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Short communication

IL-33 enhances retinoic acid signaling on CD4+ T cells

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ABSTRACT

Several molecules have been described as CD4+ T cells differentiation modulators and among them retinoic acid (RA) and more recently, IL-33, have been studied. Due to the similarities in T helper cell skewing properties between RA and IL-33, we asked whether IL-33 intersects, directly or indirectly, the RA signaling pathway. Total CD4+ T cells from DR5-luciferase mice were activated in the presence of RA with or without IL-33, and RA signaling was visualized using *ex vivo* imaging. Our results demonstrate that IL-33 itself is able to trigger RA signaling on CD4+ T cells, which is highly increased when IL-33 is added in conjunction with RA. This study presents IL-33 as a potential player that may synergize with RA in controlling T cell differentiation, and suggests that IL-33 may be an attractive target in controlling T cell differentiation *in vivo*.

1. Introduction

Retinoic acid (RA), a vitamin A metabolite, has been widely studied due to its ability to drive CD4+ T cell differentiation. In early years, RA was ascribed as playing a role in regulating Th2-type responses, but with the acquisition of new technologies, it is now clear that RA signaling, specifically on CD8+ and CD4+ T cells, is required for T cell differentiation/polarization. In CD8+ T cells, RA is needed for these cells to proliferate and to mount an effective cytotoxic activity, having a relevant effector function as an anti-tumor agent [1]. In the case of CD4+ T cells, RA in conjunction with TGF-β is able to convert Foxp3+ regulatory T cells (Tregs) from CD4+Foxp3− T cell precursors *in vitro* and *in vivo* models of gut immunity [2–4]. Conversely, RA signaling is necessary for CD4+ T cells to produce Th1 and Th17-type cytokines, since CD4+ T cells expressing a dominant negative RA receptor (dnRAR) produced low amounts of these cytokines, disabling them from rejecting a skin allograft [5]. Even more, Brown and colleagues showed that RA is necessary to stabilize Th1 phenotype while restraining conversion toward Th17 cells, thus helping to avoid the occurrence of pathogenic Th17 cells [6].

Similarly to the immunoregulatory properties of RA, the *alarmin* IL-33, an IL-1 family member, was first identified as a Th2-type response modulator, but currently it is recognized that it may also affect Tregs biology and Th1/Th17 differentiation as demonstrated in various models including transplantation, experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis (CIA), among others [7].

Given the similarities between RA and IL-33 functions, we evaluated whether there is a crosstalk between RA and IL-33 during CD4+ T cells activation. Our results show that IL-33 has the ability to trigger RA signaling by itself; which is enhanced in the presence of both molecules, suggesting that CD4+ T cell activation, and further differentiation, could be modulated by IL-33 and RA. This becomes relevant during the initiation and development of an inflammatory response, where both molecules may be present to skew CD4+ T cell polarization.

2. Materials and methods

2.1. Reporting cells

Spleens from DR5-Luciferase reporter mice were used in this study, which were kindly provided by Dr. Randolph J. Noelle (Dartmouth College, New Hampshire, USA).

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2.2. Reporting assay

CD4+ T cells were isolated from the spleens DR5-Luciferase reporter mice using the CD4+ T cell isolation kit (StemCell technology, USA). 1×10^5 cells were seeded in 96-well plates previously coated with 10 μg/mL of anti-CD3 and 1 μg/mL of anti-CD28 (clones 2c11 and PV-1, respectively, both from BioLegend, USA); in the presence or not of 1, 10 and 100 nM all-*trans* RA, and 10 or 50 ng/mL of IL-33, for 3 days at 37 °C. After this time, cell culture media was removed and replaced with 150 μg/mL of D-Luciferin in PBS 1X (GoldBiotech, USA). Luminescence was detected using the *in vivo* imaging IVIS system (Xenogen, CA, USA), and the collected data was analyzed using the Living Image software (version 2.6.1).

2.3. Statistical analysis

Data were analyzed using an unpaired Student's *t*-test or a Mann-Whitney test (two-tailed). In all cases, $p < 0.05$ was considered with statistical significance. For data analysis, Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used.

3. Results

Due to the *in vitro* and *in vivo* antecedents stating that molecules such as RA and IL-33 have an impact on CD4+ T cell differentiation [7,8], we decided to evaluate the impact of these two molecules during *in vitro* CD4+ T cell activation. Overall, RA and IL-33 have been implicated with all different types of T helper responses (from Th1, Th17 to Th2, including Tregs linked responses), and in our experience, RA seems to be required for the differentiation of pro-inflammatory responses mediated by both CD4+ and CD8+ T cells [1,3,5,6]. Historically, the immune function of IL-33 has been similar to RA's, from having an activity in innate immunity and later also involved in adaptive immune responses, in which the involvement in all types of T helper responses is very well sustained. Thus, based on this information we decided to study, using a sensitive and simple assay, whether these two molecules interplay in CD4+ T cell biology.

Previous studies from Pino-Lagos et al. and Guo et al. indicated that RA signaling is triggered upon CD4+ and CD8+ T cell activation in models of allograft rejection and tumor *in vivo*, respectively. In addition, a recent report by Brown et al. demonstrated that RA signaling is involved in the process of differentiation through Th1 and helps to fix the phenotype in the late phase of differentiation.

Based on this evidence, we decided to visualize whether IL-33 could be involved in RA signaling. For this purpose, we used a sensitive reporting system in which the reporter enzyme luciferase is expressed upon RA binding to the nuclear RA response element (RARE, or DR5 repeat). When the substrate (luciferin) is available, the enzyme catabolizes the reaction generating a luminescent product, which may be detected (and quantified) using an equipment such as the IVIS imaging. Using this sensitive approach, one can track RA signaling overtime with no need to plate cells for every time point, for example. Thus, for our purpose, splenic CD4+ T cells isolated from the DR5-reporter mice were polyclonally activated in the presence of different concentrations of RA, IL-33 or both. Our data indicates that IL-33 itself is able to activate RA signaling, which seems to be dose dependent. Interestingly, T cell activation in the presence of both RA and IL-33 showed an evident enhancement in RA signaling (Fig. 1A and B), suggesting that their signaling pathways interact, synergizing their effects.

4. Discussion

Many lines of evidence show significant similarities between RA and IL-33 in controlling immune function. Both molecules were originally shown to modulate innate immunity, however later, a role in the regulation of the adaptive immune response became apparent. For example, RA and IL-33 are molecules released during the initiation of inflammation, and their effect can impact the biology of several leukocyte lineages, including macrophages, basophils, NK cells, neutrophils, among others [9–11]. In general terms, both RA and IL-33 function as mediators of inflammation with enhancing or amplification roles. More recent work has postulated that RA and IL-33 may target T cells activity in inflammatory and anti-inflammatory scenarios, since both players can tailor CD4+ T cell polarization, and therefore, modulate the immune response. Interestingly enough is the fact that RA is involved in Th1, Th2, Th17 and Treg differentiation, affecting a plethora of immunological events. On the other hand, IL-33 has been recently reported as an inducer of Treg cells as well (*in vitro* and *in vivo*), adding to the previous data stating that IL-33 may drive either Th1 or Th2 responses [12].

In the current study we sought to investigate whether RA signaling, which activation is required for CD4+ T cell differentiation, may be linked upon IL-33 recognition. As previously published, we have been able to quantify RA signaling using a sensitive reporting system in which all cells are able to transcriptionally up-regulate the enzymatically active reporter protein Luciferase when RA is sensed in the nucleus. By adding the corresponding substrate (luciferin) one can measure, and visualize, luciferase activity by *ex vivo* imaging. Exploiting these tools, we activated RA-reporting CD4+ T cells in the presence of IL-33, RA and both. Interestingly, our data indicates that IL-33 alone triggers RA signaling, suggesting that T

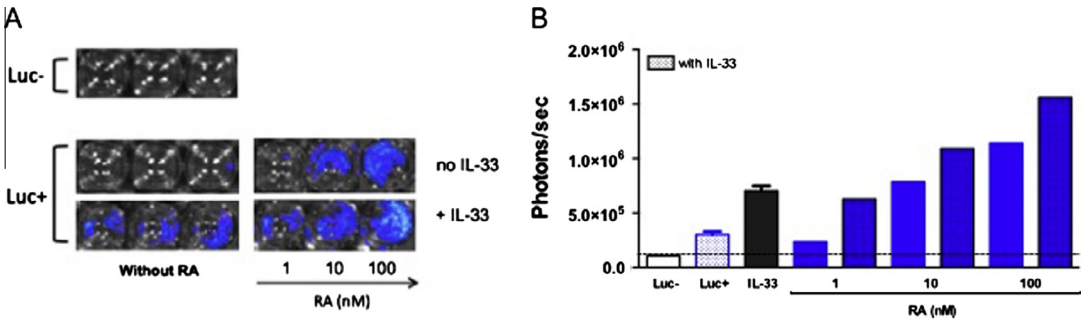

Fig. 1. IL-33 triggers RA signaling on CD4+ T cells. CD4+ T cells isolated from the spleens of DR5-Luciferase reporter mice were polyclonally activated for 72 h in the presence of IL-33 (10 ng/mL) and RA (at indicated doses). A. Luciferase activity was measured from *in vitro* activated CD4+ T cells using *in vivo* imaging. B. Luminescence (photons/s) was quantified using the same equipment than in part A. Bars represent SEM. Representative data of at least 3 experiments.

cell stimulation (αCD3 plus αCD28) in the presence of this cytokine may mobilize intracellular RA to the nucleus in CD4+ T cells. As expected, the addition of increasing amounts of RA shows increasing RA signaling, and the inclusion of IL-33 enhances this signal. Using a complementary approach, we isolated splenic CD11c+ DCs and treated them with GM-CSF and IL-4 to induce RALDH activity, in presence or absence of IL-33. Next, the modulated DCs were co-cultured with splenic CD4+ T cells to measure cytokine production. These experiments indicated that RALDH+ DCs are able to trigger both IFN-γ and IL-17, but the inclusion of IL-33 to DCs treatment greatly up-regulated the amounts of these two cytokines, indicating that RA and IL-33 signaling synergize and may affect the differentiation/polarization of CD4+ T cells (Supplementary Fig. 1). Translating this observation to an *in vivo* situation, and considering that RALDH activity by CD4+ T cells has not been reported (and in our hands this cell type does not synthesize RA [unpublished results]), one could suspect that the source of the detected reporting (nuclear) RA could be from other cell type, such as DCs or stromal cells (and acquired when the CD4+ T cells resided in the spleen). Alternatively, IL-33 could be able to stimulate RALDH activity in activated CD4+ T cells. Based on the available literature and the strong data demonstrating that IL-33 and RA similarly modulate the outcome of the immune response, we believe this data is novel and relevant for the understanding of CD4+ T cell biology and the implications associated with the use of these molecules as potential modulators of CD4+ T cell-mediated immunity in an array of diseases in which the role of CD4+ T cells (regardless of their T helper signature) is linked to pathologies. Clearly, more studies need to be performed to fully understand the interplay of IL-33 and RA, however this short communication sets the stage for future investigation taking into account RA and IL-33 as possible modulators of T cell function.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2016.06.016.

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