dently generated a similar *Tnfaip3*$^{CD11c\text{-}KO}$ mouse model that had an IBD phenotype[18]. We confirmed that the *Tnfaip3*$^{DNGR1\text{-}KO}$ mice differed from these phenotypes, since we found no intestinal or kidney inflammation. It is of note that cDC activation in the lungs of *Tnfaip3*$^{DNGR1\text{-}KO}$ mice, could result in a pulmonary hypertension phenotype (Koudstaal et al., manuscript submitted). Differences in phenotype between laboratories, using a similar *Tnfaip3*$^{CD11c\text{-}KO}$ model are likely caused by microbiome differences, although effects of genetic background or minor differences in the targeting construct cannot be excluded. How the microbiome affects genetic models deserves future attention, because they can cloud the conclusions of genetic murine studies.

A20/TNFAIP3, MYELOID CELLS AND TH17-IL-23 AXIS IN CHRONIC DISEASES

Since the original discovery of the Th17 cell lineage in 2005[19], the Th17-IL-23 axis has been a prime topic in autoimmune research. Arthritis, IBD and psoriasis are classic ex-

amples with deregulations in this axis[20]. Other chronic diseases such as COPD or asthma should deserve this attention too. Patients with severe asthma may have a neutrophilic infiltrate[21] and elevated serum IL-17 levels[22] in comparison to moderate asthma patients. In an HDM-driven mouse model of airway inflammation, our group demonstrated that myeloid deficiency of A20 lead to a neutrophilic rather than an eosinophilic airway infiltrate[23]. Since IL-17 levels coordinate neutrophil chemoattractants such as CXCL1 from airway epithelial cells[24], we hypothesized that HDM-induced neutrophil airway inflammation would be reduced in *Tnfaip3*$^{LysM\text{-}KO}$ mice when crossed onto an *Il17ra*^{KO} background (**Figure 2**). Surprisingly, this did not seem to be the case (**Chapter 4**). An equal extent of neutrophilic airway infiltration was seen in the broncho-alveolar lavage (BAL) fluid in HDM-exposed *Il17ra*^{WT} and *Il17ra*^{KO} *Tnfaip3*$^{LysM\text{-}KO}$ mice. Although IL-17Rα-signaling was absent, chemokines such as CXCL1, CXCL2 and CXCL12 were still formed, possibly due to high unaltered levels of IL-1β and IL-6. The clinical relevance of A20 in asthma patients was recently highlighted by reports of reduced TNFAIP3 mRNA and protein levels, isolated from PBMCs from asthmatic children compared to healthy controls[25]. DCs also showed a non-significant reduction of TNFAIP3 mRNA levels in asthmatic children[25].

Increased numbers of Th17 cells were present in spleens and inguinal lymph nodes of *Tnfaip3*$^{LysM\text{-}KO}$ mice[26] and these mice were originally demonstrated to have paw inflammation resembling arthritis. Although the arthritis was independent of T or B cells[26], myeloid cells such as monocytes[27] or neutrophils[28] can also produce IL-17. We thus wanted to examine the role of IL-17Rα signaling on arthritis. Surprisingly, despite having no IL-17Rα-signaling, there was equal paw inflammation in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice (**Supplementary Figure Chapter 4, Figure 2**), which was explained in a subsequent paper by evidence that IL-1β is the responsible cytokine[29]. A novel function for A20 was deciphered: it regulates the inflammasome NLRP3 and secondary IL-1β release from myeloid cells[29]. The effect of A20 on the inflammasome was confirmed in a few families around the world that had a loss-of-function mutation in A20. These patients have a rare disease called "A20 Haploinsufficiency"[30] and their PBMCs have elevated inflammasome activation, NLRP3 and IL-1β protein and mRNA production[30, 31]. Phenotypes ranged from autoinflammatory diseases such as Behçets disease[30] to autoimmune disorders[32].

In the same *Tnfaip3*$^{LysM\text{-}KO}$ mouse, another role of A20 was discovered: A20-deficiency in IFN-γ-stimulated bone marrow derived macrophages (BM-DM), resulted in higher STAT1 mRNA transcription in comparison to STAT3 mRNA transcription, indicating that A20 may regulate STAT gene transcription ratios[33]. Understanding the potent molecular functions of A20 is still of high interest, illustrated by two recent papers that studied the functional domains of A20. The Zinc Finger (ZF) 7 domain was most important to prevent a spontaneous autoimmune psoriatic-arthritis/like phenotype[34, 35], whereas abrogating the ZF4 or OTU domain of A20 surprisingly did not reveal any phenotype[36, 37]. The

psoriatic-arthritis like symptoms were dependent on T cells, IL-17 and TNFα[35]. Although DC co-stimulatory molecules were not assessed (except for MHC-II), this does suggest their involvement to activate the adaptive immune system.

In vitro, it was shown that A20-deficient BM-DCs[16], produce high levels of IL-23 and IL-6 (Vroman et al[23] and this thesis, **Chapter 6**), and could elicit IL-17 production from naïve T cells. We wondered whether *in vivo* Th17 cells could be reduced, and consecutive B cell activation as well, since Th17 cells can stimulate immunoglobulin production[38]. We thus crossed *Tnfaip3*$^{CD11c\text{-}KO}$ mice to *Il23*^{KO} mice and analyzed the resulting phenotype (**Chapter 7, Figure 2**). We surprisingly did not see a reduction of Th17 cells or IL-17^{+} CD4^{+} T-cells in the spleen when we abrogated IL-23 in *Tnfaip3*$^{CD11c\text{-}KO}$ mice. This corresponds with the literature that IL-23 is mostly important for maintenance of Th17 cells and inducing its pathogenicity, but that IL-6 and TGF-β cause Th17 cell induction[39]. Since Th17 cells influence plasma cell class switch by cytokines[38], we also assessed serum immunoglobulins. We found no differences in *Tnfaip3*$^{CD11c\text{-}KO}$ mice concerning serum IgM, IgG2b, IgG2c or IgG3 in absence of IL-23. IgA which was elevated in *Tnfaip3*$^{CD11c\text{-}KO}$ mice was lowered in *Tnfaip3*$^{CD11c\text{-}KO}$*Il23*^{KO} mice. The increase of total IgG1 levels in the serum of *Tnfaip3*$^{CD11c\text{-}KO}$ mice was independent of IL-23, nor was the amount of autoreactive IgG1 and kidney glomerulonephritis. We therefore concluded that the SLE-phenotype in *Tnfaip3*$^{CD11c\text{-}KO}$ mice acts independently from the IL-23/Th17 axis. These findings are in contrast to studies that show a crucial role of IL-23 in other SLE models, such as the lupus prone MRL.*Fas*^{lpr} mice[40, 41]. Therefore, distinct immunological mechanisms may lead to a similar SLE phenotype: in the *lpr* mice there is a greater dependency on the Th17/IL-23 axis. In our model, future studies to identify the primary responsible cytokine may include blocking IL-6, TNF-α or IFN-γ since these were elevated in *Tnfaip3*$^{CD11c\text{-}KO}$ mice *in vivo*[16-18].

A20/TNFAIP3 AND DIRECT ACTIVATION OF B CELLS BY DCS, INDEPENDENTLY FROM T CELLS

The most classic route of adaptive immune cell activation is by antigen presenting cells (APCs), via T cells, which in turn activate B cells. However, a direct interaction between APCs and B cells is also described[42], whereby DCs are shown to interact directly with B cells and present antigen to them[43]. Our group was able to demonstrate *in vitro* that BM-DCs from *Tnfaip3*$^{CD11c\text{-}KO}$ mice in combination with only naïve B cells, stimulated plasmablast and plasma cell formation leading to IgA and IgG1 release[16]. Interestingly, characteristic T cell independent activators of plasma cells such as BAFF and APRIL[44], were not necessary in this *in vitro* activation of plasma cells[16]. Only IL-6 was found to stimulate IgA release[16]. Using mRNA sequencing, we compared *Tnfaip3*^{KO}, *Tnfaip3*^{HZ} and *Tnfaip3*^{WT}

BM-DCs[23] for a comprehensive genome-wide identification of differentially transcribed genes including candidate factors that may orchestrated B cell activation by DCs (**chapter 6**). However, even by gene-set enrichment analysis (GSEA), we did not find new targets except IL-6, IL-1α and IL-1β. Factors such as BAFF and APRIL were not significantly differentially expressed in our analysis. We proceeded to analyze whether activated DCs in *Tnfaip3*$^{CD11c\text{-}KO}$ mice could also activate B cells *in vivo* and could direct class-switched plasma cells without T cell help by crossing these mice onto a *Cd40lg*^{KO} background (**Chapter 6, Figure 2**). GC B cells are dependent on CD40 ligand interaction[45]. However, while *Tnfaip3*$^{CD11c\text{-}KO}$*Cd40lg*^{KO} displayed a detectable elevation of GC-like B cells on flow cytometry in comparison to *Cd40lg*^{KO} mice, GC structures containing these cells could not be visualized using immunohistochemistry (**Chapter 6**). These findings indicating that these GC-like B cells were most likely activated B cells, rather than true GC B cells. While some studies provided evidence that GC B cells can be formed without T cell help[46, 47], these were short-lived GC B cells that did not allow the immunoglobulin variable regions to undergo somatic hypermutation. Interestingly, despite having no regular GC B cells, we did see equal number of plasma cells in double KO mice, compared to *Tnfaip3*$^{CD11c\text{-}KO}$ mice, suggesting that extrafollicular B cell activation was likely responsible for plasma cell formation. Within lupus models, the short-lived plasmablasts are the main source of autoantibodies[48, 49], which derive from extrafollicular loci instead of long-lived plasma cells that originate from germinal centers. In these extrafollicular loci, DCs are in close proximity to autoantibody-positive B cells and are thought to provide survival signals to them[49]. In accordance, it has been reported that constitutive DC depletion in MRL.*Fas*^{lpr} mice reduced plasma blast numbers, autoantibodies and glomerulonephritis[50]. Interestingly, despite the absence of T-B cell communication, IgG formation or glomerular IgG deposition, we still found glomerulonephritis in *Cd40lg*^{KO}*Tnfaip3*$^{CD11c\text{-}KO}$ mice. Possibly, autoreactive IgA contributed to the basement membrane thickening, as IgA depositions were detected in ~40% of the double KO mice. This knowledge is valuable, since therapies that inhibit T-B cell communication in patients with SLE, may not be sufficient to prevent end-stage renal disease.

A20/TNFAIP3, DCS AND B-CELL DEVELOPMENT IN THE BONE MARROW

The function of DCs in the lymph nodes, the splenic compartment and mucosa are pronounced and well-studied, but what could the roles of DCs be in the bone marrow (BM)? BM-resident DCs have been identified and are known to resemble cDC2s, but simultaneously differ from splenic cDC2s in their chemokines and chemokine receptors[51]. They are thought to provide survival signals to mature B cells in the BM[52]**.** Could DCs that are resident in the BM influence the BM environment and thereby also affect developing

B cells or play a role in their development? And what will be the effects of DCs in the BM on the functionality of developing B cells later in their life once they have left the BM? We specifically asked these questions, because we found reduced numbers of mature B cells in the spleen, but also in BM of 24-week-old Tnfaip3$^{CD11c\text{-}KO}$ mice.

In young 6-week-old mice we therefore analyzed the different stages of B cell development in BM and found a disorder around the small pre-B cell stage in **Chapter 5** (**Figure 2**). Using an *in vitro* co-culture with IL-7, we identified that the CD19^{negative} BM fraction from *Tnfaip3*$^{CD11c\text{-}KO}$ mice could negatively influence development of B cells that were derived from both WT or from *Tnfaip3*$^{CD11c\text{-}KO}$ mice. This implies that there is a non-cell intrinsic defect in B cell differentiation in these mice. Using several washing steps, we also hypothesize that cytokines are not likely responsible for defects in B cell differentiation. We however cannot exclude that during the *in vitro* study, new cytokines were produced that hampered B cell development. Future experiments may be conducted to explore the presence of any elevated inhibitory cytokines in such IL-7-driven co-cultures, including IFN α/β/γ, IL-1 α/β, IL-4 or TGF-β, or stromal cell chemokines such as CXCL12[53] that are known to influence B cell development. It is attractive to speculate that local DCs in the BM of *Tnfaip3*$^{CD11c\text{-}KO}$ mice have critical effects on B cell differentiation in the BM. It would be informative to assess activation markers and cytokines on BM-resident DCs in *Tnfaip3*$^{CD11c\text{-}KO}$ mice.

Since BM is a compartment that is difficult to access in humans, relatively little is known about possible defects in B cell development in autoimmune patients. A small cohort of SLE patients, however, revealed reduced BM CD20^{+} B cells[54], which may parallel our findings of reduced mature B cells in the BM of *Tnfaip3*$^{CD11c\text{-}KO}$ mice. Unstimulated peripheral B cells from MS patients and stimulated B cells from SLE patients had elevated CD80/CD86 expression in comparison to healthy donor B cells[55, 56]. This also corresponds to our *in vitro* findings in **Chapter 5** where peripheral mature B cells from *Tnfaip3*$^{CD11c\text{-}KO}$ mice expressed higher levels of CD80/CD86 on different stimuli. In an SLE cohort, it was found that more apoptotic cells were present in the BM, together with elevated numbers of T cells, macrophages and pDCs, thus reflecting a more pro-inflammatory environment[54]. Signs of a type 1 IFN-rich environment in the BM of SLE patients have been found[57] and it was recently shown that transitional B cells in SLE patients indeed have a type 1, and also a type 2 IFN-stimulated signature derived from the BM environment[58]. All in all, understanding of abnormal BM and B cell development in SLE patients may contribute to knowledge that is important for the future development of new therapies for this autoimmune disease.

CONCLUSIONS AND FUTURE DIRECTIONS

Immune cell activation may tip the balance from tolerance to autoimmunity. In all our murine studies we deleted A20/TNFAIP3, a negative regulator of NF-κB signaling, to lean different immune cells towards activation (**Figure 2**). How relevant is A20 deficiency in humans? Besides a very rare disorder, haploinsufficiency of A20 (HA20)[30], it is more reasonable that subtle defects in A20 expression levels or its induction is affected than general A20 deficiency. One *TNFAIP3* missense mutation near the OTU domain with the most subtle effect on function (T108A/I207L), was seen in Australian families from Maori ancestry[59]. The allele variation was also identified in Denovisan archaic human species, but not present in Neanderthals, which lived in the same Siberian cave, suggesting that this allele arose after Denovisan and Neanderthal lineage divergence more than 170,000 years ago[59]. What could be the advantage of this allele? A heightened immune response with e.g. release of TNF-α or CXCL2 could be seen in stimulated PBMCs from these families versus controls. Using a humanized mouse model with CRISPR-Cas9 genome editing, the authors elegantly demonstrate that the T108A/I207L mutation increases survival by 1.8x in male mice compared to WT mice when exposed to coxsackie virus infection[59]. Mice with more severe missense mutations, one of which was based on the HA20 phenotype in humans[30], never died of this coxsackie virus infection. But this came at a price: a range of subclinical spontaneous tissue inflammation was detectable in one variation, whereas fatal intestinal inflammation was seen in another. Thus, protection from foreign invaders may be more efficient, at the price of suffering from responses e.g. to microbes that should be tolerated.

One of A20's functions is thus to create a balanced immune system, which is primarily important in DCs. We were most interested to study the role of A20/Tnfaip3 in DCs. Given the division of labor it is important to study DC subsets specifically, and not as a whole population. We have made a first step using the DNGR1-cre model to decipher the role of A20/Tnfaip3-depletion primarily in the cDC1 subset. However, more specific cre models such as the XCR1-cre or Karma-cre can be used in future experiments to ablate A20 in only cDC1s, since the DNGR1-cre model also targets a proportion cDC2s and moDCs. When translating findings from murine studies to patients, it is crucial to consider this subdivision of DCs. Although human DCs have been properly classified[60], to date only a few studies on cDC changes in autoimmune patients have been reported. The *Tnfaip3*$^{CD11c\text{-}KO}$ model demonstrated the consequence of active DCs, but it raised several more questions. When we crossed *Tnfaip3*$^{CD11c\text{-}KO}$ mice to *Cd40lg*^{KO} mice, we saw no class switched IgG, but we did find autoreactive IgA and also IgA depositions in the kidneys. Two clinical diseases are known with similar findings: X-linked Hyper-IgM syndrome patients lack CD40LG and therefore have drastically reduced class-switched immunoglobulins, including reduced levels of IgA[61]. IgA nephropathy is characterized by a

dominance of IgA that leads to deposition in the kidney glomeruli, in contrast to IgG[62]. Although the pathogenesis of these two disorders is different, it appears that they may merge in our *Cd40lg*^{KO}*Tnfaip3*$^{CD11c\text{-}KO}$ mice, because we did find IgA depositions in the kidney glomeruli without IgG immunoglobulins present. There are case reports available of IgA nephropathy and SLE occurring in the same patients[62, 63]. Several clinical trials are currently performed with anti-CD40L or anti-CD40 biologicals in SLE[64], whereby clinical activity scores are improved over placebo controls that also include kidney function parameters. Our findings, however, suggest that in a diseased state with activated DCs, such inhibitors may still result in unexpected outcomes of kidney glomerulonephritis.

The Th17-IL23-axis was contra intuitively not of importance in our model of Tnfaip3-deficiency in DCs and other myeloid cells, concerning endpoints of kidney glomerulonephritis (**Chapter 7**, **Figure 2**), paw inflammation and neutrophilic airway inflammation (**Chapter 4, Figure 2**). Ustekinumab, the biological anti-p40 subunit against both IL-23 and IL-12 has been approved for diseases such as Crohn's disease, psoriasis and psoriatic arthritis. It is still under investigation for SLE, although promising results have been reported[65]. Ustekinumab does not seem to be effective in RA[66]. There are no ongoing clinical trials in asthma, which reflects our findings of similar neutrophilic airway inflammation with or without IL-17RA-signaling in a HDM-induced airway inflammation model in *Tnfaip3*$^{LysM\text{-}KO}$ mice. It has been demonstrated in one case study, however, that Ustekinumab also relieved an asthmatic patient of her chronic airway medication, while she was treated for psoriasis[67], suggesting that Th17 responses could be underlying asthma pathogenesis in some patients. Further investigations are needed to characterize the group of asthma patients that may benefit from IL-17 blockade, before ustekinumab may be tested in clinical trials for a subgroup of therapeutically refractive patients.

In conclusion, our studies have shown that activated DCs lie at the initiation phase of the autoimmune response, with extensive secondary consequences. Blocking an intermediate cytokine sometimes may not inhibit a primary endpoint such as kidney glomerulonephritis, because activated DCs can activate multiple cells prior to the release of the cytokine in question. Future autoimmunity treatments could be focused on inhibiting NF-κB activation. However, these treatment strategies need to be designed carefully as they might reduce acute inflammation, while causing long-lasting side effects. For example, interference with NF-κB in enterocytes prevented systemic inflammation in an intestinal-ischemia reperfusion model, but led to apoptotic mucosal damage as well[68], since NF-κB acts as an anti-apoptotic factor. For that reason, NF-κB blockade has already been an attractive candidate in cancer therapy NF-κB[69]. While IKK inhibitors pose toxicity issues, a lot of research is conducted to pharmacologically modulate ubiquitination and degradation of NF-κB components[69], thus nearing the functions of A20. Another therapeutic option in accordance to our findings, would be to treat autoimmunity with tolerogenic DCs. To date, two studies with the induction of tolerogenic DCs with an

AMPK activator and an AhR antagonist showed promising results in SLE[70]. Perhaps the induction of tolerogenic-DCs holds a future in the treatment or prevention of autoimmune diseases, and should thus be investigated.

REFERENCES

1. Steinman RM, Witmer MD. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proceedings of the National Academy of Sciences of the United States of America* 1978; 75(10): 5132-5136.
2. Steinman RM. Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol* 2012; 30: 1-22.
3. Tan JK, O'Neill HC. Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. *J Leukoc Biol* 2005; 78(2): 319-324.
4. Steinman RM, Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A* 2002; 99(1): 351-358.
5. Hume DA. Differentiation and heterogeneity in the mononuclear phagocyte system. *Mucosal immunology* 2008; 1(6): 432-441.
6. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU *et al.* Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nature reviews Immunology* 2014; 14(8): 571-578.
7. Murphy TL, Grajales-Reyes GE, Wu X, Tussiwand R, Briseño CG, Iwata A *et al.* Transcriptional Control of Dendritic Cell Development. *Annual review of immunology* 2016; 34: 93-119.
8. Schraml BU, van Blijswijk J, Zelenay S, Whitney PG, Filby A, Acton SE *et al.* Genetic tracing via DNGR-1 expression history defines dendritic cells as a hematopoietic lineage. *Cell* 2013; 154(4): 843-858.
9. Abram CL, Roberge GL, Hu Y, Lowell CA. Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. *Journal of immunological methods* 2014; 408: 89-100.
10. Mittrücker HW, Matsuyama T, Grossman A, Kündig TM, Potter J, Shahinian A *et al.* Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. *Science (New York, NY)* 1997; 275(5299): 540-543.
11. Salvermoser J, van Blijswijk J, Papaioannou NE, Rambichler S, Pasztoi M, Pakalniškytė D *et al.* Clec9a-Mediated Ablation of Conventional Dendritic Cells Suggests a Lymphoid Path to Generating Dendritic Cells In Vivo. *Frontiers in immunology* 2018; 9: 699.
12. Brown CC, Gudjonson H, Pritykin Y, Deep D, Lavallée VP, Mendoza A *et al.* Transcriptional Basis of Mouse and Human Dendritic Cell Heterogeneity. *Cell* 2019; 179(4): 846-863.e824.
13. Sharma MD, Rodriguez PC, Koehn BH, Baban B, Cui Y, Guo G *et al.* Activation of p53 in Immature Myeloid Precursor Cells Controls Differentiation into Ly6c(+) CD103(+) Monocytic Antigen-Presenting Cells in Tumors. *Immunity* 2018; 48(1): 91-106.e106.
14. Mattiuz R, Wohn C, Ghilas S, Ambrosini M, Alexandre YO, Sanchez C *et al.* Novel Cre-Expressing Mouse Strains Permitting to Selectively Track and Edit Type 1 Conventional Dendritic Cells Facilitate Disentangling Their Complexity in vivo. *Frontiers in immunology* 2018; 9: 2805.
15. Toyonaga T, Hino O, Sugai S, Wakasugi S, Abe K, Shichiri M *et al.* Chronic active hepatitis in transgenic mice expressing interferon-gamma in the liver. *Proc Natl Acad Sci U S A* 1994; 91(2): 614-618.
16. Kool M, van Loo G, Waelput W, De Prijck S, Muskens F, Sze M *et al.* The ubiquitin-editing protein A20 prevents dendritic cell activation, recognition of apoptotic cells, and systemic autoimmunity. *Immunity* 2011; 35(1): 82-96.
17. Xuan NT, Wang X, Nishanth G, Waisman A, Borucki K, Isermann B *et al.* A20 expression in dendritic cells protects mice from LPS-induced mortality. *European journal of immunology* 2015; 45(3): 818-828.

18. Hammer GE, Turer EE, Taylor KE, Fang CJ, Advincula R, Oshima S *et al.* Expression of A20 by dendritic cells preserves immune homeostasis and prevents colitis and spondyloarthritis. *Nature immunology* 2011; 12(12): 1184-1193.
19. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *The Journal of experimental medicine* 2005; 201(2): 233-240.
20. Tang C, Chen S, Qian H, Huang W. Interleukin-23: as a drug target for autoimmune inflammatory diseases. *Immunology* 2012; 135(2): 112-124.
21. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 2002; 57(10): 875-879.
22. Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an independent risk factor for severe asthma. *Respiratory medicine* 2010; 104(8): 1131-1137.
23. Vroman H, Bergen IM, van Hulst JAC, van Nimwegen M, van Uden D, Schuijs MJ *et al.* TNF-alpha-induced protein 3 levels in lung dendritic cells instruct TH2 or TH17 cell differentiation in eosinophilic or neutrophilic asthma. *J Allergy Clin Immunol* 2018; 141(5): 1620-1633 e1612.
24. Wonnenberg B, Jungnickel C, Honecker A, Wolf L, Voss M, Bischoff M *et al.* IL-17A attracts inflammatory cells in murine lung infection with P. aeruginosa. *Innate immunity* 2016; 22(8): 620-625.
25. Krusche J, Twardziok M, Rehbach K, Böck A, Tsang MS, Schröder PC *et al.* TNF-α-induced protein 3 is a key player in childhood asthma development and environment-mediated protection. *The Journal of allergy and clinical immunology* 2019; 144(6): 1684-1696.e1612.
26. Matmati M, Jacques P, Maelfait J, Verheugen E, Kool M, Sze M *et al.* A20 (TNFAIP3) deficiency in myeloid cells triggers erosive polyarthritis resembling rheumatoid arthritis. *Nat Genet* 2011; 43(9): 908-912.
27. Starnes T, Robertson MJ, Sledge G, Kelich S, Nakshatri H, Broxmeyer HE *et al.* Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *Journal of immunology (Baltimore, Md : 1950)* 2001; 167(8): 4137-4140.
28. Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. *Journal of immunology (Baltimore, Md : 1950)* 2003; 170(4): 2106-2112.
29. Vande Walle L, Van Opdenbosch N, Jacques P, Fossoul A, Verheugen E, Vogel P *et al.* Negative regulation of the NLRP3 inflammasome by A20 protects against arthritis. *Nature* 2014; 512(7512): 69-73.
30. Zhou Q, Wang H, Schwartz DM, Stoffels M, Park YH, Zhang Y *et al.* Loss-of-function mutations in TNFAIP3 leading to A20 haploinsufficiency cause an early-onset autoinflammatory disease. *Nature genetics* 2016; 48(1): 67-73.
31. Rajamäki K, Keskitalo S, Seppänen M, Kuismin O, Vähäsalo P, Trotta L *et al.* Haploinsufficiency of A20 impairs protein-protein interactome and leads into caspase-8-dependent enhancement of NLRP3 inflammasome activation. *RMD open* 2018; 4(2): e000740.
32. Kadowaki T, Ohnishi H, Kawamoto N, Hori T, Nishimura K, Kobayashi C *et al.* Haploinsufficiency of A20 causes autoinflammatory and autoimmune disorders. *The Journal of allergy and clinical immunology* 2018; 141(4): 1485-1488.e1411.
33. De Wilde K, Martens A, Lambrecht S, Jacques P, Drennan MB, Debusschere K *et*

al. A20 inhibition of STAT1 expression in myeloid cells: a novel endogenous regulatory mechanism preventing development of enthesitis. *Annals of the rheumatic diseases* 2017; 76(3): 585-592.
34. Polykratis A, Martens A, Eren RO, Shirasaki Y, Yamagishi M, Yamaguchi Y *et al.* A20 prevents inflammasome-dependent arthritis by inhibiting macrophage necroptosis through its ZnF7 ubiquitin-binding domain. *Nature cell biology* 2019; 21(6): 731-742.
35. Razani B, Whang MI, Kim FS, Nakamura MC, Sun X, Advincula R *et al.* Non-catalytic ubiquitin binding by A20 prevents psoriatic arthritis-like disease and inflammation. *Nature immunology* 2020; 21(4): 422-433.
36. De A, Dainichi T, Rathinam CV, Ghosh S. The deubiquitinase activity of A20 is dispensable for NF-κB signaling. *EMBO reports* 2014; 15(7): 775-783.
37. Lu TT, Onizawa M, Hammer GE, Turer EE, Yin Q, Damko E *et al.* Dimerization and ubiquitin mediated recruitment of A20, a complex deubiquitinating enzyme. *Immunity* 2013; 38(5): 896-905.
38. Mitsdoerffer M, Lee Y, Jäger A, Kim HJ, Korn T, Kolls JK *et al.* Proinflammatory T helper type 17 cells are effective B-cell helpers. *Proceedings of the National Academy of Sciences of the United States of America* 2010; 107(32): 14292-14297.
39. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006; 24(2): 179-189.
40. Kyttaris VC, Kampagianni O, Tsokos GC. Treatment with anti-interleukin 23 antibody ameliorates disease in lupus-prone mice. *BioMed research international* 2013; 2013: 861028.
41. Kyttaris VC, Zhang Z, Kuchroo VK, Oukka M, Tsokos GC. Cutting edge: IL-23 receptor deficiency prevents the development of lupus nephritis in C57BL/6-lpr/lpr mice. *Journal of immunology (Baltimore, Md : 1950)* 2010; 184(9): 4605-4609.
42. Xu W, Banchereau J. The antigen presenting cells instruct plasma cell differentiation. *Frontiers in immunology* 2014; 4: 504.
43. Qi H, Egen JG, Huang AY, Germain RN. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science (New York, NY)* 2006; 312(5780): 1672-1676.
44. Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. *Annual review of immunology* 2003; 21: 231-264.
45. Han S, Hathcock K, Zheng B, Kepler TB, Hodes R, Kelsoe G. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *Journal of immunology (Baltimore, Md : 1950)* 1995; 155(2): 556-567.
46. Dianda L, Gulbranson-Judge A, Pao W, Hayday AC, MacLennan IC, Owen MJ. Germinal center formation in mice lacking alpha beta T cells. *European journal of immunology* 1996; 26(7): 1603-1607.
47. Lentz VM, Manser T. Cutting edge: germinal centers can be induced in the absence of T cells. *Journal of immunology (Baltimore, Md : 1950)* 2001; 167(1): 15-20.
48. Hoyer BF, Moser K, Hauser AE, Peddinghaus A, Voigt C, Eilat D *et al.* Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice. *The Journal of experimental medicine* 2004; 199(11): 1577-1584.
49. William J, Euler C, Christensen S, Shlomchik MJ. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science (New York, NY)* 2002; 297(5589): 2066-2070.
50. Teichmann LL, Ols ML, Kashgarian M, Reizis B, Kaplan DH, Shlomchik MJ. Dendritic cells in lupus are not required for activation of T and B cells but promote their expansion,

resulting in tissue damage. *Immunity* 2010; 33(6): 967-978.

51. Zhang J, Supakorndej T, Krambs JR, Rao M, Abou-Ezzi G, Ye RY *et al.* Bone marrow dendritic cells regulate hematopoietic stem/progenitor cell trafficking. *The Journal of clinical investigation* 2019; 129(7): 2920-2931.
52. Sapoznikov A, Pewzner-Jung Y, Kalchenko V, Krauthgamer R, Shachar I, Jung S. Perivascular clusters of dendritic cells provide critical survival signals to B cells in bone marrow niches. *Nature immunology* 2008; 9(4): 388-395.
53. Ueda Y, Yang K, Foster SJ, Kondo M, Kelsoe G. Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression. *The Journal of experimental medicine* 2004; 199(1): 47-58.
54. Park JW, Moon SY, Lee JH, Park JK, Lee DS, Jung KC *et al.* Bone marrow analysis of immune cells and apoptosis in patients with systemic lupus erythematosus. *Lupus* 2014; 23(10): 975-985.
55. Fraussen J, Claes N, Van Wijmeersch B, van Horssen J, Stinissen P, Hupperts R *et al.* B cells of multiple sclerosis patients induce autoreactive proinflammatory T cell responses. *Clinical immunology (Orlando, Fla)* 2016; 173: 124-132.
56. Sim JH, Kim HR, Chang SH, Kim IJ, Lipsky PE, Lee J. Autoregulatory function of interleukin-10-producing pre-naïve B cells is defective in systemic lupus erythematosus. *Arthritis research & therapy* 2015; 17(1): 190.
57. Palanichamy A, Bauer JW, Yalavarthi S, Meednu N, Barnard J, Owen T *et al.* Neutrophil-mediated IFN activation in the bone marrow alters B cell development in human and murine systemic lupus erythematosus. *Journal of immunology (Baltimore, Md : 1950)* 2014; 192(3): 906-918.
58. Dieudonné Y, Gies V, Guffroy A, Keime C, Bird AK, Liesveld J *et al.* Transitional B cells in quiescent SLE: An early checkpoint imprinted by IFN. *Journal of autoimmunity* 2019; 102: 150-158.
59. Zammit NW, Siggs OM, Gray PE, Horikawa K, Langley DB, Walters SN *et al.* Denisovan, modern human and mouse TNFAIP3 alleles tune A20 phosphorylation and immunity. *Nature immunology* 2019; 20(10): 1299-1310.
60. Collin M, Bigley V. Human dendritic cell subsets: an update. *Immunology* 2018; 154(1): 3-20.
61. Conley ME, Larché M, Bonagura VR, Lawton AR, 3rd, Buckley RH, Fu SM *et al.* Hyper IgM syndrome associated with defective CD40-mediated B cell activation. *The Journal of clinical investigation* 1994; 94(4): 1404-1409.
62. Saha MK, Julian BA, Novak J, Rizk DV. Secondary IgA nephropathy. *Kidney international* 2018; 94(4): 674-681.
63. Perazzio SF, Granados Á, Salomão R, Silva NP, Carneiro-Sampaio M, Andrade LE. High frequency of immunodeficiency-like states in systemic lupus erythematosus: a cross-sectional study in 300 consecutive patients. *Rheumatology (Oxford, England)* 2016; 55(9): 1647-1655.
64. Karnell JL, Rieder SA, Ettinger R, Kolbeck R. Targeting the CD40-CD40L pathway in autoimmune diseases: Humoral immunity and beyond. *Advanced drug delivery reviews* 2019; 141: 92-103.
65. Larosa M, Zen M, Gatto M, Jesus D, Zanatta E, Iaccarino L *et al.* IL-12 and IL-23/Th17 axis in systemic lupus erythematosus. *Experimental biology and medicine (Maywood, NJ)* 2019; 244(1): 42-51.
66. Smolen JS, Agarwal SK, Ilivanova E, Xu XL, Miao Y, Zhuang Y *et al.* A randomised phase II study evaluating the efficacy and safety of subcutaneously administered ustekinumab and guselkumab in patients with active rheumatoid arthritis despite treatment with methotrexate. *Annals of the rheumatic diseases* 2017; 76(5): 831-839.

67. Amarnani A, Rosenthal KS, Mercado JM, Brodell RT. Concurrent treatment of chronic psoriasis and asthma with ustekinumab. *The Journal of dermatological treatment* 2014; 25(1): 63-66.
68. Chen LW, Egan L, Li ZW, Greten FR, Kagnoff MF, Karin M. The two faces of IKK and NF-kappaB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. *Nature medicine* 2003; 9(5): 575-581.
69. Herrington FD, Carmody RJ, Goodyear CS. Modulation of NF-κB Signaling as a Therapeutic Target in Autoimmunity. *Journal of biomolecular screening* 2016; 21(3): 223-242.
70. Ritprajak P, Kaewraemruaen C, Hirankarn N. Current Paradigms of Tolerogenic Dendritic Cells and Clinical Implications for Systemic Lupus Erythematosus. *Cells* 2019; 8(10).