The RUNX complex: Reaching beyond haematopoiesis into immunity

The RUNX Complex: Reaching beyond Hematopoiesis Into Immunity

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SUMMARY

Of their diverse roles as transcriptional regulators during development and cell fate specification, the RUNX transcription factors are best known for their parts in hematopoiesis. RUNX proteins are expressed throughout all hematopoietic lineages, being requisite for the emergence of the first hematopoietic stem cells to their terminal differentiation. While much progress has been made since their discoveries almost two decades ago, current appreciation of RUNX in hematopoiesis is largely grounded on their lineage specifying roles. In contrast, the importance of RUNX to immunity has been mostly obscured for historic, technical and conceptual reasons. However, this paradigm is likely to shift over time, since a primary purpose of hematopoiesis is to resource the immune system. Furthermore, recent evidence suggests a role for RUNX in the innate immunity of non-hematopoietic cells. This review takes a hematopoiesis-centric approach to collate what is known of RUNX's contribution to the overall mammalian immune system and discuss their growing prominence in areas such as autoimmunity, inflammatory diseases and mucosa immunity.

INTRODUCTION*: The RUNX family of transcription factors*

The RUNX proteins are evolutionarily conserved transcription factors that share the Runt-related domain for DNA binding and heterodimerization with a common partner, CBFβ [1]. In diverse metazoans, RUNX proteins function as critical lineage determinants and in mammals are represented by RUNX1, 2 and 3 (also known as AML1/3/2, PEBP2αB/A/C, CBFα1/2/3). Mammalian RUNX genes share additional common features, which include the use of two promoters, termed P1 (distal) and P2 (proximal), through which different RUNX isoforms are derived [1]. RUNX1 was originally identified as a frequent target of leukemogenic chromosomal translocations in human acute myelogenous leukemia (AML) [2]. RUNX1 is requisite for the generation and maintenance of hematopoietic stem cells (HSC) and the differentiation of diverse hematopoietic lineages [3]. RUNX2 is a master regulator of osteoblast differentiation and necessary for bone and cartilage development and maintenance [4]. Haploinsufficiency in RUNX2 due largely to mutations in its DNA-binding Runt-domain results in human cleidocranial dysplasia (CCD) [1]. The expression of RUNX3 is necessary for the differentiation of TrkC positive dorsal root ganglion neurons and observed in a range of epithelial tissues [1]. Of relevance to this review, RUNX3 is expressed across hematopoietic lineages where its distribution overlaps significantly with RUNX1 while remaining distinct. In comparison, the expression of RUNX2 in hematopoietic lineages is less studied, except in specific contexts, while CBFβ isoforms are ubiquitously expressed across many tissues at approximately the same ratio [5, 6]. Due to their profound involvement in hematopoiesis and the maturation of cell lineages involved in virtually all facets of immunology, RUNX proteins hold important roles in host immunity. These functions will be highlighted and discussed in the following sections that describe RUNX's contribution to each major hematopoietic lineage.

RUNX and hematopoietic stem cells

The hematopoietic stem cells (HSC) are the multipotent stem cells from which all hematopoietic lineages are derived. Developmentally, the mammalian hematopoietic system can be demarcated into three discrete phases: 1) primitive hematopoiesis during embryogenesis; 2) definitive hematopoiesis in late fetal development; and 3) adult hematopoiesis. The importance of RUNX proteins to hematopoiesis was first revealed in the complete absence of definitive hematopoiesis in *Runx1* knockout mice. The loss of Runx1 completely abolished the transition of the first definitive HSC from hemogenic endothelial cells at the aorta-gonadal-mesonephros (AGM) region [7-12]. Runx1 was also necessary for the maintenance of HSC in adult hematopoiesis, though not essential for their biogenesis. Several studies showed that conditional targeting of *Runx1* in bone marrow (BM) HSC in adult mice by *Mx1-Cre* resulted in defective T and B lymphocyte development at various stages and a blockade of megakaryocyte maturation [13-15]. Unexpectedly, some studies reported an initial expansion of the Runx1-deficient HSC that was followed by their progressive exhaustion [13-17]. These paradoxical phenotypes were attributed in part to the premature exit of HSC from its cellular niche due to the mis-regulation of the chemokine receptor *Cxcr4* [17, 18]. These HSC defects were strongly accentuated when *Runx3* was

concurrently deleted, suggesting that Runx proteins served overlapping functions in the homeostatic maintenance of HSC [19]. Indeed, *Runx1*;*Runx3* deletion in the BM led to profound differentiation and proliferative disorders across all hematopoietic lineages, eventually causing bone marrow failure or myeloproliferative disorder (MPD) [19]. Similarly, pan-hematopoietic deletion of *Cbfb* severely impaired differentiation of all hematopoietic lineages and resulted in proliferative disorder in myeloid cells [20, 21]. Interestingly, *Mx1-cre* targeting of *Cbfb* did not cause lethal bone marrow failure observed in *Runx1;Runx3* double KO mice, concordant with a Cbfβ-independent role for Runx1 and 3 in DNA repair [19].

The role of RUNX in thymocytes differentiation

A major aspect of RUNX's contribution to immune function is in the differentiation and maturation of T cells, which has been investigated in detail through mouse genetics (reviewed in Collins *et al.* [22]). In all stages of T cell development, RUNX proteins are expressed in overlapping but distinct patterns and differential expression levels [5] (Table 1). The expression of Runx proteins in the thymus goes beyond the hematopoietic system and extends to the thymic epithelial cells (TEC) (Sela A & Abramson J, personal communication). Runx1 is most highly expressed in the thymic cortex where $CD4^-CD8^-$ double negative (DN) thymocytes reside, reaching an apex at the DN3 stage before a sharp decline [5, 23-25]. Thereafter, Runx1 expression is maintained in $CD4^+CD8^+$ double positive (DP) and $CD4^+$ and $CD8^+$ single positive (SP) cells, with the expression in the $CD4^+$ SP cells being higher [25-27]. In line with this, the targeting of *Runx1* in BM by *Mx1-Cre* and thymocytes by *lck-Cre* resulted in a maturation block of DN3 and DN4 thymocytes, respectively. Moreover, the ablation of *Runx1* using *Cd4-cre* disrupted DP to SP transition [13, 26]. In human and mouse, these events coincide with the involvement of Runx1 in T cell receptor (TCR) γδ and TCR αβ rearrangement, respectively (Figure 1) [28-31]. Runx1 orchestrates TCR rearrangement events by binding to the corresponding TCR chains enhancers and, in human Dδ2 to Dδ3 rearrangement, resulting in the recruitment of Recombination Activating Gene 1 (RAG1) through physical interaction [32]. Collectively, these studies firmly establish Runx1 as a key driver of early T cell development.

Compared with Runx1, Runx3 gains prominence later in T cell differentiation. It plays a dominant role in the specification of $CD8^+$ cytotoxic T (Tc) cells from immature $CD4^+CD8^+$ DP cells. This is achieved through a number of mechanisms. Firstly, Runx3, and also Runx1, bind to the silencer element in the *Cd4* locus to suppress its expression [26, 33]. Secondly, it binds to the silencer element of *Thpok,* a key CD4 lineage determinant [33]. Runx3 also directs the activity of other participating transcription factors that regulate *Cd4* and *Thpok*, such as TCF-1 and LEF-1 [34]. Lastly, the binding of Runx proteins to the *Cd4* and *Cd8* loci promotes their association and enables the long-range epigenetic regulation that underlies their reciprocal expression patterns [35]. In line with these important functions, the genetic ablation of the Runx complex resulted in the blockade of $CD8^+$ cytotoxic T lymphocytes differentiation and a redirection of their development to a $CD4^+CD8^-$ phenotype [26, 33].

RUNX in the differentiation of effector T cell subsets

Importantly, Runx1 and Runx3 are further involved in the maturation of naïve $CD4^+$ T cells into various effector T lineages following TCR activation and exposure to environmental cues. In detail studies of these lineages, a recurring theme has been the functional cooperation amongst Runx proteins and primary lineage-specifying transcription factors [36]. During Th1 differentiation, Runx3 expression increases with a corresponding reduction in Runx1 expression. Accordingly, Th1 differentiation and cytokine production were found to be impaired in *Runx3*-deficient mice [37]. Reprising its dualistic role in silencing *Cd4* while activating *Cd8*, Runx3 cooperated with T-bet, a Th1-specific transcription factor, to fortify the Th1 phenotype. Together with Runx1, Runx3 concurrently activated the hallmark Th1 cytokine *Ifng* while suppressing the Th2-specific cytokine *Il4* [37-39]. Though not studied in detail, increase in *Runx1* and decrease in *Runx3* expression were observed during Th2 specification, suggesting a role for Runx1 in Th2 functions [37, 38].

Regulatory T (Treg) cells co-expressing CD4 and CD25 may arise spontaneously in the thymus (naturally occurring Treg or nTreg) or when induced by TGF-β, IL-2 and other signaling cues in the periphery (induced Treg or iTreg) [40, 41]. The Treg phenotype is driven by the transcription factor Foxp3. Runx proteins are essential for the maintenance of Foxp3 expression in nTreg and its induction during iTreg differentiation [42-45]. Furthermore, Runx proteins physically interact with Foxp3 to maintain the Treg genetic programme, which includes the repression of *Il2* [42, 45, 46]. Consequently, the conditional ablation of *Runx1, Runx3* or *Cbfb* in mice strongly disrupted nTreg and iTreg differentiation, maintenance and function [42-44]. In particular, Treg defects due to Runx/Cbfβ-deficiency resulted in lymphoproliferative syndrome, hyper-production of IgE, and autoimmunity in mucosal tissues, such as stomach and lung [42, 43]. Of note, these phenotypes are reminiscent to the severe gastrointestinal and lung inflammation reported in a Runx3-deficient mouse model, which had been attributed to spontaneous maturation of dendritic cells (DC) [47, 48].

In addition to the induction of iTreg cells, TGF-β is also essential for the differentiation of Th17, a potent mediator of inflammation [41, 49, 50]. This is dependent on concurrent stimulation by proinflammatory cytokines, including IL-6, TNFα and IL-1β [51]. While Th17 cells are important in providing immunity against bacteria and fungus at mucosal surfaces, deregulation and hyperactivity of Th17 cells are linked strongly to the development of autoimmune diseases (reviewed in Ivanov *et al.* [49] and Singh *et al.* [52]). As Runx proteins are important downstream mediators of TGF-β, their involvement in the reciprocal induction of Treg and Th17 was extensively investigated [41, 50]. Analogous to their regulation of Foxp3, Runx proteins acts upstream of Th17-specifying transcription factor RORγt [53-55]. Furthermore, Runx proteins physically interact with RORγt for the maximal induction of *Il17*, in a manner independent of their DNA-binding activities [55]. Remarkably, Foxp3 also interacts with RORγt, which leads to the suppression of *Il17* transcription [55]. A picture therefore emerges of a tripartite relationship between Runx, Foxp3 and RORγt in Th17 lineage specification and effector function, namely the secretion of IL-17 [55]. It is now recognized that Th17-associated autoimmune inflammation is mediated by a pathogenic subset of Th17 characterized by high IL-23 receptor expression and the production of

IFN-γ, normally a Th1 cytokine [56]. Recently, it was shown that IFN-γ production by pathogenic IL23R^{high} Th17 cells required T-bet and Runx1/3, hence linking Runx proteins in the pathogenesis of certain autoimmune conditions [57]. Consistent with this, increased Runx1 and decreased Runx2 levels were associated with salt-induced pathogenic Th17 [58-60].

In the intestinal mucosa where strong environmental cues such as TGFβ and retinoic acid (RA) influence T cell differentiation, plasticity in the CD4$^+$ T cell lineage helps maintain homeostasis by preventing exaggerated responses to commensal microbiota [61-63]. During their migration to the intestinal intraepithelial compartment, progenitors of intestinal epithelial lymphocytes (IELs) express Runx3 in response to IL-15 signaling to endow a CD4$^+$ CD8αα$^+$ phenoytpe [64-66]. Moreover, Runx3 confers CD4$^+$ IELs cytotoxic T lymphocyte (CTL) and innate-like lymphocyte properties and attenuates Th17 differentiation in a TGFβ- and RA-dependent manner [65]. As in the case of Th1 differentiation, Runx3 is positively regulated by T-bet and partners it in executing the IEL differentiation program, such as the down-regulation of ThPOK [37, 66, 67].

While the necessity of Runx3 for the specification and TCR-mediated expansion of SP CD8$^+$ cytotoxic T cell was a seminal discovery, the role of Runx proteins in the effector functions of mature CD8$^+$ T cell awaits further investigation [26, 33, 68]. Nevertheless, it is evident that Runx proteins are integral components of lineage specifying transcriptional circuit. In particular, Runx3 is requisite for the induction of T-box protein Eomesodermin (Eomes) during the differentiation of cytotoxic T cells (Tc/CTL). Runx3-deficient CD8$^+$ Tc cells showed reduced cytolytic activity due to weakened TCR-induced proliferation [26, 69] and reduced expression of key Tc effector genes, including granzyme, perforin and IFN-γ [39].

Runx is important also in the development of NKT cells, which are specialized T lymphocytes that are CD1d-restricted and co-express TCRα/β and NK maturation markers, like NK1.1 (CD161) [70-72]. In particular, Runx1 (together with RORγT) is indispensable for the differentiation of iNKT, a subclass of NKT cells with an invariant TCR α chain [71]. These invariant NKT cells have been implicated in diverse immunological processes, including immune regulation, cytokine production, microbial immunity and autoimmunity [70, 73, 74]. However, due to the lack of NKT-specific Cre mouse line, the precise contribution of Runx complex in NKT cell functions remains to be determined.

Lastly, Runx3 is critical for the development of dendritic epidermal T cells (DETC), a distinct skin-associated intraepithelial γδ T cells marked by a dendritic morphology [75]. Runx3 regulates IL-2Rβ and CD103, which mediate IL-2/IL-15-induced cell proliferation and the migration of thymic DETC to skin during late fetal development, respectively [75, 76]. As DETC are a major component of cutaneous immunity and homeostasis, the effects of their complete absence in Runx3-deficient mice on immune modulation, surveillance and repair warrant further investigation [75, 77].

RUNX in B cell determination and functions

A role for Runx proteins in B cell function was first revealed in a series of *in vitro* and *ex vivo* experiments, in which Runx3 was shown to cooperate with Smad

proteins to mediate the induction of germline Ig α promoter by TGF-β [78-82] (Figure 2). This is a key event of IgA class switching when naïve B cells are activated by antigen. In agreement with these observations, Runx3- and Runx2;Runx3-deficient splenocytes displayed varying degrees of class switching defects *ex vivo* and *in vivo* [82, 83]. In addition to Runx3, Runx1 is requisite for early B lineage specification in mouse. The targeting of *Runx1* by *Mx1-Cre* in adult BM resulted in a blockade of $B220^+$ B cell differentiation from common lymphocyte progenitors (CLP) [13, 14]. This early involvement stemmed in part from Runx1's cooperation with the EBF transcription factor in its regulation of *mb-1* (also *Cd79a*), a component of the B cell receptor [84]. Consequently, conditional ablation of *Runx1* and *Cbfb* (but not Runx3) by *mb1-Cre* blocked early B lymphopoiesis. Of note, pre-proB to pro-B transition was impaired, resulting in a loss of IgM^+ B cells and reduced V_H to DJ_H recombination [85]. Although not fully elucidated, Runx1 appears an integral component of early B lineage specification circuit through its regulation of key lineage determinants, such as the pre-B cell receptor [84-86].

In contrast, Runx3 gains prominence in later stages of B cell differentiation. An example of this was the strong induction of Runx3 observed in mouse B cell lines by TGF-β in naïve B cells during IgA class switching and the differentiation of effector B cells [78-81, 87]. Increased RUNX3 expression could also be observed following mitogenic or antigenic stimulation of primary B cells, as well as during Epstein-Barr virus (EBV) induced immortalization [88]. As with T cell development, cross regulation between Runx proteins and their reciprocal expression during B cell differentiation has been reported [89]. This is most pronounced in the suppression of RUNX1 by RUNX3 during EBV immortalization of resting B cells to generate proliferating B lymphoblastoid cells [88, 90, 91]. This is achieved by the silencing of RUNX1 distal P1 promoter through the VWRPY domain within RUNX3 [92]. Furthermore, RUNX3 is recruited by EBNA2 and EBNA3C to co-occupy promoter and enhancer elements to modulate key regulatory proteins, such as $CDKN2A/p14^{ARF}$ and B-cell maturation antigen (BCMA) [91-93]. BCMA plays an important role in the survival of B cells, particularly in mature memory B cells (B_{mem}) and plasma B cells [94].

In human, a distinct subpopulation of CD27-independent $FCRL4^+$ B_{mem} cells resides in the palatine tonsils, crypt epithelium and intestine-associated lymphoid tissues. The expression of RUNX1 was found correlated to the maintenance of these B_{mem} cells in an undifferentiated $FCRL4^-$ state through its physical occupancy *FCRL4* promoter [95]. As in the case of naïve B cell activation and EBV immortalization, the maturation and expansion of $FCRL4^+$ B_{mem} cells coincided with a loss of RUNX1 expression [95]. Taken together, RUNX1 appears to be necessary for the maintenance of immature and undifferentiated B cells while RUNX3 and RUNX2 expression are observed during terminal specialization of effector B cells [96].

RUNX in lymphoid organogenesis

Recently, the Runx/Cbfβ complex has been implicated in the development of lymphoid tissue inducers (LTi) cells [72]. These are specialised innate lymphoid cells expressing Rorγt and IL-7Rα in the absence of additional lineage markers.

They are essential for the biogenesis of secondary lymphoid tissues, such as lymph nodes (LN) and Peyer's patches by initiating anlagen formation [97]. Runx1c, a Runx1 isoform derived from the P1 promoter, and Cbfβ2 are involved in LTi differentiation at two distinct development stages [72]. Specifically, Runx1c/Cbfβ2 are needed for the early specification of LTi differentiation and during the formation of LN anlagen [72]. Consequently *Runx1c/Cbfb2* knockout mice showed defective LTi differentiation and impaired lymphoid tissue organogenesis, particularly Peyer's patches and periphery LN [72]. Runx1c/Cbfβ2 are likely to mediate these functions through their regulation of *Rorγt* [72]. This is reminiscent of the functional interaction between Runx/Cbfβ and Rorγt during T helper and iNKT differentiation, providing a clear example of recurrent utilization of transcriptional axes during hematopoiesis [55, 71, 72].

RUNX proteins in Natural Killer cells development

Natural Killer (NK) cells are a distinct subset of cytotoxic lymphocytes lineage that plays important roles in innate immunity, particularly against viral infection and tumour cells [98]. They are unique in their ability to recognize target cells in the absence of antibodies or MHC molecules, relying instead on a separate set of receptors for immune recognition and self tolerance [99]. NK cells develop in multiple sites, including fetal and adult liver, BM, spleen and thymus. Their differentiation from the NK progenitor, marked by the expression of CD122 (IL-2/IL-15 receptor β chain) is reliant on IL-15 signalling [98]. The first evidence of Runx complex having a role in NK cells came in a hypomorphic Cbfβ mouse model, in which an early block in NK differentiation was observed. At low Cbfβ dosage, the fetal liver cells displayed a profound defect in their ability to generate $NK1.1^{-}CD122^{+}$ NK progenitor cells in *ex vivo* culture [100, 101]. This was likely due to a loss of Runx complex-mediated *CD122* transcription, thereby blocking the crucial IL-15-induced genetic programme [102]. Runx proteins were shown to occupy and regulate the *CD122* promoter in mouse NK progenitors, while a dominant negative Runx mutant suppressed CD122, Ly49 family, Mac-1 and CD43 expression [102, 103]. Due to its dominant expression, these studies implicated an important role for Runx3 during NK cell development [101, 102]. Direct evidence was provided in a recent study of Runx3-deficient mice. Although Runx3 was found largely dispensable for NK cell development and function in resting conditions, it was requisite for IL-15-induced NK cell proliferation and maturation *in vivo* and *ex vivo* [104]. In particular, the loss of Runx3 resulted in the complete loss of IL-15-dependent uterine NK cells in pregnant mice [104]. However, due to the limitation in current mouse models, it remains unresolved whether Runx proteins are further necessary for NK functions, such as IFN-γ production and tumor immunity [101, 102, 104].

RUNX in myeloid cell specification and functions

Relatively little is known of the contribution of RUNX to myeloid cell development and functions. Initial mouse studies showed that the deletion of Runx1 in adult HSC severely impaired megakaryocyte maturation but displayed no discernable defect in the differentiation of other myeloid lineages, including neutrophils, monocytes and erythrocytes [13-15]. This comparatively mild phenotype is likely due to a greater degree of redundancy shared between Runx

proteins in myeloid differentiation [19-21]. Indeed, clearer phenotypes were observed upon the pan-hematopoietic targeting of *Cbfb* by *Vav1-iCre*, which disrupted the function of all Runx proteins. This led to severe reductions of classical and plasmacytoid dendritic cells (DC) in the spleen and peripheral tissues, including the lung and intestinal lamina propria. Cbfβ was found requisite for the development of $Flt3^+$ DC progenitors, as well as erythroid progenitors in the BM [20]. Further analysis revealed that Runx1 was the primary driver of the Cbfβ knockout DC phenotype, with Runx2 playing a minor role later in DC maturation. However, no role was ascribed to Runx3, in contrast with earlier reports of aberrant DC development and spontaneous maturation in a *Runx3* knockout mouse [20, 48, 83]. Notwithstanding this discrepancy, Runx3 is needed Langerhans cells (LC), which are a distinct skin epidermal DC lineage derived from myeloid progenitors early in embryogenesis [83, 105]. Here, Runx3 acts downstream of PU.1 to promote LC differentiation induced by TGFβ signaling [105].

In addition to DC differentiation and maturation, RUNX3 regulates CD11c and chemokine expression in an isoform-specific manner *in vitro* [106]. As DC are central coordinators of adaptive immune responses and self-tolerance, the importance of Runx proteins in their differentiation will have far-reaching effects in the immune system. In addition to DC, a recent study showed that specific ablation of the *Runx1c* isoform in mice resulted in a drastic reduction in granulocytic basophils, owing to a block in the transition of granulocyte progenitors to basophil progenitors. Furthermore, Runx1c-deficiency specifically attenuated basophil (and not mast cells) functions, including IgE-mediated chronic allergic skin reaction, and their expansion in response to IL-3 or nematodes [107].

In monocytes and macrophages, RUNX1 cooperates with PU.1 to regulate macrophage colony-stimulating factor (M-CSF/CSF-1) receptor, which is essential for the survival, differentiation and expansion of macrophages [108, 109]. RUNX1 and RUNX3 are also important controllers of adhesive interactions through their regulation of integrin-like LFA-1/CD11a, CD11c, CD49d and ICAM-3 in macrophage and monocytic cell lines [110-112]. Of note, LFA-1 and ICAM-3 interaction contribute to the polarization of Th1 cells, highlighting the multifaceted contribution of RUNX to T cell differentiation and function [111]. The microglia are unique phagocytic cells that reside within the parenchyma of the central nervous system (CNS), where they serve as inflammatory cells that safeguard the CNS against microbes and injuries. Akin to the LCs, microglia are derived from $Runx1^+$ yolk sac primitive myeloid progenitors and are seeded in the CNS during embryonic and perinatal stages of development [113-115]. Postnatally, Runx1 was reported to inhibit proliferation of immature microglia and promote their maturation [115]. Interestingly, Runx1 expression was reactivated following injury to the nervous system, suggesting a role in the function of mature microglia in CNS surveillance and repair [115].

RUNX in inflammation and autoimmunity

In the thymus, the induction of self-tolerance by nTreg is essential for the maintenance of immune homeostasis, the loss of which would lead to autoimmunity [116]. Given the heavy involvement of RUNX proteins in differentiation and function of nTreg and iTreg [42-46], as well as that of Th17 cells [53, 55], it is likely that the interference of RUNX function would contribute to autoimmune and inflammatory disease states. This possibility is supported by several extensive studies of genetic association in human autoimmune conditions. Firstly, an intronic SNP within the *PDCD1* locus that alters a RUNX binding site was associated with systemic lupus erythematosus (SLE) in an ethnically diverse cohort. The alteration in RUNX site disrupted RUNX1 binding *in vitro* and led to aberrant expression of PDCD1, which is important to self-tolerance and the suppression of hyperactivity in SLE [117]. Of note, PDCD1 (also called PD-1) is a negative regulator of tumor immunity and an important target of cancer immunotherapy [118]. Similarly, a RUNX site variant between a PDZ-domain phosphoprotein *SLC9A3R1* and N-acetyltransferase *NAT9* was associated with psoriasis, a chronic inflammatory skin disorder [119]. In addition, the *RUNX1* locus and a RUNX site-ablating SNP in the transporter gene *SLC22A4* were found to be significantly associated with rheumatoid arthritis [120]. It bears highlighting that the disease-associated RUNX sites could function as the binding targets of all RUNX proteins as they recognize the same cognate sites [117, 119, 120]. Consistent with this, a *Runx3* knockout mouse model spontaneously developed an inflammatory bowel disease (IBD)-like condition characterized by infiltration of leukocytes with mixed Th1/Th2 response and hyperplastic mucosa [47]. In human, the chromosomal region 1p36 where RUNX3 resides is a susceptibility region for IBD [121-123]. More recently, genome-wide association studies (GWAS) identified variants in the *RUNX3* locus to be associated with two subtypes of IBD, namely celiac disease and ulcerative colitis [124, 125]. Lastly, SNPs within the *RUNX1* locus and *RUNX1* levels were also found associated with pediatric asthma [126]. Together, the above findings suggest that alteration of RUNX functions could lead to dysregulated self-tolerance, chronic lymphocyte hyperactivity and autoimmunity in organs.

Established and emerging roles of RUNX in mucosal immunity

Although direct evidence is only beginning to emerge, RUNX proteins are strongly implicated in innate and adaptive immunity in mucosal systems. RUNX family members play important roles in the development, maturation and effector functions of every major immune lineage within the mucosal system (illustrated in Figure 3). Importantly, RUNX is functionally integrated into the overall immune response at multiple levels. An instance of this is in the biogenesis, organization and function of mucosal lymphoid tissues, like Peyer's patches [72]. In addition to their formation, Runx proteins also participate in the activation of periphery T and B cells within them by regulating DC maturation for antigen presentation [20,48, 83]. Following activation, the terminal differentiation of naïve peripheral T cells is coordinated by Runx proteins in partnership with various lineage-defining transcription factors, into effector T helper and cytotoxic lineages [22, 127]. Of particular relevance in the mucosal system is the role of Runx1/3 in the differentiation of IELs in response to environmental cues and pathogenic challenges [65-67]. Runx1 is further involved in the development of

iNKT lymphocytes, which orchestrate microbial immunity through cytokine production [71, 72]. Concurrently, Runx3 and Runx2 are essential for IgA class switching in mature B cells, a crucial event in the configuration of mucosal immunity in response to microbial pathogens [128]. Lastly, RUNX1 is implicated in the maturation of FCRL4$^+$ memory B cells that resides in gastrointestinal epithelial niche [95].

Though less understood, Runx proteins function through innate leukocytes and lymphocytes to mount innate immunity at the mucosa. Of note, Runx3 is necessary for the expansion and maturation of NK cells, which serve important cytotoxic functions independent of antigen presentation [104]. Similarly, Runx1 is needed for the expansion of basophils and maximal anti-parasitic and proinflammatory activities [107]. In macrophages and monocytes, RUNX transcriptionally regulates adhesion complexes to promote their migration to the sites of infection to perform their phagocytic functions [111, 112].

In addition to hematopoietic cells, a major component of mucosal immunity is the epithelial cells that constitute the foremost barrier to the mucosal microbiota. The precise contribution of this cell type to mucosal immunity through antigen processing and cytokine production is currently under-explored. Likewise, while RUNX proteins are important in a diverse range of epithelial tissues, the potential for their immune contribution has not been experimentally tested [1]. Recently, RUNX3 was reported to transcriptionally regulate *IL23A* in gastric epithelial cells [129]. IL23A is a subunit of IL-23, a proinflammatory cytokine best known for driving Th17 activities [130]. Furthermore, RUNX3 strongly augmented the secretion of IL23A in the presence of the proinflammatory cytokines TNF-α and IL-1, and the gastric pathogen *Helicobacter pylori* [129]. These findings are conceptually significant as they provide evidence that RUNX proteins can further participate in innate immunity through epithelial cytokine production.

Concluding remarks

It is evident that RUNX proteins are profoundly involved in the development, organization and function of the mammalian immune system. Much of these are accomplished through their multifaceted contribution to definitive and adult hematopoiesis. In addition, RUNX is often recurrently involved in cell fate decision within a lineage in response to extracellular cues, through interplays with other primary lineage-determining factors. This is most apparent during T cell differentiation, where an antagonistic interplay between Runx complex and Th-POK determines the fate of CD8$^+$ and CD4$^+$ SP T cells. This is revisited in activated periphery T cells, where RUNXs concurrently promote one T helper phenotype while suppressing another through molecular interplays with T-bet, FoxP3 or RORγT. Such observations are consistent with the RUNX loci being a part of a finely tuned high-order transcriptional circuit. Importantly, this circuit is functionally oriented and extends beyond hematopoietic lineage decision. For example, the RORγT-RUNX axis impacts diverse immune functions, notably inflammation (via Th17 cells), commensal microbe tolerance (iNKT cells) and lymph node biogenesis (LTi cells). These observations indicate that

RUNX proteins are amongst the primary building blocks of the immune system during evolution [131]. Therefore, elucidating the spatial, temporal and functional contribution of RUNX to the dynamics and complexity of the immune system is an attractive goal. However, that RUNX functions are deeply woven into the immune processes presents a significant technical challenge. Indeed, direct evidence of their contribution to overall immunity is lacking and awaits studies combining lineage-specific gene targeting with immunologic challenges. It is hoped that this immune oriented survey of current knowledge will stimulate future studies of RUNX functions beyond hematopoiesis into immunity.

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Disclosure of Competing Interests

The authors declare that they have no competing interests.

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FIGURE LEGENDS

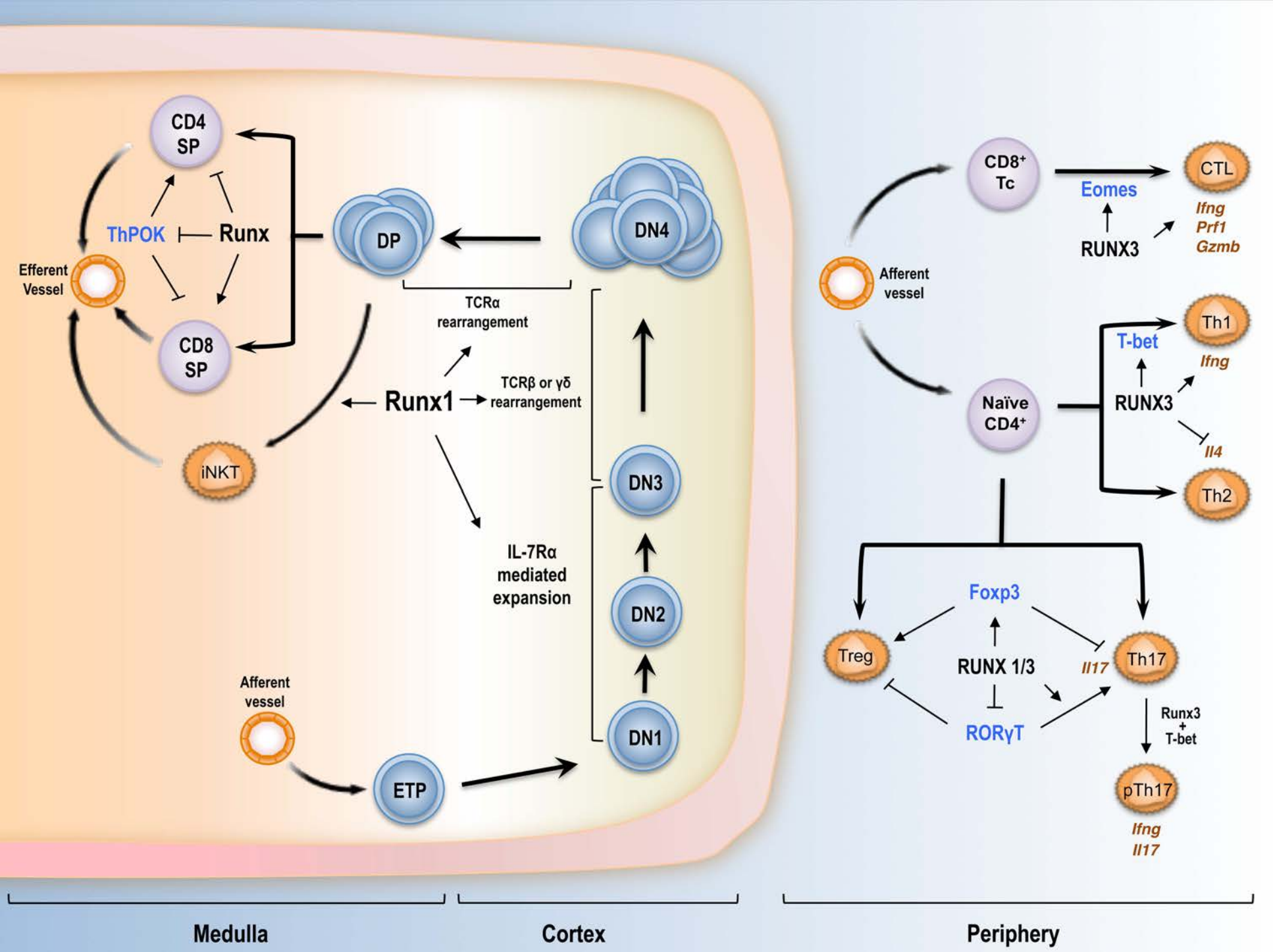
Figure 1: RUNX and T lymphocytes differentiation. In the thymic cortex, Runx1 is expressed in $CD4^-CD8^-$ double negative (DN) thymocytes, reaching a maximum at DN3 before declining during DN3 to DN4 transition. Runx1 transcriptionally orchestrates IL-7Rα-mediated expansion, T cell receptor (TCR) γδ and TCR αβ rearrangement during these developmental stages. In addition, Runx1 is also a key factor for the differentiation of invariant NKT (iNKT) cells in the medulla cortex of the thymus. Following TCR-mediated selection, Runx3 gains prominence and is a major driver of $CD8^+$ T cell differentiation through the silencing of *Cd4* and *Thpok*, master regulator of $CD4^+$ differentiation. In the periphery, Runx3 promotes the maturation of $CD8^+$ T cells into cytotoxic T lymphocytes (CTL) via its regulation of Eomes and key effector genes. In TCR-activated $CD4^+$ T cells, Runx3 cooperates with T-bet to fortify the Th1 phenotype by activating *Ifng* expression while suppressing Th2-specific cytokine *IL4.* Runx proteins are also important in the differentiation and functions of Treg cells through its regulation and interaction with Foxp3. Lastly, Runx proteins influence the development of Th17 in distinct ways. Runx could suppress or activate *Rorγt* depending on the presence of Foxp3, while interact with Rorγt to activate *Il17* expression. Moreover, Runx1/3 are needed for the production of IFN-γ in a subset of Th17 cells important in the pathogenesis of autoinflammatory conditions. In this figure, key T lineage determinants that functionally interact with RUNX are labeled in blue and notable effector genes are labeled brown. ETP denotes early thymocyte progenitors.

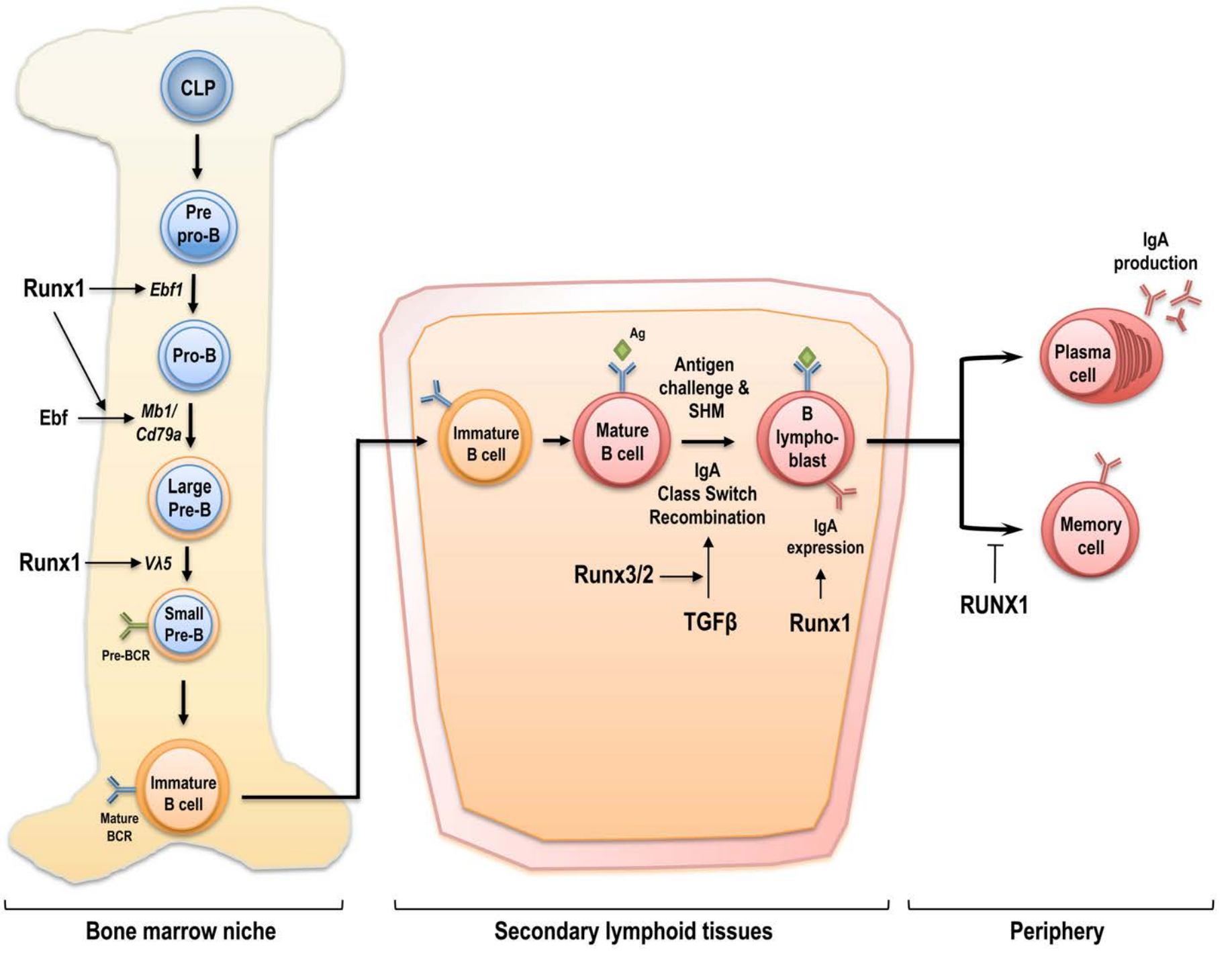
Figure 2: RUNX in B cells development and humoral immunity. In adult bone marrow, Runx1 is involved in the differentiation of B cell from common lymphoid progenitors (CLP). Specifically, Runx1 regulates and cooperates with Ebf for the transition of pre-proB cells to pro-B cells. Runx1 also takes part in the transition of large pre-B cells to small pre-B cells via V_H to DJ_H recombination as well as regulation of pre-B cell receptor (BCR). Runx3 is necessary in the later stage of B cell development in secondary lymphoid tissues. Runx3 and Runx2 function downstream of TGFβ-mediated IgA class switching, a key event in the development of B lymphoblast. Runx1 is also needed to maintain high IgA expression on the surfaces of activated lymphoblasts. In human, RUNX1 further influences the differentiation of $FCRL4^+$ memory B cells that normally reside in mucosal tissues.

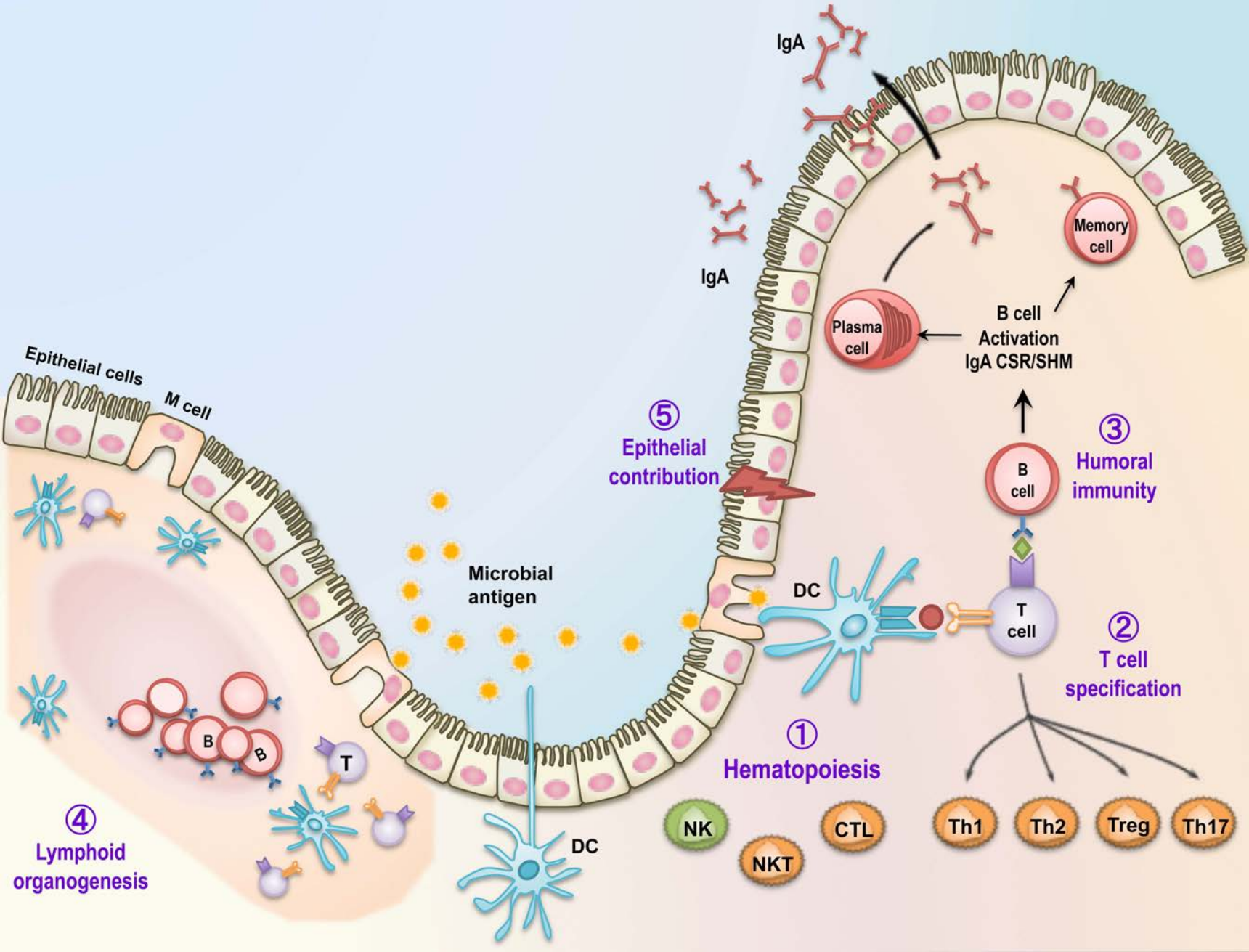
Figure 3: The multifaceted contribution of RUNX in mucosal immunity. (1)RUNX proteins are essential for the differentiation and effector functions of diverse cell lineages that participate in the mucosal immune system. These include T cells, B cells and dendritic cells (DC) for adaptive immunity; as well as macrophages, NK cells and epithelial cells for innate immunity. (2) Runx1 and Runx3 are critically involved in the specification of CD8+ cytotoxic T (Tc) and naïve $CD4^+$ helper T (Th) lymphocytes prior to their entrance into the periphery. Following antigen engagement and TCR activation, Runx1/3 coordinate the terminal differentiation of $CD4^+$ T cells into Th1, Th2, Th17 and Treg, and $CD8^+$ T cells into CTLs effector T lineages. (3) In B lymphocytes, Runx play a crucial role

in TGFβ-mediated IgA class switching following antigen engagement. Runx proteins are also necessary for the maximal surface expression of IgA on activated B lymphoblast and the maintenance of specialized memory B (Bmem) cells distributed at the mucosa. (4) The Runx complex is required for the formation of anlagen that initiate Peyer's patches and periphery lymph node biogenesis. These secondary lymphoid tissues are home to naïve periphery lymphocytes and dendritic cells and are necessary for maintaining surveillance and homeostasis. (5) The mucosal epithelium is in direct contact of the microbial load and functions as a physical as well as immune barrier. RUNX proteins are functionally important in a diverse range of mucosal epithelial cells, including those in lung and gastrointestinal tract. Runx3 is requisite for the homeostasis of an intact mucosal epithelium by regulating the proliferation and apoptosis of epithelial cells [1]. RUNX proteins may further contribute to innate immunity within the epithelium by regulating cytokine production (such as IL23A) during infection and inflammation [129].

Lineage	Differentiation step	RUNX/CBFβ	Description	Immune functions affected when RUNX/CBFβ is disrupted	References
T lymphocytes	Thymocytes	Runx1	DN2 to DN3 transition by regulating IL7Rα, TCRγδ rearrangement	Defective TCR rearrangement and thymocytes maturation	13-15, 26, 28, 29, 34
	CD4/CD8	Runx3,1	DP to $CD8^+$ SP differentiation, TCRαβ rearrangement	Reduced $CD8^+$ Tc/CTL numbers	26, 30, 31, 33, 132
		Runx1	DP to $CD4^+$ TCR-αβ rearrangement	Reduced Il7r and survival	132
	Th1/2	Runx3	Promotes Th1 phenotype in cooperation with T-bet	IFN-γ production, IL-4 suppression	37, 38
	Treg	Runx1 Runx1,3, Cbfβ	Cooperates with Foxp3 in nTreg function Induction and function of iTreg	Repression of IL-2 Repression of IL-2 Reduced immune tolerance especially in mucosal surfaces.	46 42-45 42, 43
	Th17	Runx1, 2	Promote Th17 differentiation by inducing *RORγT* and *Il17* transcription Inhibits Th17 by cooperating with Foxp3 to suppress RORγT	IL-17 production	55
		Runx1, 3	Promotes pathogenic Th17 and secretion of IFN-γ with T-bet	IL-17 and IFN-γ	57
	IEL	Runx3	Necessary for $CD8\alpha\alpha^+$ expression. Cooperates with T-bet to suppress Th-POK	Accentuated Th17 differentiation	64-67
	Tc/CTL	Runx3	Necessary for $CD8^+$ Tc differentiation	Reduced CTL activity	26, 69, 132
			Regulates Eomes, granzyme, perforin and IFN-γ expression	Reduced CTL activity	39
	NKT	Runx1, Cbfβ	Needed for iNKT differentiation	Undetermined	71, 72
	Dermal dendritic T cells	Runx3	Promotes maturation of γ3 thymocyte via CD103 and IL-2Rβ	Loss of adult skin DETC	75
B lymphocytes	Immature B cells	Runx1 Runx1/Cbfβ	Early B cell maturation prior to Pre- and Pro-B stage Regulates pre-B cell receptor Interacts with Ebf to activate *mb-1*/*cd79a* Promote pre-proB to pro-B transition by inducing *Ebf1*	Defective B cell expansion and maturation. Reduced IgM^+ B cells and V_H to DJ_H recombination	13-15, 84, 85 15 , 86 84 85
	B cell maturation	Runx3	Cooperates with TGF-β for activating germline Ig α promoter	Defective IgA class switching	78-82
		Runx1	Promotes surface IgA expression in activated primary B cells	Defective IgA class switching	82
		Runx3, Runx2	Necessary for IgA expression in peripheral B cells	Reduced IgA production	82
	Memory B cells	RUNX1	Maintains undifferentiated state by silencing FCRL4	Undetermined	95
	Primary B cells	RUNX1	Suppresses proliferation of resting B cells	Undetermined	88
		RUNX3	Immortalizes B cells via silencing of RUNX1	Undetermined	88, 89, 91, 92
NK cells	NK differentiation	Cbfβ	Needed for $NK1.1^-CD122^+$ progenitor	Undetermined	100, 101
		Runx3	Regulates *CD122*, *Ly49* family, *Mac-1* ,*CD43* and *IFN-γ* Needed for IL-15-induced NK cell proliferation and maturation	Undetermined	102, 103 104
	Uterine NK cells	Runx3	Essential for IL-15-dependent uterine NK cells	Loss of uterine NK cells	104
LTi cells	LTi cells	Runx1c, Cbfβ2	Necessary for early specification of LTi lineage Induction of Rorγt at anlagen	Loss of Peyer's patches and periphery LNs.	72
		Runx1	Necessary in BM cells for LTi differentiation	Absent or defective Peyer's patches	15
Granulocytes	Dendritic cells (DC)	Cbfβ, Runx1,2 but not Runx3	Required for $Flt3^+$ DC progenitors.	Loss of classical and plasmacytoid DC	20
		Runx3	Cooperates with TGFβ to suppress DC maturation Restricts DC migration by suppressing *CCR7* expression.	Spontaneous DC maturation Allergic airway inflammation; severe gastritis.	48, 83 47, 48, 83
	Langerhans cells (LC)	Runx3	Mediates TGF-β-induced LC differentiation	Loss of Langerhans cells	83, 105
	Basophils	Runx1c	Required for generation of basophil progenitors	Attenuated basophil expansion and functions.	107
	Monocytes/macrophage	RUNX1	Cooperates with PU.1 to induce M-CSFR	Reduced macrophage survival, differentiation and expansion	108, 109
		RUNX3,1	Regulates *LFA- 1*/*CD11a, CD11c*, *CD49d* and *ICAM-3*	Undetermined	110-112
	Microglia	Runx1	Runx1 promotes microglia maturation Runx1 is induced during nerve injury	Runx1 restricts iNOS production *in vitro*	115
	Gastric epithelial cells	Runx3,Runx1	Response to inflammation and infection	Secretion of IL23A	129


CD4 SP
ThPOK
Runx
CD8 SP
Efferent Vessel
iNKT
DP
DN4
TCRα rearrangement
Runx1
TCRβ or γδ rearrangement
IL-7Rα mediated expansion
DN3
DN2
DN1
Afferent vessel
ETP
Medulla
Cortex
CD8⁺ Tc
Eomes
CTL
Ifng
Prf1
Gzmb
RUNX3
Afferent vessel
Naïve CD4⁺
T-bet
Th1
Ifng
RUNX3
Il4
Th2
Foxp3
Treg
RUNX 1/3
Il17
Th17
RORγT
Runx3 + T-bet
pTh17
Ifng
Il17
Periphery


CLP
Pre pro-B
Runx1
Ebf1
Pro-B
Ebf
Mb1/ Cd79a
Large Pre-B
Runx1
Vλ5
Small Pre-B
Pre-BCR
Immature B cell
Mature BCR
Ag
Immature B cell
Mature B cell
Antigen challenge & SHM
IgA Class Switch Recombination
B lympho-blast
IgA expression
Runx3/2
TGFβ
Runx1
IgA production
Plasma cell
Memory cell
RUNX1
Bone marrow niche
Secondary lymphoid tissues
Periphery


IgA
IgA
Memory cell
Plasma cell
B cell
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IgA CSR/SHM
Epithelial cells
M cell
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Epithelial
contribution
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Humoral
immunity
B cell
Microbial
antigen
DC
T cell
②
T cell
specification
①
Hematopoiesis
NK
NKT
CTL
Th1
Th2
Treg
Th17
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Lymphoid
organogenesis
DC