

Ageing affects the CD4⁺ T cell polarization and mucosal tropism induced by TLR2/TLR4-activated dendritic cells

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Abstract

Toll-like receptor (TLR)2 activation induces aldehyde dehydrogenase enzymes in nonmucosal dendritic cells (DCs) enabling them to metabolize vitamin A into all-trans retinoic acid, which induces the expression of mucosal homing molecules ($\alpha 4\beta 7$ and CCR9) in the activated T cells. Recently, we have shown that the simultaneous activation of nonmucosal DCs through TLR2 and TLR4 maintains such capacity while reinforcing the polarization of primed CD4⁺ T cells towards Th1. Here, we observed that TLR2/TLR4 stimulation of aged DCs leads to the production of less TNF α and more IL-10 and that CD4⁺ T cells primed by those DCs express lower levels of the mucosal homing receptor CCR9 and produce less type-1 (IFN γ) and more type-2 (IL-4 and IL-13) cytokines. These results emphasize the importance of considering the age-related alterations in DC function when developing novel immunomodulation strategies that rely on the DC-T cell crosstalk through stimulation of pattern recognition receptors.

Keywords: aging, dendritic cells, intestine, toll-like receptor 2, toll-like receptor 4

1. Introduction

Effective subunit vaccines rely not only on the identification of relevant target antigens, but also on the ability to tailor the immune response to the nature of the infectious agent. This requires programming the appropriate protective effector mechanisms while controlling their localization to the pathogen's port of entry or replication sites. Dendritic cells (DCs) play a central role in T cell polarization upon priming and the possibility to influence this process through the activation of pattern recognition receptors (PRRs) on DCs has long been recognized.¹ More recently, DCs were also shown to play a critical role in directing lymphocyte homing to different tissues. For instance, mucosal-associated DCs, unlike DCs from other locations, express aldehyde dehydrogenase (ALDH) enzymes that convert vitamin A into all-trans retinoic acid, which functions as a signal for T cells to express surface molecules involved in mucosal homing.² These homing-imprinting properties can also be modulated through PRRs,^{3–5} and TLR2 has emerged as a key receptor for the induction of mucosal immunity. Extra-intestinal DCs were shown to acquire gut-specific imprinting properties when stimulated through TLR2/1 by Pam₃CSK₄, but not by signals from other TLRs.⁶ Recently, we found that the simultaneous activation of non-mucosal DCs through TLR2 and TLR4 maintains the gut homing instructing capacity while reinforcing the polarization of primed CD4⁺

T cells towards a Th1 profile,⁷ suggesting that the inclusion of TLR2/TLR4 agonists in vaccines and immunotherapy could be explored to promote the gut localization of Th1 cells. These strategies could be particularly impactful to the most vulnerable populations, including the elderly, for whom vaccination and immunotherapies are more necessary and often less effective. Despite the success of several new T cell-dependent vaccines, the decline in vaccine effectivity with age is now well recognized.^{8–10} Although the cellular and molecular mechanisms remain unclear, defects in the function of both DCs^{11–13} and T cells^{14–16} have been identified, including alterations in PRR function in aged DCs.^{17,18} However, the changes in DC functions that may affect the tissue homing instructions imprinted on T cells are largely unknown.

In this Brief Report, we present results showing an intrinsic dysfunction of aged DCs to respond to simultaneous activation via TLR2/TLR4, affecting CD4⁺ T cell polarization and the expression of molecules associated with tissue tropism upon priming. To our knowledge, this is the first time that an age-related alteration in the DC's capacity to imprint signals for mucosal homing receptors on T cells has been described. The results extend our previous findings highlighting the importance of considering age-related changes when developing novel vaccine and immunotherapy strategies that rely on modulation of DC-T cell crosstalk through PRR activation.

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2. Materials and Methods

2.1 Mice and animal procedures

C57BL/6 mice were purchased from IGC (Lisbon, Portugal). OT2.Rag2^{-/-} mice were bred and provided by the LGraça laboratory (GIMM, ULisboa). The mice were maintained at the FMV Animal Facility (FMV, ULisboa) under specific pathogen-free conditions, in cages with sawdust as bedding, in temperature-controlled rooms (22 to 25 °C) with a 12-h light/dark cycle, provided standard diet and water ad libitum. The mice were females aged 5 to 6 mo—OT2.Rag2^{-/-} mice and young groups—or 19 to 20 mo—old groups. Experiments compared cells from young and old mice tested simultaneously. All procedures followed Portuguese (Decreto-Lei 113/2013) and European Commission (Directive 2010/63/EU) guidelines and were approved by FMV's Ethics and Animal Welfare Committee (ORBEA, FMV, ULisboa) and the Portuguese National Agency for Animal Health (DGAV licence number 0421/000/000/2020).

2.2 DC isolation

Spleens were washed in sterile PBS, cut into pieces in 6 cm petri dishes and incubated in 10 mL of prewarmed RPMI medium (ThermoFisher, #11875093) with DNase I (Roche, #11284932001; 1 µg/mL) and collagenase IV (Gibco, #17104-019, 200 U/mL) at 37 °C for 30 min with occasional stirring. Splenocytes were then collected by grinding the spleen pieces through sterile 70 µm strainers (Corning, #431751) using a syringe plunger. Filtered splenocytes were centrifuged (300 × g, 6 min), resuspended in red cell lysis buffer (Invitrogen, #00-4333-57), 5 mL/spleen, and incubated at room temperature for 5 min. After two washes with magnetic cell sorting (MACS) buffer (1X PBS + 2% FCS + 2 mM EDTA), DCs were isolated by magnetic negative selection using the MojoSort Mouse Pan Dendritic Cell Isolation Kit (Biolegend, #480097) according to the manufacturer's instructions.

2.3 DC stimulation

TLR ligands (Invivogen: P₃CSK₄, #tlrl-pms; MPLA, #tlrl-mpls) were diluted in X-Vivo 15 medium (Lonza, #LONZBE02-060F). Negatively selected DCs were stimulated with the TLR agonists at 1 µg/mL for 24 h in serum-free X-Vivo 15 medium. Cell suspensions were collected and centrifuged (300 × g, 8 min). Supernatants were stored at -80 °C for later cytokine evaluation, and pelleted DCs were washed twice with PBS and used for the Aldefluor assay or cocultured with CD4⁺ T cells.

2.4 ALDH activity

The Aldefluor assay (Stemcell Technology, #01700) was used to assess ALDH activity in DCs stimulated with TLR ligands following the manufacturer's protocol with slight modifications. Cells (10⁵/well in 96-well plates) were washed in Aldefluor Assay Buffer, resuspended in 200 µL of the same solution containing Aldefluor Reagent and incubated for 40 min at 37 °C. After washing, cells were stained for immunophenotyping by flow cytometry and resuspended in 0.5 mL Aldefluor Assay Buffer.

2.5 CD4⁺ isolation for DC cocultures

Spleens from OT2.Rag2^{-/-} mice were washed in sterile PBS and disrupted using a syringe plunger over a moistened 70 µm strainer coupled to a 50 mL tube (Corning, #734-1812). After erythrocyte lysis (Invitrogen, #00-4333-57, 5 min at room temperature), cells were washed in MACS buffer and processed for magnetic separation

using the mouse Naïve CD4⁺ T cell Isolation kit (Miltenyi Biotec, #130-104-453), following the manufacturer's recommendations.

2.6 DC-T cell cocultures

Stimulated DCs were pulsed with MHC class II Ova₃₂₃₋₃₃₉ peptide (Invivogen, #vac-isq; 100 mM) for 2 h at 5% CO₂ and 37 °C, then washed in PBS and cocultured with naïve CD4⁺ cells from OT2.Rag2^{-/-} mice at a 1:1 ratio (0.5 × 10⁶/mL each) in X-Vivo 15 medium with freshly added retinol (Sigma-Aldrich, #R7632) to a final concentration of 50 nM. Cocultures were incubated for 5 d at 37 °C with 5% CO₂.

2.7 Flow cytometry

Single-cell suspensions from DC stimulations and DC-T cell cocultures were surface stained in PBS with 2% FBS (Gibco, Premium Plus, #A4766801): DCs for 30 min at 4 °C, and cocultures with OT2 cells for 15 min at 37 °C plus 15 min at room temperature. Staining was performed with anti-CD11c (clone N418, eBioscience), anti-MHCII (I-A/I-E) (clone M5/114.15.2, eBioscience), anti-CD19 (clone 1D3, Invitrogen), anti-Thy1.2 (clone 53-2.1, eBioscience), anti-CD8a (clone 53-6.7, Invitrogen), anti-CD4 (clone RM4-5, eBioscience), anti-CD44 (clone IM7, Biolegend), anti-α4β7 integrin (clone DATK32, Invitrogen), anti-CCR9 (clone eBioCW-1.2, Invitrogen), anti-TLR4/CD284-MD2 (clone MTS510, Biolegend), and anti-TLR2/CD282 (clone QA16A01, Biolegend). Cell viability was assessed using a LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen, #L34975) to exclude dead cells. If necessary, cells were fixed with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, #00-5523-00). Flow cytometry was performed on an Attune NxT cytometer (ThermoFisher), and data were analyzed using FlowJo v10 software (TreeStar).

2.8 Cytokine quantification

Cytokine levels in culture supernatants were assessed by ELISA using mouse Ready-Set-Go kits (eBiosciences, #88-7064-88; #88-7105-88; #88-7121-88; #88-7324-88) for IL-6, IL-10, IL-12(p70), TNF-α, or by the Legendplex multiplex assay (Biolegend) for cytokines associated with Th1 (IFNγ), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17A), and Treg (IL-10) in the same sample. Manufacturer's instructions were followed in both cases. The experiment comparing cytokines in cocultures with young vs old DCs was performed alongside a comparison between "FLT3L-expanded young DCs" vs "bona fide young DCs" reported in our publication describing the TLR2/TLR4 effect on DCs.⁷ Therefore, the multiplex assay results from bona fide young DCs in Fig. 1E are also reported there in figure 3C, bottom row—Bona fide DCs—P3C + MPLA group.

2.9 Quantification and statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0. Data are presented as mean ± standard error of the mean (s.e.m.) of three replicates. Group differences between young and old mice were assessed using unpaired two-tailed Student's t-test, with significance ranked as follows: ns, not significant; *P < 0.05, **P < 0.01, and ***P < 0.001.

3. Results

We investigated how aging affects DCs' ability to induce signals for mucosal tropism while at the same time influencing the polarization of primed CD4⁺ T lymphocytes. DCs from aged vs young animals were stimulated via TLR2/TLR4, using the TLR2 agonist Pam₃CSK₄ and the TLR4 agonist MPLA, and cocultured with

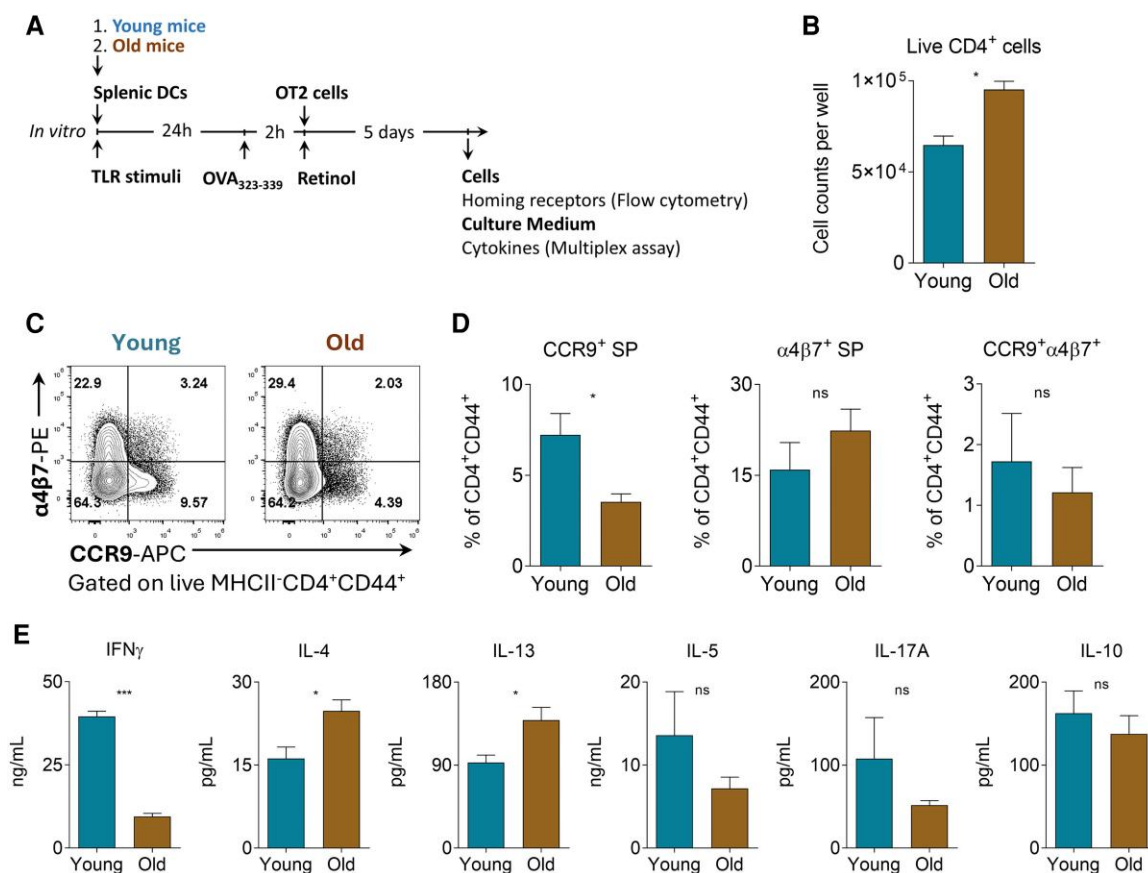


Fig. 1. CD4⁺ T cells primed by TLR2/TLR4-activated DCs from old animals express lower levels of the gut homing receptor CCR9 and produce less type 1- and more type 2-cytokines. Experimental design of the *in vitro* DC-T cell coculture system (A). DCs isolated by MACS negative selection from the spleens of young and old C57BL/6 mice were stimulated with the TLR2 agonist Pam₃CSK₄ together with the TLR4 agonist MPLA. After 24 h, the DCs were loaded with MHC class II OVA peptide and cocultured with OT2 cells in the presence of retinol. After 5 d, the culture medium and cells were collected and analyzed by flow cytometry and multiplex assay. Absolute quantification of live CD4⁺ cells at the end of coculture by flow cytometry (B). Representative contour plots (C) and quantification (D) of gut homing receptors (CCR9 and α4β7; SP—single positive) surface expression on activated OT2 cells (MHCII⁺CD4⁺CD44⁺) analyzed by flow cytometry. Quantification of the cytokines IFN_γ, IL-4, IL-13, IL-5, IL-17A, and IL-10 in the culture medium by multiplex assay (E). Note that the unit for the IFN_γ graph is ng/mL, whereas for the remaining cytokines it is pg/mL. Data are presented as mean ± s.e.m. and analyzed by Student's t-test: *P < 0.05, ***P < 0.001; ns, not significant.

naïve ovalbumin (OVA)-specific CD4⁺ (OT2) cells in the presence of retinol (Fig. 1A). By flow cytometry, we observed that T cells proliferated more (Fig. 1B) when activated in culture with aged DCs and that the induction of CCR9 was reduced in T cells primed by aged DCs stimulated via TLR2/TLR4 when compared with DCs from young animals (Fig. 1C and D).

We then quantified cytokines in the medium of the cocultures using a multiplex assay, testing Th1 (IFN_γ), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17A), and regulatory (IL-10)-related cytokines (Fig. 1E). While no differences were observed for the IL-5, IL-17A, and IL-10 cytokines, we found a striking reduction of IFN_γ and increased IL-4 and IL-13 levels in cocultures with aged DCs stimulated via TLR2/TLR4. These results indicate a skew toward Th2 or a less-polarized Th1 differentiation. To clarify whether these alterations were specific to the double stimulation via TLR2/TLR4, we assessed the same parameters in DCs activated via TLR2 alone and TLR4 alone (Fig. 2). In both cases, CCR9 expression was not reduced (Fig. 2A), whereas a reduction of IFN_γ and IL-13 production was observed for TLR2 activation and a reduction of IL-4 for TLR4 activation (Fig. 2B). Therefore, our results suggest that the defect in aged DCs in the priming of Th1 responses with a concomitant defect in the induction of mucosal tropism receptors, specifically CCR9, is characteristic of double stimulation by TLR2 plus TLR4.

To investigate if the observed differences resulted from altered TLR expression in aged DCs, we evaluated the expression of TLRs in DCs of young vs aged animals by flow cytometry. We established a panel to test TLR2 and TLR4/MD2 surface expression in mouse DCs and performed an antibody titration that showed the expected staining pattern for the anti-TLR2 antibody and a poor staining for the anti-TLR4 antibody (not shown). We then tested the TLR expression in *ex vivo* DCs after enrichment by MACS (Fig. 3A and B) and found no differences in the percentage of positive cells and fluorescence intensity of cell staining (mean fluorescence intensity [MFI]) between DCs from young and aged animals (Fig. 3B).

To functionally evaluate the activity of ALDHs in splenic DCs stimulated via TLR2/TLR4, we incubated MACS-sorted DCs from young vs old animals with Pam₃CSK₄ plus MPLA and performed the Aldefluor assay. In this assay, cells are incubated with a fluorescent nontoxic ALDH substrate, which diffuses into viable cells and when metabolized is retained inside the cell, leading to a fluorescence intensity proportional to the ALDH enzymatic activity. No differences were found in the ALDHs activity of DCs from old vs young animals under TLR2/TLR4 combined stimulation (Fig. 3C and D).

Finally, we looked at whether cytokine production by DCs in our system was altered by aging. Since the aged immune system

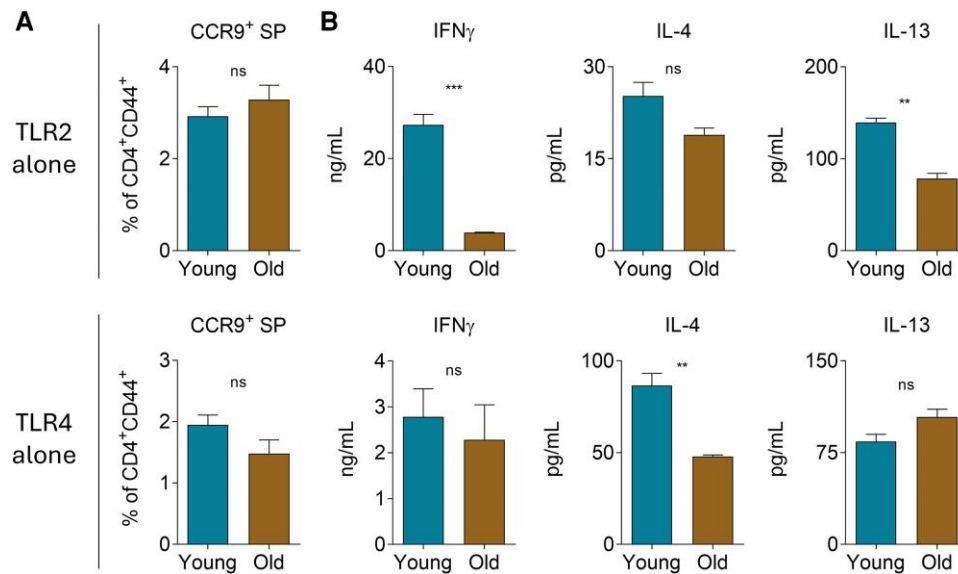


Fig. 2. The defect of aged DCs in priming Th1 responses with a concomitant defect in inducing mucosal tropism is specific to double stimulation by TLR2 plus TLR4. DCs isolated by MACS negative selection from the spleens of young and old C57BL/6 mice were stimulated with the TLR2 agonist Pam₃CSK₄ alone (top row) or with the TLR4 agonist MPLA alone (bottom row). After 24 h, the DCs were loaded with MHC class II OVA peptide and cocultured with OT2 cells in the presence of retinol. After 5 d, the culture medium and cells were collected and analyzed by flow cytometry and multiplex assay. Quantification of the surface expression of the gut homing receptor CCR9 (SP) on activated OT2 cells (MHCII⁺CD4⁺CD44⁺) analyzed by flow cytometry (A). Quantification of the cytokines IFN_γ, IL-4 and IL-13 in the culture medium by multiplex assay (B). Note that the unit for the IFN_γ graph is ng/mL, whereas for the remaining cytokines it is pg/mL. Data are presented as mean ± s.e.m. and analyzed by Student's t-test: **P < 0.01, ***P < 0.001; ns, not significant.

is often characterized by an inflammatory landscape under homeostatic conditions, we quantified the pro-inflammatory cytokines TNF α and IL-6 in supernatants of unstimulated DC cultures, but observed no differences between aged and young DCs (Fig. 3E). We then measured by ELISA a set of cytokines relevant for T cell activation and polarization upon TLR2/TLR4 stimulation (Fig. 3F). Under these activation conditions, we found a slight reduction in TNF α and a strong increase in IL-10 secretion by DCs from aged animals compared with cells from young animals (Fig. 3F).

4. Discussion

Our results show that age influences the response of DCs to dual stimulation via TLR2/TLR4, as well as the subsequent polarization and induction of tissue homing receptors on CD4⁺ T cells primed by these DCs. In our system, the effects on the primed T cells—reduction in CCR9 expression and pronounced decline in IFN_γ production—could not be attributed to diminished TLR2 expression levels on aged DCs. This finding aligns with studies quantifying TLR expression on aged conventional DCs, which show stable TLR expression with age,¹⁹ including TLR2 and TLR4 in both human monocyte-derived and conventional DCs^{20,21} and in mouse bone marrow-derived DCs.¹⁹

Regardless of surface expression levels, the functional responses of TLRs have been shown to be altered in some circumstances in aged animals, as was the case in the present study. However, the results reported in the literature regarding the cytokine response of aged DCs to PRR agonists are conflicting.^{19–23} In our study, using bona fide mouse splenic DCs, we observed no difference in IL-6 production in aged DCs stimulated via TLR2/TLR4, but TNF α levels were slightly reduced. However, the most notable alteration was an exceptional elevation in IL-10 production by aged DCs. In older individuals, lower IL-10 levels together with higher IFN_γ and granzyme B activity determined upon *ex vivo*

restimulation of peripheral blood mononuclear cells have been shown to be a good correlate of protection for an influenza vaccine,²⁴ suggesting a possible role for IL-10 in the reduced vaccine response in the elderly. In fact, IL-10 levels in human serum²⁵ and mouse lymphoid and nonlymphoid tissues²⁶ are increased in aging individuals and IL-10-producing Tfh cells have been shown to be a likely important IL-10 source in aged mice.²⁶ However, the contribution of DCs to this overall increase has not been documented and the effect of aging on the IL-10 response of antigen-presenting cells when stimulated by pathogens or pathogen-derived molecules remains unclear, with both increases^{27,28} and reductions²⁹ in IL-10 production reported. These variations may reflect differences in the animal species and antigen-presenting cell models used, and the fact that the signaling pathways downstream of the different PRRs studied, including TLR2 and TLR4, are not fully coincident. Interestingly, the functional defects in aged DCs in our system are prominent specifically upon combined TLR2/TLR4 stimulation, while responses to individual agonists show different patterns of alteration. This suggests that the age-related dysfunction may lie not merely in the capacity to respond to a single TLR, but rather in the integration, synergistic crosstalk, or sustained coordination of signals from these distinct pathways. Several potential cellular mechanisms could underpin this loss of coordination in aged DCs. For instance, alterations of the PI3K/AKT–MAPK axis, a regulator of TLR signaling,³⁰ has been reported in aged DCs.²¹ Furthermore, age-associated metabolic reprogramming and oxidative stress³¹ or epigenetic modifications, such as altered histone methylation patterns affecting promoter accessibility,³² might differentially impact the DCs' capacity to mount a coordinated response to multiligand stimuli vs singular agonists.

The observed marked decrease in IFN_γ and increase in IL-4 and IL-13 in our study is possibly related with the enhanced DC production of IL-10, as it cannot be attributed to alterations in IL-12, which

Since in our coculture system, only the DCs are from old animals, the decreased imprinting of the gut homing receptor CCR9 could be due to an aged environment created in the coculture by the DCs. This could be due to the altered cytokine environment or due to changes in antigen presentation, which are known to occur in old DCs.¹⁷ It is known that the antigen dose has an impact on the expression of homing receptors,³⁵ therefore altered TCR signals transmitted by the aged DCs could contribute to the observed effect. It will also be important in the future to investigate whether an aging environment can influence the sensitivity and signaling of T cells in response to retinoic acid *in vivo*. For example, changes in the expression levels of retinoic acid receptors, retinoid X receptors and their binding to retinoic acid responsive elements³⁶ should be evaluated.

Our results emphasize the importance of considering age-related alterations in DC functions when developing vaccine or immunotherapy strategies that rely on the modulation of DC-T cell crosstalk through PRR activation.

Author contributions

A.P.B. and A.L. conceptualized the study. S.Z., I.L.S.D., M.T.R., A.C.M., D.S., S.N., and A.P.B. performed research. S.Z., M.F., I.L.S.D., S.N., L.G., A.L., and A.P.B. contributed to designing research, analyzing data, and writing the paper. All authors read and approved the final manuscript.

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Conflicts of interest. The authors declare no conflict of interest

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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