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Editorial

Editorial: **An ATRA oddity: new questions revealed on retinoid synthesis in bone marrow cells**

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Retinoids are derivatives of vitamin A (retinol) and function as essential nuclear-targeting hormones that activate heterodimers of RAR (α, β, and γ) and RXR proteins. The activated RAR/RXR complexes control multiple developmental pathways during early organogenesis, including the differentiation of hematopoietic, bone, skin, and certain neural tissues [1, 2]. The importance of retinoids to human physiology is revealed by the diverse phenotypes attributed to vitamin A deficiency, including anemia, atrophy of the thymus and spleen, and increased susceptibility to infection. One well-studied retinoid in hematopoiesis is ATRA, which supports HSC self-renewal by activating RARγ. This role was revealed in RARγ null mice that exhibited decreased HSC numbers associated with increased production of myeloid and lymphoid progenitors [3]. ATRA also acts through RARγ to drive the expression of genes important to hematopoietic cell functions, including those encoding effector proteins in T cells and inflammatory cytokines in macrophages [4]. During myelopoiesis, ATRA can override the developmental block created by RARα fusion proteins that act in a dominant-negative fashion, now established as the underlying cause of APL [5, 6]. Pharmacologic levels of ATRA cause the release of transcriptional corepressors (e.g., N-CoR, SMRT, and HDAC) at RAREs and subsequent recruitment of coactivators that up-regulate the expression of genes required for promyelocyte differentiation (**Fig. 1**). Thus, intracellular ATRA and the expression of the enzymatic machinery required for its synthesis (as depicted in Fig. 1 and described in the accompanying legend) are considered critical to the maintenance of HSC populations and the differentiation of myeloid and lymphoid lineages. However, results from the current study by Niu et al. [7] raise 2 important questions regarding ATRA metabolism during hematopoiesis: 1) to what extent is intracellular ATRA actually synthesized by HSCs and mature myeloid lineages, and 2) do different developmental stages of hematopoiesis use the same enzymatic machinery to maintain required levels of ATRA?

Niu et al. [7] used a dual vector reporter system designed to provide constitutively expressed Gal4-RARα fusion proteins that are activated by intracellular ATRA and trigger expression of a UAS-GFP transgene [8]. The UAS-GFP/Gal4-RARα reporter system was tested initially in transfected human embryonic kidney 293T cells, in which serum-available retinols supported ATRA synthesis (quantified by GFP expression) that decreased upon addition of ALDH1-specific inhibitors (DEAB or WIN 18446). The study then transitioned to the analysis of mouse bone marrow-derived, ex vivo-cultured Kit$^+$ progenitors transduced with the same reporter system, but the authors discovered that ATRA synthesis from serum-derived retinols was undetectable. Addition of ATRA or its metabolites (retinyl acetate or RAL) significantly increased the percentage of GFP$^+$ cells, which declined when the progenitors were treated with DEAB or WIN 18446. These results verified that Kit$^+$ progenitors can synthesize ATRA, and this process is ALDH1 dependent, but GFP is detected only when cells are cultured with superphysiologic concentrations of ATRA precursors. Additionally, transcripts encoding 3 class 1 Aldh1 isoforms (Aldh1a1, -2, or -3) were detected in the Kit$^+$ progenitors, consistent with previous findings of their high-level expression in HSCs and early myeloid progenitors [9]. However, the lack of detectable ATRA synthesis in Kit$^+$ progenitors cultured in serum alone suggests that only low levels of ATRA are maintained in early hematopoietic precursors, a notion supported by the observation that inhibition of ALDHs delays HSC differentiation [10]. The

Abbreviations: ADH = alcohol dehydrogenase, ALDH = aldehyde dehydrogenase, APL = acute promyelocytic leukemia, ATRA = all-*trans*-retinoic acid, BMMΦ = bone marrow macrophage, CRABP = cellular retinal binding protein, CRBP = cellular retinol binding protein, Cyp = cytochrome p450, DEAB = diethylaminobenzaldehyde, Gal4 = galactose-responsive transcription factor 4, HDAC = histone deacetylase, HSC = hematopoietic stem cell, N-CoR = nuclear receptor corepressor 2, RA = retinoic acid, RAL = retinal, RAR = retinoic acid receptor, RARE = retinoic acid response element, RBP = retinol binding protein, ROS = reactive oxygen species, RXR = retinoid X receptor, SMRT = silencing mediator for retinoid or thyroid-hormone, STRA6 = stimulated by retinoic acid 6, UAS = upstream activating sequence, WIN 18446 = *N,N'*-bis (dichloroacetyl)-1, 8-octamethylenediamine

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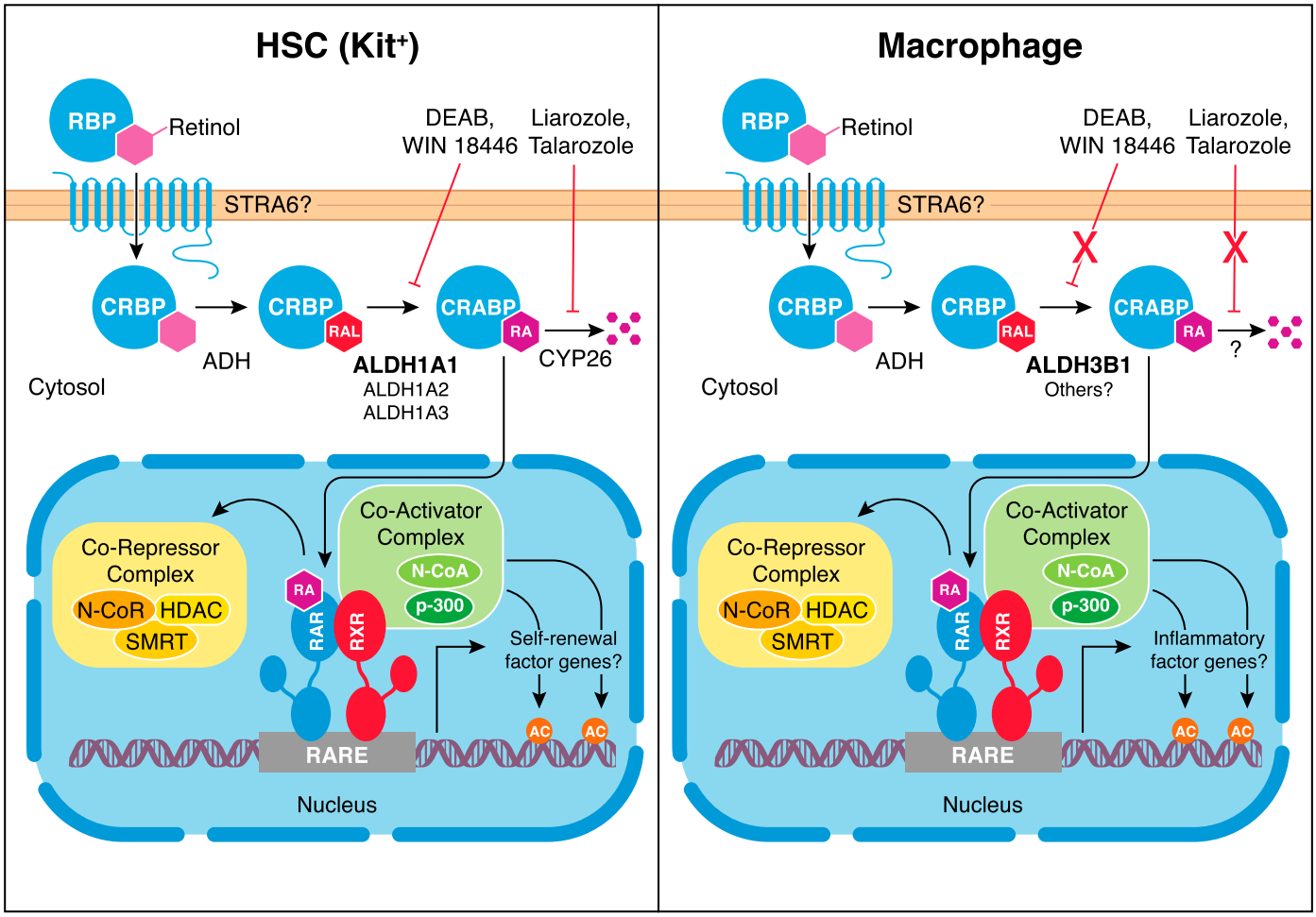

Figure 1. Depiction of the proposed retinoid signaling pathway in a HSC or macrophage. Retinol is delivered in blood serum as bound to RBP and enters the cell through the RBP receptor, STRA6. Intracellular retinol then binds to CRBP and is enzymatically converted by ADH to RAL. RAL is released from CRBP and then binds CRABP, where it is oxidized by ALDH to RA (all-*trans* or 9-*cis*). Three ALDH isotypes are found in HSCs, among which ALDH1A1 (in bold) is expressed at the highest level. By comparison, macrophages lack expression of class 1 ALDHs but express high levels of the class 3 ALDH, ALDH3B1. Upon entering the nucleus, RA binds to a RAR/RXR complex, which undergoes a conformational change that triggers the release of the corepressor complex, followed by the recruitment of coactivator complex proteins. Histone acetyltransferase (e.g., p300), bound to the coactivator complex, opens the nearby chromatin structure, allowing binding of the basal transcriptional machinery to promoters of genes encoding self-renewal (HSC) or function-specific (macrophages) factors. Meanwhile, residual cytoplasmic RA is degraded in HSCs by the actions of CYP26; however, the mechanism for RA degradation in macrophages is unknown. The schematic also illustrates the inhibitory effects of drugs known to disrupt ALDH1 (DEAB and WIN 18446) or CYP26 (Liarozole and Talarozole) activities in HSCs, neither of which affected RA metabolism in macrophages.

importance of Cyp26 for maintaining ATRA was also investigated, as this p450 cytochrome is expressed in HSCs and causes ATRA catabolism. Two Cyp26-specific inhibitors, Liarozole or Talarozole, caused increased ATRA levels in Kit$^+$ progenitors when supplied along with the ATRA metabolites. Interestingly, the *CYP26* gene contains a canonical RARE, suggesting a positive-feedback mechanism for RA catabolism that is crucial for regulating ATRA levels [11].

Application of the same detection system on ex vivo-cultured BMMΦ or neutrophils yielded results similar to those obtained from Kit$^+$ progenitors; neither myeloid lineage synthesized sufficient ATRA from serum-derived retinols to activate GFP expression, whereas addition of exogenous RAL produced abundant numbers of positive cells. However, and quite surprisingly, the addition of DEAB or Cyp26-specific inhibitors with BMMΦ had no effect on RAL-stimulated Gal4-RARα activity. Furthermore, BMMΦ lacked expression of all 3 ALDH1 isoforms, which was unexpected, given the requirement of ATRA for RARγ activation and its role in driving the expression of multiple inflammatory cytokines (e.g., IL-6, IL-12, and TNF-α) expressed in stimulated macrophages [4]. Further analyses using Affymetrix array profiling and RT-PCR assays demonstrated that an alternative ALDH, *Aldh3b1*, was highly expressed in BMMΦ. Endogenous enzymatic activity of Aldh3b1 was much greater in the BMMΦ compared with Kit$^+$ progenitors. Furthermore, small interfering RNAs targeting *Aldh3b1* transcripts in transduced BMMΦ suppressed Gal4-RARα activity,

confirming that Aldh3b1 supports ATRA generation in macrophages. Continued studies with neutrophils suggested that these cells use both classes of ALDHs; addition of DEAB reduced Gal4-RARα activity, and *Aldh1a1* expression was identified, but *Aldh3b1* was also expressed in ex vivo-cultured granulocytes and polymorphonuclear neutrophils from mice and humans. Despite these intriguing ex vivo results, perhaps the most significant finding was that neither in vivo bone marrow progenitors nor peritoneal macrophages exhibited evidence of ATRA synthesis, even when mice were supplied vitamin A-sufficient diets or exposed to hematopoiesis-stimulating reagents. A lack of the retinol importer protein STRA6 could not explain deficient ATRA synthesis, because BMMΦ overexpressing this protein still lacked Gal4-RARα activity.

Therefore, hematopoietic progenitors maintain robust levels of ALDH enzymatic activity, but the question remains as to why macrophages and neutrophils express an alternative class of ALDHs compared with immature progenitors. In addition, BMMΦ may use other Cyp family members to catabolize ATRA, 2 of which were identified in these cells (Cyp20a1 and Cyp4v3), but their function in ATRA degradation was not investigated. The lack of ATRA production in maturing neutrophils is particularly perplexing, given the well-established promyelocyte response to ATRA during terminal differentiation. Interestingly, there is only limited evidence that ATRA-induced activation of RARα/RXR complexes directly control the expression of neutrophil lineage-specifying genes, excluding those that encode the C/EBP family transcription factor, C/EBPε [5]. Nonetheless, ATRA synthesis by hematopoietic cells in vivo may not even occur, despite abundant ALDH activity and its proposed role as the rate-limiting step in this process [12]. Therefore, gene expression changes previously attributed to activated RAR/RXR complexes in maturing blood cell lineages may require additional mechanisms. Another important consideration is that some ALDH proteins perform functions unrelated to ATRA synthesis, specifically metabolism of ROS and reactive aldehydes: ROS levels are generally lower in quiescent cells compared with proliferating and differentiating cells, and both ROS and elevated reactive aldehyde levels have been implicated in multiple diseases, including myeloid leukemias [12, 13]. ALDHs may protect rapidly proliferating multipotent progenitors that are susceptible to leukemogenic transformation induced by ROS or reactive aldehydes. Why macrophages express a class 3 ALDH is unclear, but perhaps there is a mechanistic advantage for the fully differentiated cells to express an ALDH that is less efficient in eliminating ROS, high levels of which are critical to bacterial destruction.

The observations by Niu et al. [7] have provided new insight into retinoid metabolism during hematopoiesis; however, there are several aspects of this study that would benefit from further investigation and improved methodologies. For example, the expression of the carrier proteins CRBP and CRABP, each thought to mediate the enzymatic actions of ADH and ALDH, respectively (Fig. 1), have not been thoroughly analyzed in the hematopoietic compartment. The regulatory potential of CYP26 in degrading ATRA during hematopoiesis also requires consideration, given that RA-mediated induction of CYP26 expression may contribute to ATRA resistance in APL patients [11]. ATRA degradation may be too rapid to detect with the current UAS-GFP/Gal4-RARα reporter system; thus, the identification of the types and activity levels of Cyp during hematopoiesis is warranted. Moreover, there are drawbacks with the current ATRA reporter system, including use of retroviral vectors and GFP for detection, which could impact retinoid synthesis and are not conducive to in vivo human studies. The sensitivity of the detection system should be increased, as ATRA may be present in hematopoietic lineages in vivo, at least at levels sufficient to support RAR/RXR activities. Comparative analysis of the ATRA metabolic machinery within homogenous populations of primary mouse versus human HSCs, rather than mixed populations or leukemic human bone marrow samples, may reveal that each species uses different ALDH classes or isoforms. A comprehensive survey of the mechanisms mediating retinoid biology throughout hematopoiesis may address some of the observed oddities of ATRA synthesis and help elucidate the pathology of ATRA resistance in an increasing population of APL patients that pose serious treatment challenges.

AUTHORSHIP

M.G.H.P. and P.G. contributed equally to the writing of the manuscript.

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Editorial: **"Invisible" MDSC in tumor-bearing individuals after antibody depletion: fact or fiction?**

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Tumors can cause an expansion of myeloid cell populations in different parts of the body, such as blood, spleen, lung, and liver, irrespective of the presence or absence of a tumor in that particular organ. In mice, $CD11b^+Gr1^+$ cells compose the majority of the tumor-induced myeloid population. MDSCs are immature $CD11b^+Gr1^+$ cells with the ability to suppress antigen-specific and nonspecific T cell responses and to promote primary tumor progression and metastatic spread [1]. Murine MDSCs can be divided into 2 major subgroups: granulocytic $CD11b^+Ly6G^+Ly6C^{low}$ (G-MDSC) and monocytic $CD11b^+Ly6G^-Ly6C^{high}$ (M-MDSC). In the absence of a marker, which can be used to distinguish G-MDSC from $CD11b^+Ly6G^+$ tumor-associated neutrophils, analysis of the suppressor function remains the "gold standard" to prove the presence of MDSCs. This can be done in vitro and in vivo.

A widely used method to study the in vivo function of the $CD11b^+Ly6G^+$ cells is antibody-mediated depletion of these cells. Two different antibodies are commonly used for this approach: anti-Ly6G (clone 1A8) and anti-Gr1 (clone RB6-8C5). Several investigators have used RB6-8C5 for the depletion of MDSCs [2, 3]. Targeted depletion of G-MDSC but not M-MDSC has been achieved using 1A8 in *KPC* mice bearing spontaneous pancreatic ductal adenocarcinoma [3]. However, recently, concerns have been raised about antibody-mediated MDSC depletion studies. Ribechini et al. [4] observed that RB6-8C5 injection failed to eliminate bone marrow MDSC. Instead, bone marrow MDSC started to proliferate upon antibody treatment. Furthermore, we reported the presence of hepatic MDSC in tumor-bearing mice after RB6-8C5 treatment. This was a result of an immediate reconstitution of the liver with MDSC upon elimination [5].

In this issue of the *Journal of Leukocyte Biology*, Moses et al. [6] carefully investigated antibody-mediated $Ly6G^+$ cell depletion in bone marrow, spleen, and tumor sites in a head and neck tumor-bearing mouse model. They used 1A8 and RB6-8C5 antibodies. $Ly6G^+$ cells were detected by flow cytometry, immunohistochemistry, and intravital imaging. The authors observed an effective depletion for 1 wk in peripheral blood, followed by a rebound phase with reappearance of immature $Ly6G^+$ cells with an immunoregulatory phenotype. Interestingly, unlike in peripheral blood, viable and depletion-resistant $Ly6G^+$ cells were found in tumor tissues, even in the early depletion phase. High systemic levels of G-CSF and KC, along with an enhanced extramedullary granulopoiesis, were detected. In line with reports that RB6-8C5 depletion fails to eliminate $CD11b^+Gr1^+$ cells in bone marrow [4] and liver [5], this study expanded the finding to $Ly6G^+$ cells in primary tumor sites.

Although 1A8 and RB6-8C5 antibody depletion has been widely used for a long time, the confirmation of depletion has often been overlooked for the following three reasons.

WHERE TO LOOK

Studies from Ribechini et al. [4], ours [5], and Moses et al. [6] consistently found that 1A8 and RB6-8C5 antibodies have compartment-dependent differential depletion capability. A nearly complete depletion of MDSCs in blood and spleen is observed during the early depletion phase. In contrast, MDSCs can still be found in bone marrow, liver, and tumor at the same time point. As

Abbreviations: G-MDSC = granulocytic myeloid-derived suppressor cell, KC = keratinocyte-derived chemokine, M-MDSC = monocytic myeloid-derived suppressor cell, MDSC, myeloid-derived suppressor cell

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