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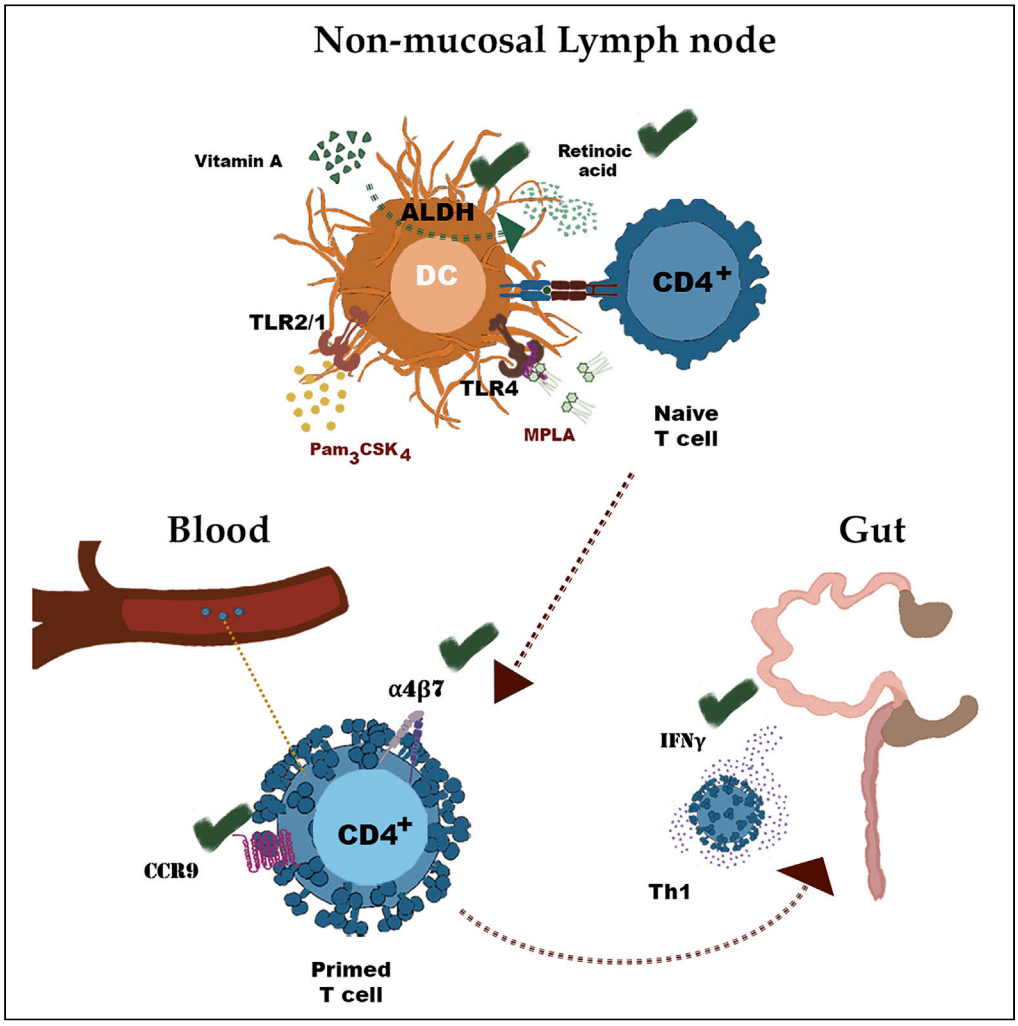

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Highlights

Combined TLR2/TLR4 activation induces ALDH activity in non-mucosal DCs

$CD4^+$ T cells primed by TLR2/TLR4 activated DCs co-express IFN-γ and α4β7/CCR9

$CD4^+$ T cells thus primed show an enhanced *in vivo* tropism to the gut

Subcutaneous immunization with TLR2/TLR4 agonists primes gut-associated Th1 cells

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Combined TLR2/TLR4 activation equip non-mucosal dendritic cells to prime Th1 cells with gut tropism

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SUMMARY

Activated CD4$^+$ T cells located at mucosal surfaces orchestrate local effector immune mechanisms. When properly polarized, these cells contribute to block infections at early stages and may be essential to restrain the local growth of mucosal tumors, playing a critical role in host protection. How CD4$^+$ T cells simultaneously integrate gut-homing instructions and Th polarization signals transmitted by TLR activated dendritic cells (DCs) is unknown. Here, we show that the combined activation through TLR2, which alone does not induce a clear Th polarization, and TLR4, which alone does not imprint mucosal tropism, equip non-mucosal DCs to prime gut-homing CD4$^+$ T cells with reinforced Th1 polarization. These results show that targeting DCs with combined innate stimuli with distinct properties is a rational strategy to program the outcome of T cell polarization and simultaneously control their tissue tropism. Exploring this strategy could enhance the efficacy of vaccines and immune cell therapies.

INTRODUCTION

Upon infection, memory CD4$^+$ T cells present in mucosal tissues are reactivated locally and play a determinant role in protection by triggering and orchestrating local immune activation.[1,2] Although the administration of antigens directly at mucosal surfaces may enhance mucosal immunity, the mucosae are often hyporesponsive to inactivated and subunit vaccine formulations due to the tight regulation of immune responses at mucosal compartments.[3,4]

Since dendritic cells (DCs) play a major role both in determining the fate of T cell differentiation and in instructing the localization of lymphocytes in different tissues, targeting DCs becomes a promising strategy to promote the traffic of relevant effector and memory mechanisms to specific organs.[5–7] The observation that non-mucosal DCs, which are inefficient at inducing T cell tropism into mucosal surfaces, can be induced to promote the homing of primed T cells to the gut mucosa when activated specifically through toll-like receptor (TLR)2 is of particular interest in the vaccinology context.[8] This capacity was especially demonstrated for CD8$^+$ T cells and relies on the TLR2 induction of aldehyde dehydrogenase (ALDH) enzymes on activated DCs that become able to metabolize vitamin A into all-trans retinoic acid, which imprints the expression of mucosal-homing molecules ($\alpha4\beta7$ and CCR9) in the primed T cells.[8,9]

A possible strategy to avail the TLR2-specific property of inducing gut homing lymphocytes in vaccines or immunotherapy protocols would be the concomitant activation with other pattern recognition receptors (PRRs) that could help to define and reinforce specific types of CD4$^+$ T cell differentiation. In theory, this strategy could be used to induce different Th cell types since, although a relation exists between the expression of some chemokine receptors and T cell polarization,[10,11] the expression of gut-homing receptors in CD4$^+$ T cells is not exclusive of one type of polarization.[12–14] Still, it is important to understand if the TLR2-specific property of equipping DCs with the potential to induce gut-tropic T cells is maintained or abrogated in the presence of other PRR stimuli. Clarifying this issue is of major relevance since retinoic acid, the main DC-derived signal imprinting mucosal tropism, has been shown to influence T cell differentiation both in regulatory and inflammatory fates depending on the stage of an immune response and on other concomitant signals acting on the T cells, such as DC-secreted

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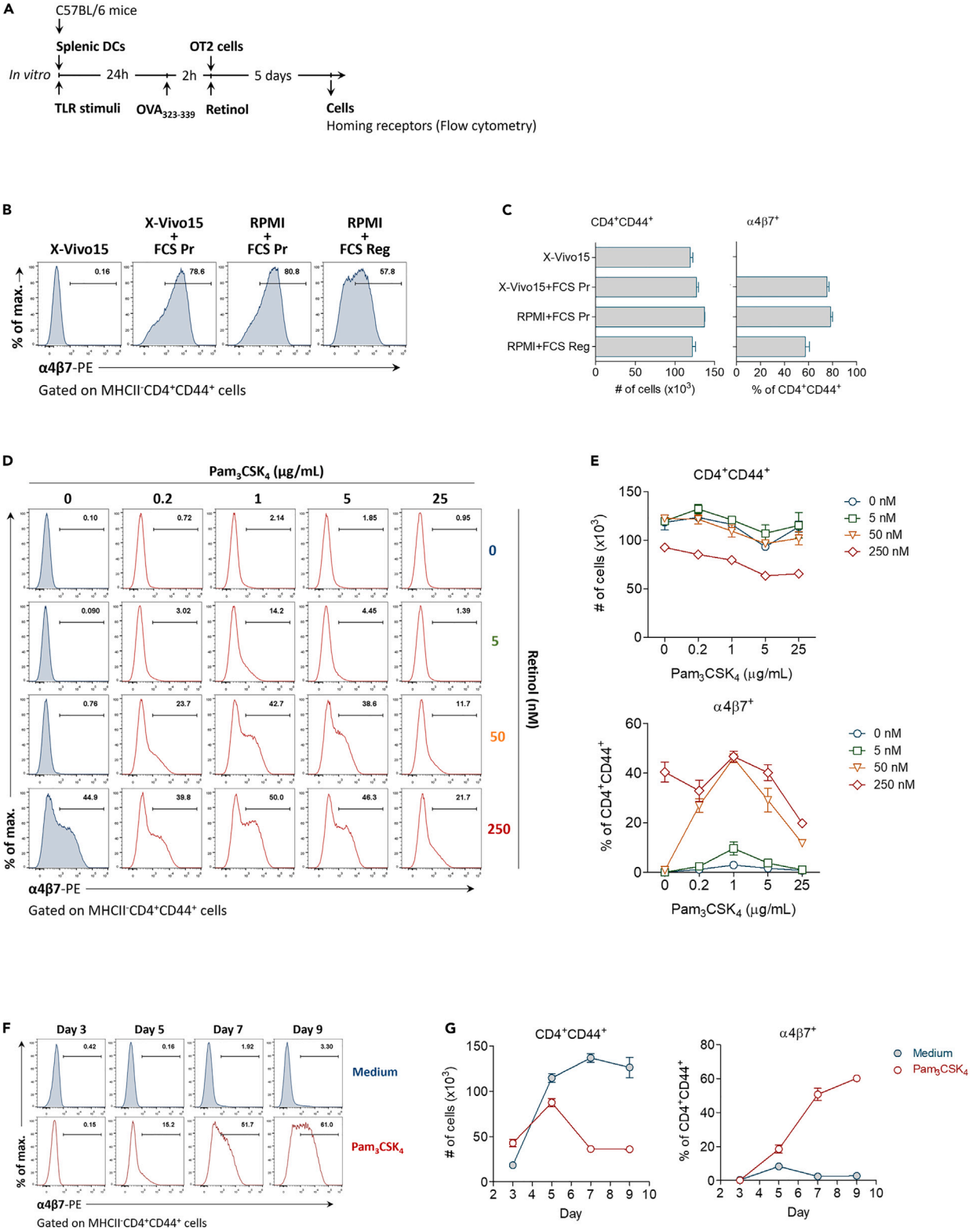

Figure 1. Optimization of experimental settings with the canonical TLR2/1 agonist Pam_3CSK_4

Experimental design of the *in vitro* DC-T cell coculture system (A). Dendritic cells isolated by MACS negative selection from spleens of C57BL/6 mice are cultured unstimulated or stimulated with TLR ligands. After 24 h, the DCs are loaded with MHC class II OVA peptide and are cocultured with OT-II cells in the presence of

Figure 1. *Continued*
retinol. Experimental settings were optimized testing different culture medium conditions (X-vivo 15: serum free medium; RPMI: RPMI medium) and serum supplementation (FCS Prm: Fetal calf serum (FCS) premium, with guaranteed low endotoxin levels; FCR reg: regular FCS) (B and C), the concentration of Pam_3CSK_4 (0.2, 1.0, 5.0, and 25.0 μg/mL) and of retinol (0, 5, 50, and 250 nM) (D and E), and the duration of coculture (3, 5, 7, and 9 days) (F-G). In B-E, the cells were collected after 5 days for analysis. Absolute quantification of activated ($CD4^+CD44^+$) $CD4^+$ T cells (C and E) and representative histograms (B, D, and F) and quantification (C, E, and G) of gut-homing receptor (α4β7) surface expression on the activated OT-II cells ($MHCII^-CD4^+CD44^+$ cells). Data shown as the mean ± s.e.m.

cytokines.[15–17] Additionally, while the activation of DCs with some pathogen-associated molecular patterns (PAMPs) clearly favors a specific type of $CD4^+$ T cell polarization, TLR2 activation is ambiguous in this respect.[18] Depending on the ligand and on the context of activation, TLR2 activation may induce immune responses with either pro- or anti-inflammatory characteristics[19,20] and has been shown to be implicated in the development of Th1,[21] Th2,[22] Th9,[23] Th17[24] and in regulatory differentiation.[25]

To investigate how the TLR2 intrinsic properties of inducing T cell mucosal tropism operate in the presence of other innate immunity stimuli, we chose the TLR4 pathway. TLR4 is canonically activated by LPS and is the only TLR that activates both the MyD88-and TRIF-dependent pathways.[26] Like other PAMPs, LPS has been shown to have a reduced capacity to stimulate ALDH activity in extra-mucosal dendritic cells compared to the TLR2/1 agonist Pam_3CSK_4 (P3C).[8] For this study, we selected the TLR4 agonist monophosphoryl lipid A (MPLA), which is a form of LPS initially extracted from *Salmonella minnesota* with a low toxicity associated with a bias toward TRIF signaling.[27] Synthetic forms of MPLA have now been developed, and the molecule has already been approved for use in clinical vaccines. It is, for example, incorporated in vaccines against the hepatitis B virus, human papillomavirus, or against varicella zoster, in which it has been shown to have a positive effect on the magnitude, profile, and durability of the induced immune response.[28] MPLA is, therefore, a good candidate for the strategy of co-activation with TLR2.

In this study, we investigated whether the TLR2 property to induce mucosal-homing molecules in $CD4^+$ T cells is maintained when associated with TLR4 agonists. We further evaluated how the simultaneous activation of non-mucosal DCs through TLR2 and TLR4 impacts on $CD4^+$ T cell polarization. Our results show that the combined TLR2/TLR4 DC activation promotes the gut localization of Th1 cells and that the TLR2-specific properties could be explored to increase the efficacy of non-mucosal vaccines and to improve DC vaccine strategies or T cell immunotherapies.

RESULTS

Characterizing and optimizing the induction of gut-homing receptors on $CD4^+$ T cells by dendritic cell activation with the TLR2/1 agonist Pam_3CSK_4

Different agonists triggering TLR2 pathways have been shown to promote the ability to metabolize retinol to retinoic acid, both on mucosal and non-mucosal DCs.[8,9] Here, we used the TLR2 agonist Pam_3CSK_4 (P3C), a small lipopeptide that, contrary to other TLR2 ligands, exclusively activates the canonical TLR2 signaling pathway through the dimerization of TLR2 with TLR1.[29]

To address the impact of combining TLR2 activation with PAMPs that do not share the property of inducing ALDH activity on DCs, and further understand how it correlates with T cell polarization, we set up an *in vitro* system where we coculture stimulated DCs pulsed with the MHC class II immunodominant OVA peptide with naive OVA-specific $CD4^+$ (OT-II) cells in the presence of retinol (Figure 1A). Different factors, such as the type of culture medium, the stimuli concentration, and the duration of DC-T cell coculture, have been previously found to affect other *in vitro* systems addressing different aspects of retinoic acid metabolism.[30,31] Therefore, to minimize confounding factors, we addressed the impact of such parameters in our DC-T cell coculture system with P3C-stimulated DCs before proceeding to the TLR2/TLR4 co-stimulations (Figure 1). First, we evaluated whether retinol, residual PRR agonists, or other unknown factors present in the serum used for medium supplementation impacted the expression of homing receptors in $CD4^+$ T cells under our experimental conditions. For that, we cocultured unstimulated non-mucosal DCs with antigen-specific $CD4^+$ T cells in serum-free medium (XVivo15) with or without low-endotoxin serum (FCS Pr) and in RPMI medium supplemented with FCS Pr or regular FCS (FCS Reg) (Figures 1B and 1C). The addition of serum to the culture medium resulted in spontaneous expression of the mucosal homing integrin α4β7 in cocultured $CD4^+$ T cells, in agreement with previous studies.[30–32] This effect was not prevented by using highly pure FCS (Figures 1B and 1C), indicating that it was not due to the presence of residual PAMPs in the supplementation serum. Then, we performed the same co-culture system using a serum-free medium to determine the concentration range of the P3C ligand that is effective in activating non-mucosal DCs to imprint the expression of α4β7 on cocultured $CD4^+$ T cells, in the presence of different concentrations of retinol. The highest α4β7 expression resulted from combining concentrations between 0.2 and 5 μg/mL of P3C with 50 nM of retinol and a marked decrease of viable activated ($CD44^+$) $CD4^+$ T cells numbers was observed when using 250 nM of retinol (Figures 1D and 1E). Using the optimized conditions, we further determined the best time-point to evaluate the homing receptor's expression. Although the expression of α4β7 steadily increased with time until day 9 of coculture, the number of activated $CD4^+$ T cells peaked at day 5 and decreased afterward (Figures 1F and 1G). We therefore fixed day 5 as the time of coculture for the following experiments.

Combined TLR2/TLR4 activation maintains the TLR2 capacity to induce aldehyde dehydrogenase activity in non-mucosal dendritic cells while synergizes in inducing dendritic cell cytokines secretion and expression of maturation markers

Having set the optimal conditions for P3C stimulation, we compared the effect of P3C concentration range side-by-side with two TLR4 agonists, LPS (ultrapure, i.e., devoid of outer membrane lipoproteins - TLR2 ligands) and MPLA, evaluating the induction of ALDH activity in non-mucosal DCs by the Aldefluor assay, a flow cytometry-based assay in which a fluorescent non-toxic substrate is converted by ALDH enzymes into a negatively charged product that accumulates in the cell cytoplasm. This experiment showed the differential ability of the TLR2 versus

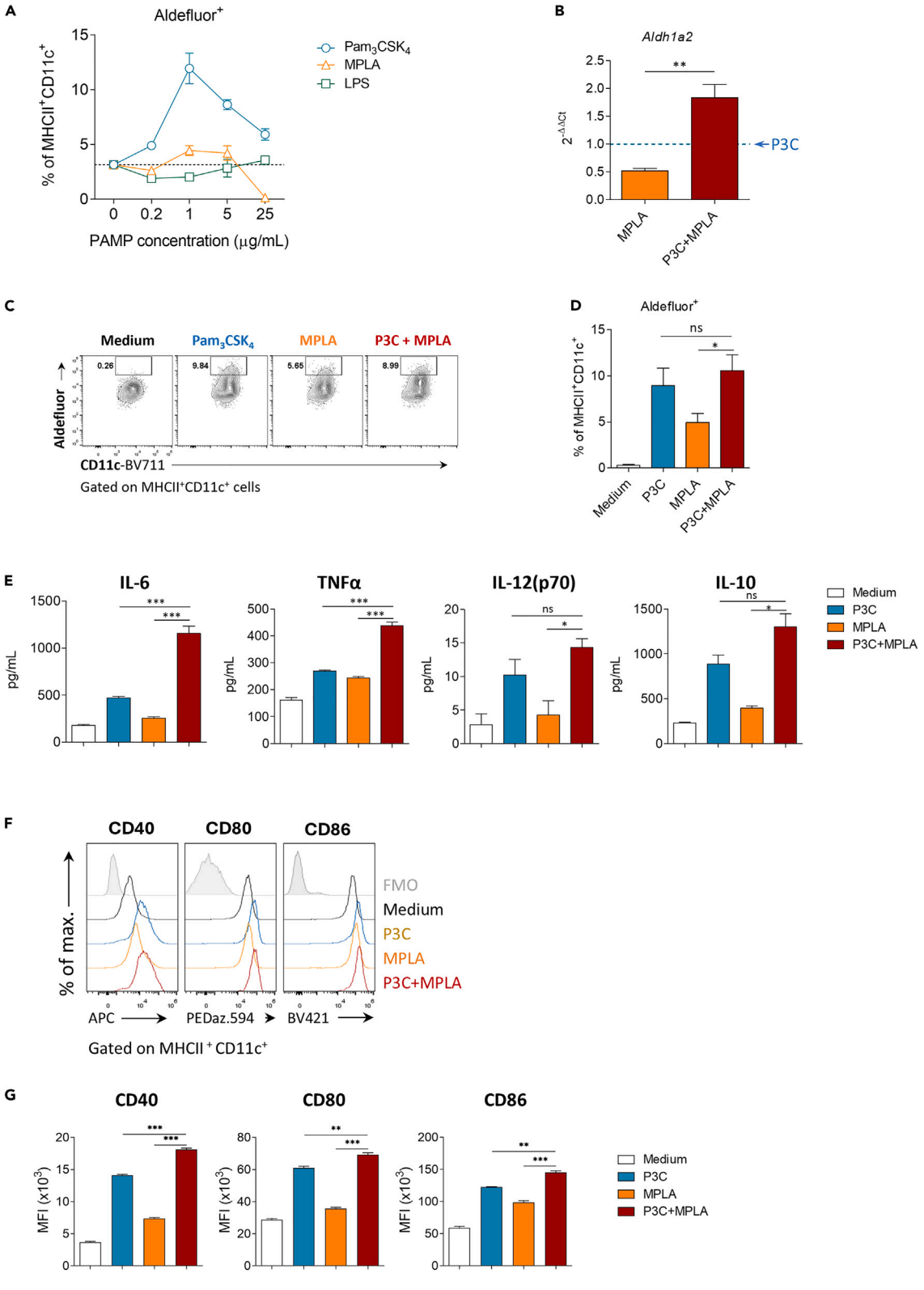
A
Aldefluor+
% of MHCII+CD11c+
PAMP concentration (μg/mL)
Pam3CSK4
MPLA
LPS
B
Aldh1a2
2-ΔΔCt
P3C
MPLA
P3C+MPLA
C
Medium
Pam3CSK4
MPLA
P3C + MPLA
Aldefluor
CD11c-BV711
Gated on MHCII+CD11c+ cells
D
Aldefluor+
% of MHCII+CD11c+
ns
Medium
P3C
MPLA
P3C+MPLA
E
IL-6
TNFα
IL-12(p70)
IL-10
pg/mL
Medium
P3C
MPLA
P3C+MPLA
F
CD40
CD80
CD86
% of max.
FMO
Medium
P3C
MPLA
P3C+MPLA
APC
PEDaz.594
BV421
Gated on MHCII+CD11c+
G
CD40
CD80
CD86
MFI (x10³)
Medium
P3C
MPLA
P3C+MPLA

Figure 2. Combined TLR2/TLR4 activation maintains the TLR2 capacity to induce ALDH activity in non-mucosal DCs while synergize in inducing DC cytokines secretion

Dendritic cells expanded *in vivo* by B16-Flt3l tumor inoculation were isolated by MACS negative selection from spleens of C57BL/6 mice, were cultured unstimulated (dashed line) or stimulated with the TLR2 agonist Pam_3CSK_4 or the TLR4 agonists MPLA or LPS in the indicated concentrations. After 24 h, ALDH activity was analyzed by flow cytometry using the Aldefluor assay (A).

Dendritic cells obtained in a similar manner were cultured unstimulated (Medium) or stimulated with the TLR2 agonist Pam_3CSK_4 (P3C), the TLR4 agonist MPLA or both (P3C + MPLA). After 24 h, the culture medium and the cells were collected for analysis.

(B) Evaluation of *Aldh1a2* gene expression by quantitative real-time PCR. The $2^{-\Delta\Delta CT}$ values were determined in reference to the *Actin b* housekeeping gene of the same sample and then normalized to the average $2^{-\Delta CT}$ values obtained for P3C-stimulated cells (blue dashed line). Representative contour plots (C) and quantification (D) of the Aldefluor reaction in cultured dendritic cells (gated on $MHCII^+CD11c^+$ cells) analyzed by flow cytometry. Quantification of cytokines in the culture medium by ELISA assay (E).

Representative histograms including fluorescence minus one (FMO) controls (F) and quantification (G) of surface expression, expressed as mean fluorescence intensity (MFI), of co-stimulatory and activation molecules (CD40, CD80, and CD86) on stimulated DCs. Data shown as the mean ± s.e.m. Significant differences between each single stimulation group and the double stimulation group were determined by unpaired two-tailed Student's t test and were ranked: ns – not significant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

the TLR4 agonists to induce ALDH activity (Figure 2A) and revealed that DC viability was maintained with concentrations up to 5 μg/mL of all the ligands tested (data not shown).

The induction of ALDH activity in non-mucosal DCs by TLR2 signals has been previously demonstrated to rely on the induction of *Aldh1a2* expression.[8,9,33] To address the effect of the combined stimulation with TLR2 and TLR4 agonists, we evaluated how the mRNA expression upon MPLA alone or combined with P3C compares with that induced by P3C stimulation on non-mucosal DCs. After 24h in culture in the presence of the stimuli, the level of *Aldh1a2* in the MPLA-stimulated cultures was half the level observed in P3C-stimulated cells (dashed line in Figure 2B), but it was around 1.5 times higher when both ligands were combined (Figure 2B).

To clarify whether the mRNA expression has correspondence to the enzyme activity metabolizing retinol, we performed the Aldefluor assay and confirmed that the capacity to induce ALDH activity by TLR2 activation is preserved when combined with TLR4 activation (Figures 2C and 2D). This result suggests that non-mucosal DCs simultaneously activated through TLR2 and TLR4 have the potential to influence the gut-homing phenotype of T cells upon priming.

Given the importance of cytokines in determining the differentiation of $CD4^+$ T cells, we wondered how this combined stimulation would affect the cytokine profile secreted by the stimulated DCs. For that, we stimulated *in vitro* splenic DCs with each PAMP alone and with both combined, and evaluated the cytokine secretion by ELISA. In these conditions, the combined stimulation led to a general increase in the secretion of IL-6, TNFα, IL12(p70) and IL10, cytokines relevant for T cell activation and differentiation (Figure 2E).

The maturation status of DCs may also influence both the signals that DCs send and receive during T cell priming. To further investigate the state of DCs upon PAMP stimulation, we assessed the expression of co-stimulatory and activation molecules in the same conditions used to assess ALDH activity and cytokine production. The results show that the surface expression of CD80, CD86, and CD40 is increased by single stimulation via TLR2 and TLR4 and further enhanced by simultaneous stimulation via TLR2/TLR4 (Figures 2F and 2G). This pattern parallels the increased ALDH activity and cytokine production by stimulated DCs (Figures 2C–2E).

Together, the results show that the combined TLR2/TLR4 activation preserves the ability of TLR2 to induce ALDH activity in non-mucosal DCs, while increasing DC cytokine secretion and upregulating the surface expression of co-stimulatory and activating molecules.

Non-mucosal dendritic cells activated through TLR2/TLR4 prime $CD4^+$ T cells to express gut-homing receptors and lead to increased levels of IFN-γ in DC-$CD4^+$ T cell cocultures

Next, to evaluate whether the enhanced ALDH activity induced on non-mucosal DCs by the TLR2/TLR4 combined stimulation was translated into the expression of gut-homing receptors in $CD4^+$ T cells upon priming, we cocultured activated DCs (from B6.Thy1.1 mice) with OT-II cells as depicted in Figure 1A. After 5 days of coculture, higher numbers of activated OT-II cells ($Thy1.2^+CD4^+CD44^+$) primed by P3C alone or by P3C + MPLA expressed the two gut-homing receptors α4β7 and CCR9, including a population expressing both molecules (Figures 3A and 3B). To get insights on the type of polarization induced by the different DC stimulation conditions, we tested the culture supernatants by a multiplex assay for cytokines associated with Th1 (IFN-γ), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17A), and Treg (IL-10) differentiation (Figure 3C upper row). The level of the Th1 cytokine IFN-γ was highly above the other cytokines (ng/mL levels) and was enhanced by the double stimulation. Although several studies have shown that *in vivo* Flt3l-expanded DCs maintain most of the features of bona fide DCs from different compartments,[8,34,35] differences in cytokine secretion and receptor expression have been reported for DCs differentiated under different conditions[36] and thus we also performed the stimulation experiment with DCs directly isolated from non-treated mice. As with DCs expanded *in vivo* by Flt3l-secreting tumor, the highest level of IFN-γ was observed when bona fide splenic DCs were stimulated simultaneously via TLR2 and TLR4 (Figure 3C lower row). Moreover, a decrease in the secretion of the Th2 cytokine IL-4 was significant for the double stimulation comparing with each single stimulation (Figure 3C lower row).

$CD4^+$ T cells primed by TLR2/TLR4 activated dendritic cells co-express IFN-γ and gut-homing receptors

The expression of homing receptors and the cytokines secreted in cocultures suggest that the combined TLR2/TLR4 stimulation promotes a Th1 differentiation of naive $CD4^+$ T cells while providing conditions for the expression of gut-homing receptors on primed T cells. However,

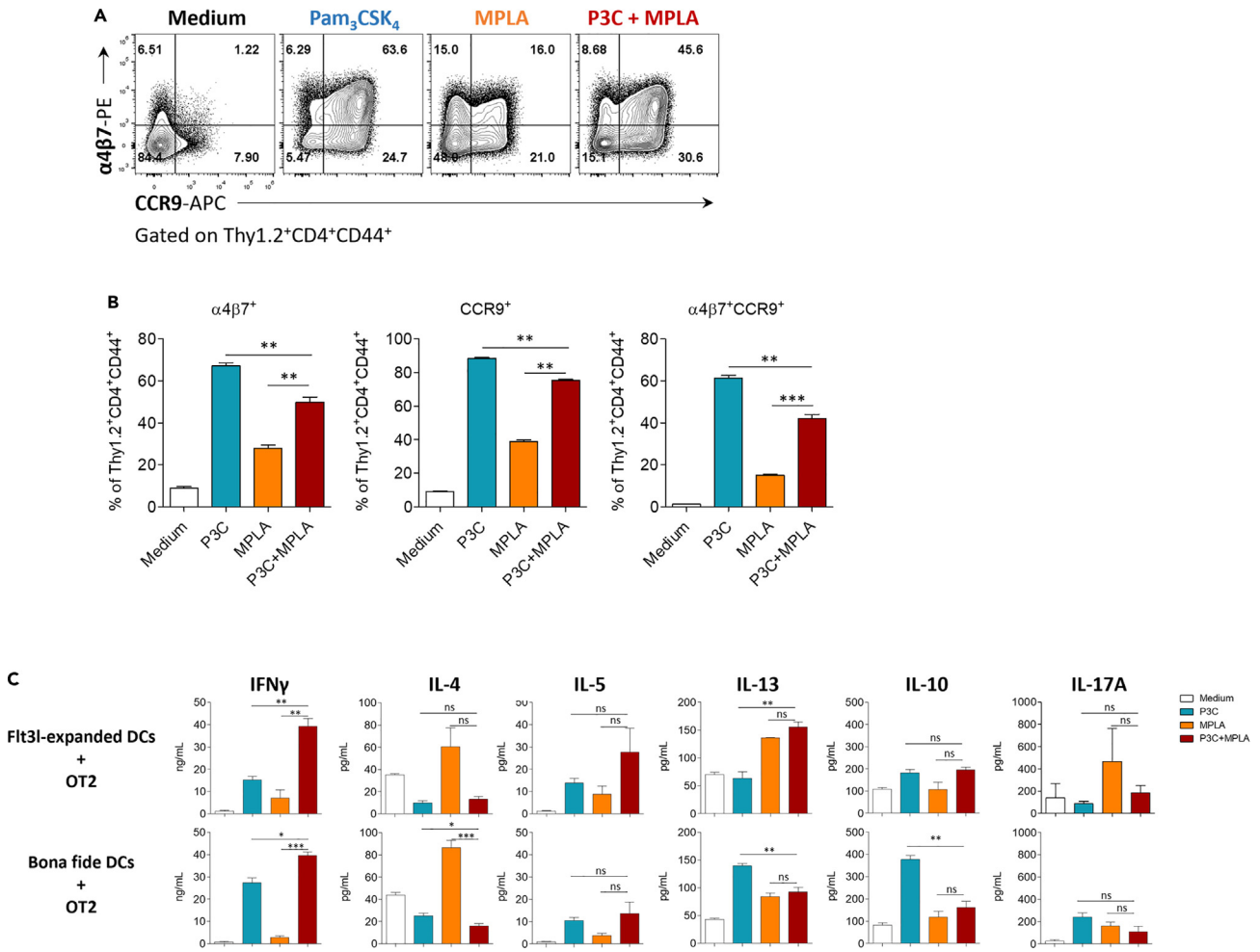

Figure 3. Non-mucosal DCs activated through TLR2/TLR4 prime CD4$^+$ T cells to express gut-homing receptors and lead to increased levels of IFN-γ in DC-CD4$^+$ T cell cocultures

Dendritic cells isolated by MACS negative selection from spleens of C57BL/6 mice, either expanded or unexpanded *in vivo* (Flt3l-expanded or bona fide, in panel C), were cultured unstimulated (medium) or stimulated with the TLR2 agonist Pam_3CSK_4 (P3C), the TLR4 agonist MPLA or both (P3C + MPLA). After 24 h, the DCs were loaded with MHC class II OVA peptide and were cocultured with OT-II cells in the presence of retinol. After 5 days, the culture medium and the cells were collected for analysis. Representative contour plots (A) and quantification (B) of the surface expression of gut-homing receptors on the activated OT-II cells (Thy1.2$^+$CD4$^+$CD44$^+$ cells) analyzed by flow cytometry.

(C) Quantification of cytokines in the culture medium by multiplex assay; note that the unit for the IFN-γ graph is ng/mL while for the remaining cytokines it is pg/mL. Data shown as the mean ± s.e.m. Significant differences between each single stimulation group and the double stimulation group were determined by unpaired two-tailed Student's t test and were ranked: ns – not significant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

the evaluation of IFN-γ secreted to the culture medium does not demonstrate that the same cells differentiating into Th1 type are simultaneously expressing gut homing receptors. To clarify this, we performed intracellular staining for IFN-γ upon phorbol myristate acetate (PMA)/ionomycin restimulation of DC-T cell cocultures together with the surface staining of homing molecules (Figure 4). The intracellular staining confirmed that the number of CD4$^+$ T cells producing IFN-γ was increased when adding the TLR2 stimulus to the TLR4 stimulus (Figures 4A and 4B) and that more than 80% of the IFN-γ–producing cells induced by the double stimulation coexpressed at least one of the gut-homing receptors, α4β7 or CCR9 (Figures 4C and 4D).

Adding a TLR2 stimulus to a TLR4 activation favors the priming of IFN-γ-producing CD4$^+$ T cells with enhanced *in vivo* tropism to the gut

To demonstrate that the enhanced *in vitro* expression of gut homing receptors observed for a combined TLR2/TLR4 DC activation is translated into a functional tropism of primed CD4$^+$ T cells to the gut *in vivo*, we performed a homing index assay. For that, we primed CD4$^+$ T cells *in vitro* with non-mucosal DCs stimulated with a TLR4 agonist alone or combined with a TLR2 stimulus and, after a differential staining with

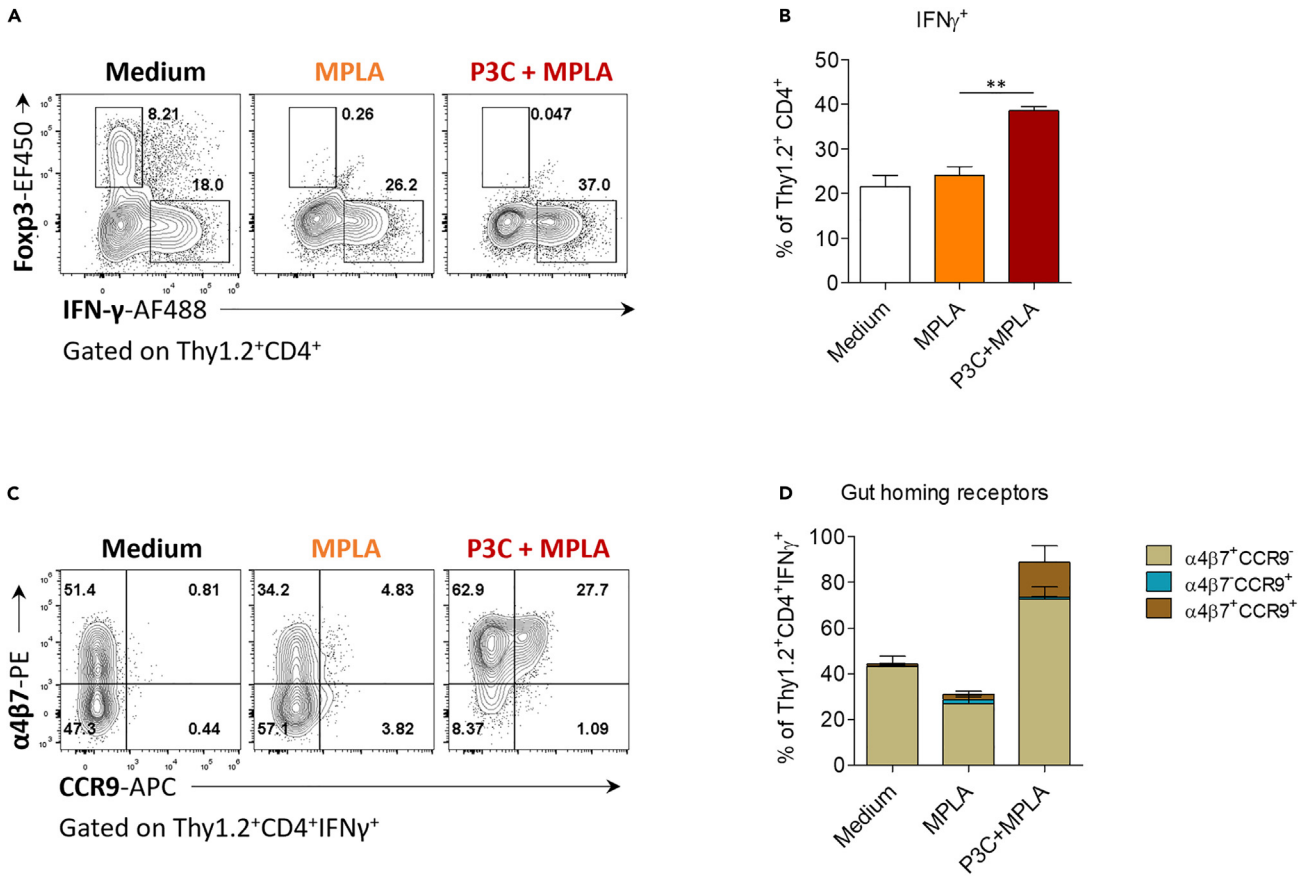

Figure 4. CD4$^+$ T cells primed by TLR2/TLR4 activated DCs co-express IFN-γ and gut-homing receptors

Dendritic cells isolated by MACS negative selection from spleens of C57BL/6 mice were cultured unstimulated (medium) or stimulated with the TLR4 agonist MPLA alone (MPLA) or together with the TLR2 agonist Pam_3CSK_4 (P3C + MPLA). After 24 h, the DCs were loaded with MHC class II OVA peptide and were cocultured with OT-II cells in the presence of retinol. After 5 days, the cells were restimulated with PMA/Ionomycin for 4 h and analyzed by flow cytometry intracellular staining. Representative contour plots and quantification of Foxp3 and intracellular IFN-γ (A and B) and of gut-homing receptors in IFN-γ-producing OT-II cells (C and D) analyzed by flow cytometry. Data shown as the mean ± s.e.m. Significant differences between the single stimulation group and the double stimulation group were determined by unpaired two-tailed Student's t test and were ranked: ns – not significant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

CFSE (carboxyfluorescein succinimidyl ester) and CTV (CellTrace Violet) dyes, transferred the primed cells intravenously, in a proportion 1:1, to recipient mice (Figure 5A). After 20h, we collected blood, skin draining lymph nodes (inguinal lymph nodes), and gut-associated secondary lymphoid organs (Peyer's patches and mesenteric lymph nodes) and determined the relative loads of stained cells in each organ. The cocultures with TLR2/TLR4 stimulated DCs resulted in OT-II cells with higher expression of α4β7 integrin and the culture supernatants contained higher levels of the Th1-associated cytokine IFN-γ (not shown). While CD4$^+$ T cells primed by DCs activated via TLR4 alone prevailed in the skin draining lymph nodes, the addition of the TLR2 stimulus favored the migration to gut associated secondary lymphoid organs (Figures 5B and 5C).

Finally, we evaluated whether the TLR2 properties denoted upon the *in vitro* stimulation of non-mucosal dendritic cells are also observed *in vivo* in an immunization context. For this, we transferred OT-II cells to Ly5.1 (CD45.1) mice and immunized the recipient animals with ovalbumin plus MPLA or with the same formulation added with P3C. To highlight the differential circulation of specific lymphocytes through different compartments of the body, between days 3 and 6 after immunization, the animals were treated daily with FTY720, a drug that inhibits the egress of lymphocytes from lymph nodes (Figure 6A). Seven days after the immunization, the animals immunized with the TLR4 agonist alone showed a slightly higher proportion of OT-II cells in the inguinal lymph nodes, both in those draining the injection site and in the contralateral ones, while the levels of OT-II cells in the mesenteric lymph nodes were similar (Figures 6B and 6C). However, upon *ex vivo* restimulation with the vaccine antigen, the production of IFN-γ in the superficial lymph nodes was higher in animals that received the TLR2 agonist and only these animals showed IFN-γ production in the mesenteric lymph nodes. These results suggest that the addition of a TLR2 agonist into vaccine adjuvants could be explored to establish specific immunity in the intestinal mucosa, for instance in prime and pull immunization protocols.

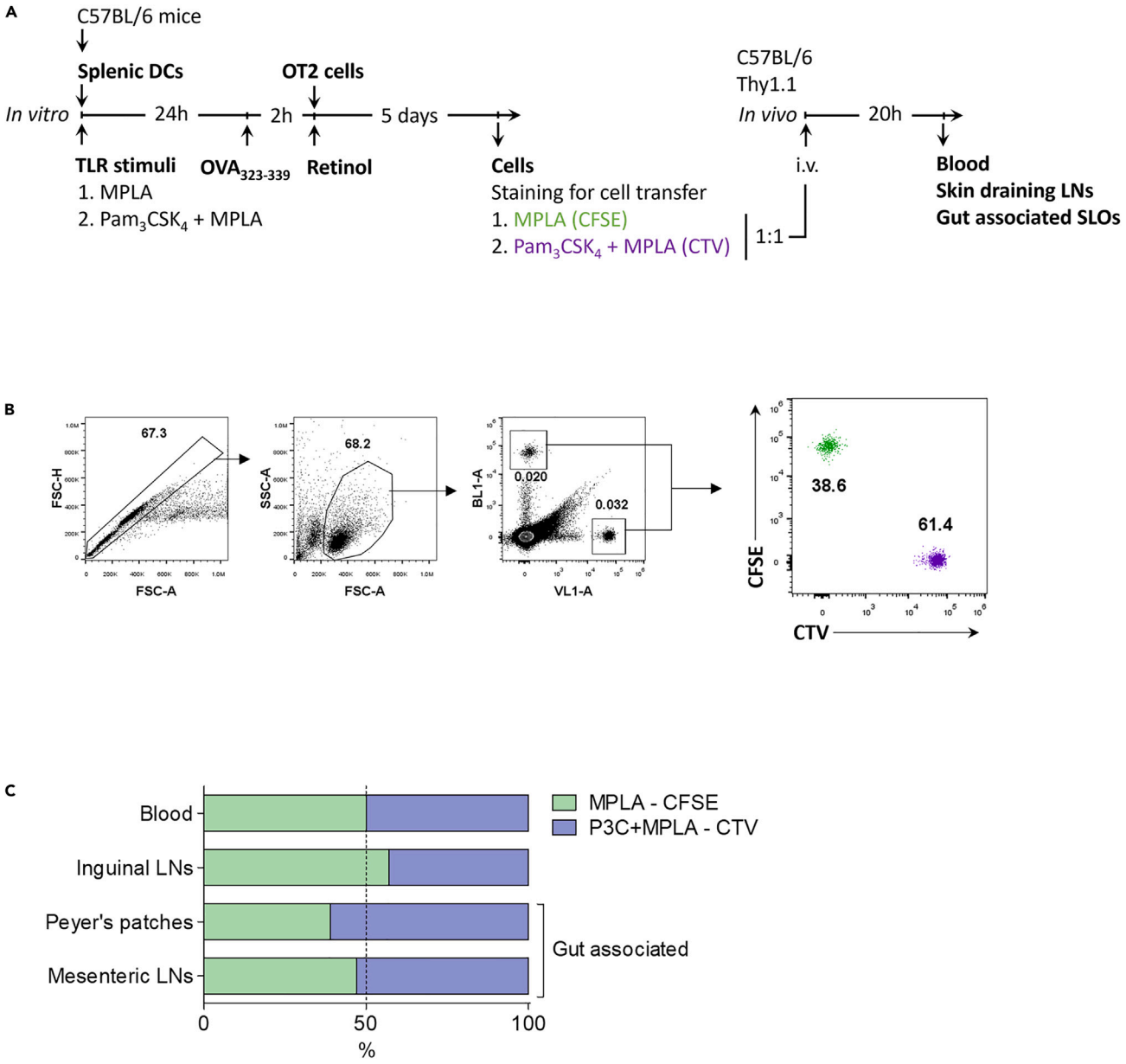

Figure 5. Adding a TLR2 stimulus to TLR4-activated non-mucosal DCs favors the priming of CD4$^+$ T cells with enhanced *in vivo* tropism to the gut

Dendritic cells isolated by MACS negative selection from spleens of C57BL/6 mice were cultured stimulated with the TLR4 agonist MPLA alone or with MPLA and the TLR2 agonist Pam_3CSK_4 (P3C + MPLA). After 24 h, the DCs were loaded with MHC class II OVA peptide and were cocultured with OT-II cells in the presence of retinol. After 5 days, the cells were collected, stained with CFSE (MPLA-stimulated) or CTV (P3C+MPLA-stimulated), and inoculated intravenously in recipient mice at a 1:1 ratio. After 20h, blood, skin draining lymph nodes (inguinal lymph nodes), and gut-associated secondary lymphoid organs (Peyer's patches and mesenteric lymph nodes) were collected (A). The organs were processed for flow cytometry, the stained cells were identified (B), and the relative loads in each organ were determined (C) and normalized to the percentage of stained cells found in the blood.

DISCUSSION

Contrary to DCs from extra-intestinal sites, gut-associated DCs induce T and B cells with tropism to the gut, whereas skin DCs selectively induce T cells that preferentially traffic to the skin.[37,38] It has been shown that vitamin A in the gut and vitamin D3 in the skin, specifically metabolized by resident DCs, are involved in these processes.[6,39] Even if the organ of origin is a primary factor determining the homing instructions transmitted by a DC to T cells,[40] the DC homing-imprinting properties can be modulated by activating DCs through specific pathways.[8,41–43] The mucosal tropism of T cells primed by TLR2-activated DCs has been well demonstrated for CD8$^+$ T cells,[8] which tend to express higher levels of gut-homing receptors when compared to CD4$^+$ T cells.[44,45] However, how the DC activation via TLR2 simultaneously impacts the CD4$^+$ T cell mucosal tropism

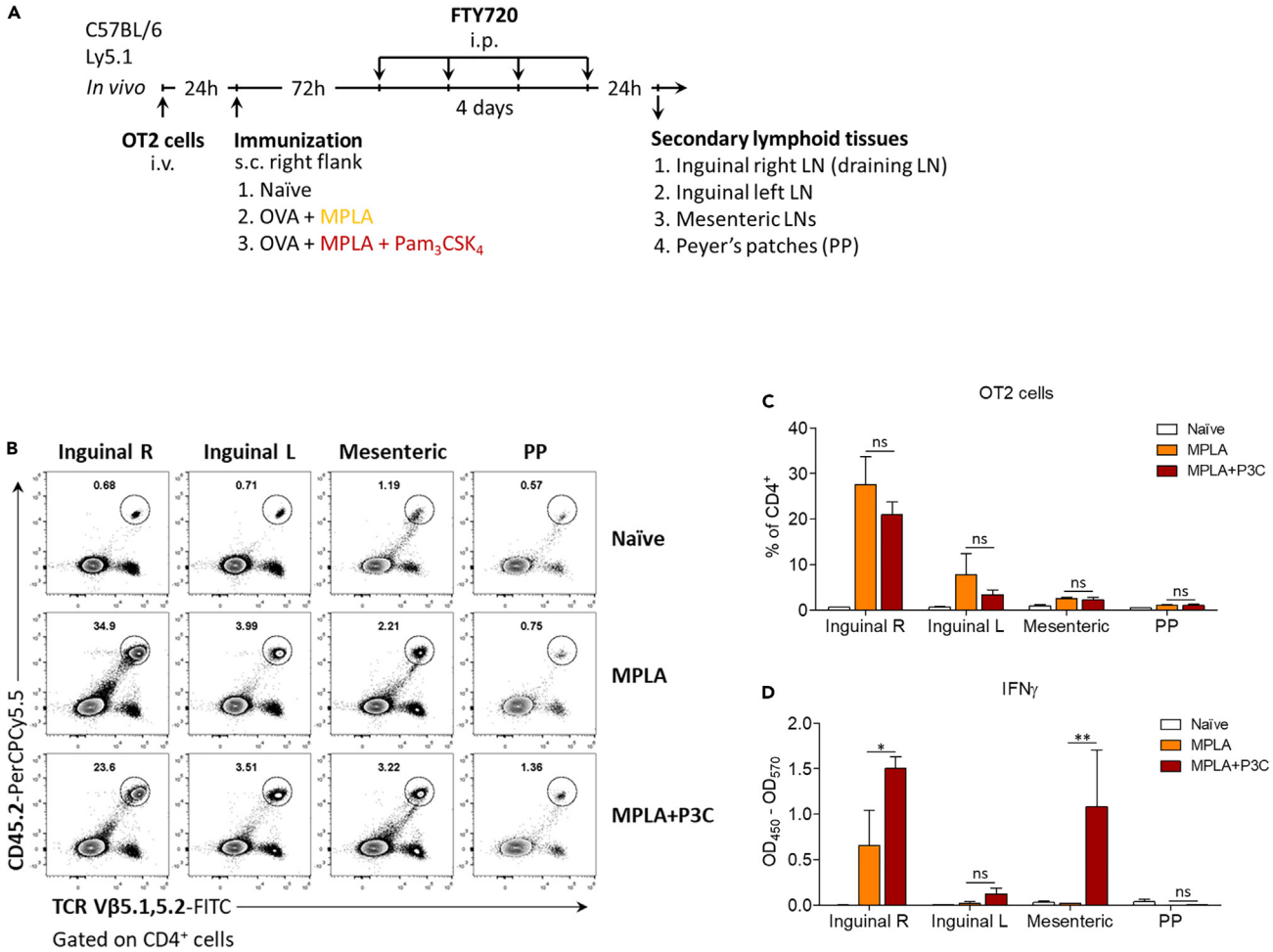

Figure 6. Adding a TLR2 stimulus to a TLR4-targeting immunization potentiates the production of IFN-γ both in superficial and gut-draining lymph nodes

OT-II cells (CD45.2) were transferred to C57BL6.Ly5.1 (CD45.1) mice and the day after the recipient mice were immunized subcutaneously (s.c.) in the right flank with ovalbumin (OVA) with the TLR4 agonist MPLA alone or with MPLA and the TLR2 agonist Pam_3CSK_4 (MPLA+P3C). After 72 h, the mice were treated daily for 4 days with FTY720, a drug that prevents the egress of lymphocytes from the lymph nodes. Seven days after the immunization, skin draining lymph nodes (inguinal lymph nodes) and gut-associated secondary lymphoid organs (Peyer's patches and mesenteric lymph nodes) were collected (A). The organs were processed for flow cytometry to identify the OVA-specific OT-II cells (B), and the relative proportions present in each organ were determined (C). The same cells were restimulated *ex vivo* with the immunizing antigen (OVA at 50 μg/mL) and the secreted IFN-γ was evaluated by ELISA (D). Data shown as the mean ± s.e.m. Significant differences between single adjuvanted group and the double adjuvanted group were determined by two-way ANOVA with Bonferroni post-tests and were ranked: ns – not significant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

and their polarization has not been investigated. Here, we show that the combined TLR2/TLR4 activation of non-mucosal DCs induces gut-homing receptors in $CD4^+$ T cells, reinforcing at the same time the polarization toward a Th1 response. We show that the levels of IFN-γ produced in DC-T cell cocultures are significantly increased by this stimuli combination and, by intracellular staining of IFN-γ, we further demonstrate that the cells induced to produce IFN-γ simultaneously express gut-homing receptors.

In agreement with previous works where the expression of gut-homing receptors was found in T-bet^+ activated T cells upon oral infection with *Toxoplasma gondii*,[46] our results reinforce the notion that the genetic programs driving Th1 polarization and the expression of gut-homing receptors are not mutually exclusive. In fact, while the expression of CXCR3 and CCR4 correlates with the Th1 versus Th2 polarization, the expression of skin- or gut-homing receptors on T cells seems to be independent of the polarization type.[31] These Th1 gut-tropic T cells may have a relevant protective role in infectious[46] or tumoral[47] settings and, importantly, we show that the combination of TLR2/TLR4 stimulation can be explored in vaccines to elicit them – by parenteral immunization or by DC vaccine strategies –, and in the *ex vivo* instruction of T lymphocytes for therapeutic purposes.

TLR2 and retinoic acid signaling have both been shown to lead to different and contradictory T cell differentiation outcomes, depending on the priming context. Specifically, retinoic acid originated by gut associated DCs and macrophages was shown to act together with

TGF-β to induce the differentiation of Treg cells,[16,48–51] while inhibiting the differentiation of Th17 cells by IL-6 and TGF-β.[50–52] Additionally, in a mouse Influenza A model, vitamin A was shown to negatively regulate Th1 responses and promote type 2 cytokines.[53] However, in apparent contradiction to these results, in a *T. gondii* infection and immunization mouse model retinoic acid was essential for the induction of mucosal Th1 and Th17 cell responses.[46] Additionally, retinoic acid production by DCs activated through zymosan-induced TLR2 signaling has been also implicated in the differentiation of regulatory T cells.[9,33] Other studies suggest that some TLR2 ligands also favor a Th2 differentiation of $CD4^+$ T cells.[54,55] However, spleen DCs stimulated with P3C in our experiments did not induce Foxp3 expression in primed T cells nor produce significant amounts of the Th2-related cytokines IL-4, IL-5 and IL-13 in DC-T cell cocultures. This is in accordance with previous studies with P3C[8] and may be related with the cytokine milieu induced by different TLR2 ligands or ligand combinations. Furthermore, the concomitant stimulation with the TLR4 ligand MPLA, not only increased the IFN-γ production in DC-T cell cocultures, but also reduced the production of the Th2 cytokine IL-4 when bona fide DCs were used. These results indicate that despite the ambiguous TLR2 role in defining Th polarization, TLR2 agonists can be explored for the induction of Th1 immunity especially if associated with other PRR ligands.

The results of the initial assays performed for setting up the coculture experimental conditions also emphasize the need for a careful optimization of several parameters that can be critical, for instance, in DC vaccine protocols or *ex vivo* T cell activation for therapeutic applications relying on the induction of homing receptors. The supplementation of the culture medium with FCS has been previously pointed as a potential source of variable amounts of vitamin A, residual endotoxins and, possibly, other factors such as GM-CSF and IL-4 that affect the expression of homing receptors generating relevant variability in these assays.[8,31,43,45] While we did not find evidence of residual endotoxins, neither when performing assays in the presence of polymyxin B (an endotoxin neutralizing compound; not shown) nor when testing an FCS lot with guaranteed low endotoxin level, we actually observed the induction of variable levels of homing receptor expression when using different lots of FCS. This variation did not occur when using a serum free medium.

Due to the low number of DCs that is possible to isolate from the different mouse organs, in most of our experiments we used DCs expanded *in vivo* by engrafting the Flt3l-secreting tumor B16-Flt3l.[56–58] DCs obtained from Flt3l-treated mice were shown to have similar properties to bona fide DCs regarding the capacity to induce gut tropism on T cells.[8] However, it is known that different DC models express distinct cytokine profiles when stimulated through PRRs. For instance, in a study on the induction of regulatory responses by zymosan-stimulated DCs, Manicassamy et al. (2009) observed the production of the proinflammatory cytokines IL-23, IL-6, IL-12, and TNF-α as well as IL-10 from zymosan-stimulated BMDCs, while zymosan induced high IL-10 production and low pro-inflammatory cytokines in splenic DCs *in vitro* and *in vivo*.[9] The enhanced production of the Th1 cytokine IFN-γ observed in our system by TLR2/TLR4 stimulation, both when expanded and bona fide DCs were used in DC-T cell cocultures, reinforces the utility of this double stimulation for the induction of gut-tropic Th1 responses independently of the DC origin.

With the aim of surpassing the mucosal delivery limitations of antigens for the induction of mucosal responses, the administration of retinoic acid has been previously explored to induce gut homing in parenteral vaccines.[59] However, retinoic acid may have off-target effects undesirable for the type of response to elicit and limiting retinoic acid production to the DC-T cell niche can avoid such limitations. In order to explore the results here presented, a possible approach could be the development of nanoparticle systems, such as liposomes,[60] designed to specifically target DCs, incorporating both antigens and ligands promoting the desired T cell polarization and the induction of DC ALDH activity, carrying also its substrate, retinol. This strategy would limit the production of retinoic acid to the targeted DCs, with the metabolite acting at the microenvironment surrounding the DC, avoiding counteracting retinoic acid effects on other cells that may occur if directly administered. The combined stimulation through different PRRs has also been shown to synergistically enhance the secretion of cytokines and promote other features of the immune response relevant for immune protection.[61–64] In this respect, this study also contributes to clarify the relation between DC activation through multiple PRRs and the integration of tissue homing signals and polarization factors by primed $CD4^+$ T cells, the basis for a rational use of PAMPs for adjuvant formulation and cell therapy protocols.

Limitations of the study

These studies have been developed in a mouse model and further studies will be needed to extend their applicability to other animal species. It would be desirable to extend the study to other antigens, as our studies are based on a single model antigen. The findings may have practical utility in the development of vaccines and immunotherapies but, further investigation in infection and disease models is required to ascertain the true applicability of these strategies.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents should be directed to and will be fulfilled by Alexandre Leitão (alexandre@fmv.ulisboa.pt).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data reported in this article will be shared by the lead contact upon request.
- This article does not report original code.
- Any additional information required to reanalyse the data reported in this work article is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

APB and AL conceptualized the study. S.Z., M.F., I.L.S.D., P.G., S.A., M.T.R., A.C.M., D.S., S.N., and A.P.B. performed the investigation. S.Z., M.F., I.L.S.D., S.N., L.G., A.L. and A.P.B. contributed to designing methodology, analyzing data, and writing the article. All authors reviewed and approved the final version of the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Mouse/Rat Thy1.1	eBiosciences	Clone HIS51; RRID: AB_906237
Anti-Mouse Thy1.2	eBioscience	Clone 53-2.1; RRID: AB_2650924
Anti-Mouse CD11c	eBioscience	Clone N418; RRID: AB_2563905
Anti-Mouse MHCII (I-A/I-E)	eBioscience	Clone M5/114.15.2; RRID: AB_465927
Anti-Mouse CD4	eBioscience	Clone RM4-5; RRID: AB_2562608
Anti-Mouse/Human CD44	Biolegend	Clone IM7; RRID: AB_2562451
Anti-Mouse CD62L	eBioscience	Clone MEL-14; RRID: AB_469409
Anti-Mouse α4β7 integrin	Invitrogen	Clone DATK32; RRID: AB_657804
Anti-Mouse CCR9	Invitrogen	Clone eBioCW-1.2; RRID: AB_2016702
Anti-Mouse TCR Vβ5.1,5.2	BD Pharmingen	Clone MR9-4; RRID: AB_394697
Anti-Mouse CD45.2	Invitrogen	Clone 104; RRID: AB_953590
Anti-Mouse CD40	Biolegend	Clone 1C10; RRID: AB_1134081
Anti-Mouse CD80	Biolegend	Clone 16-10A1; RRID: AB_2564174
Anti-Mouse CD86	Biolegend	Clone GL-1; RRID: AB_10898329
Anti-Mouse Foxp3	eBioscience	Clone FJK-16s; RRID: AB_1518812
Anti-Mouse IFN-γ	eBioscience	Clone XMG1.2; RRID: AB_469932
Chemicals, peptides, and recombinant proteins		
Aldefluor Assay	Stemcell Technology	Cat#01700
Penicillin-Streptomycin	Invitrogen	Cat#15140-122
0.05% trypsin-EDTA	Gibco	Cat#25300-054
PBS	Gibco	Cat#14190-169
RPMI medium	ThermoFisher	Cat#11875093
FBS	Gibco	Cat#A5256701
FBS Premium Plus	Gibco	Cat#A4766801
DNAse I	Roche	Cat#11284932001
Collagenase, Type IV	Gibco	Cat#17104-019
Red cell lysis buffer	Invitrogen	Cat#00-4333-57
P_3CSK_4	Invivogen	Cat#tlrl-pms
MPLA	Invivogen	Cat#tlrl-mpls
LPS	Invivogen	Cat#tlrl-pb5lps
X-Vivo 15 medium	Lonza	Cat#LONZBE02-060F
$Ova_{323-339}$ peptide	Invivogen	Cat#vac-isq
OVA EndoFit	Invivogen	Cat#vac-pova
Retinol	Sigma-Aldrich	Cat#R7632
RNase-free DNase Set I	Omega Bio-Tek Inc	Cat#E1091-02
FTY720	Sigma-Aldrich	Cat#SML0700
PMA	Invivogen	Cat#tlrl-pma
Ionomycin	Invivogen	Cat#inh-ion-3
Brefeldin A	Invivogen	Cat#inh-bfa

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Monensin	Biolegend	Cat#420701
Critical commercial assays		
Mouse Naïve $CD4^+$ T cell Isolation kit	Miltenyi Biotec	Cat#130-104-453
MojoSort Mouse Pan Dendritic Cell Isolation Kit	Biolegend	Cat#480097
CD4 (L3T4) MicroBeads	Miltenyi Biotec	Cat#130-049-201
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit	Invitrogen	Cat#L34965
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	Invitrogen	Cat#L34975
Foxp3/Transcription Factor Staining Buffer Set Foxp3 Staining Set	eBioscience	Cat#00-5523-00
Mouse IL-6 Uncoated ELISA Kit	eBioscience	Cat#88-7064-88
Mouse IL-10 Uncoated ELISA Kit	eBioscience	Cat#88-7105-88
Mouse IL-12 p70 Uncoated ELISA Kit	eBioscience	Cat#88-7121-88
Mouse TNF alpha Uncoated ELISA Kit	eBioscience	Cat#88-7324-88
Mouse IFN gamma Uncoated ELISA Kit	eBioscience	Cat#88-7314-88
E.Z.N.A. Total RNA kit I	Omega Bio-Tek Inc	Cat#R6834-02
SOLIScript kit	Solis BioDyne	Cat#06-33-00050
SensiFAST SYBR Hi-ROX kit	Meridian Bioscience	Cat#BIO-92020
CellTrace CFSE Cell Proliferation Kit	Invitrogen	Cat#C34554
CellTrace Violet Cell Proliferation Kit	Invitrogen	Cat#C34557
Legendplex	Biolegend	Mix and Match
Experimental models: Cell lines		
B16-Flt3l	Paula Videira, NOVA School of Science and Technology, Universidade NOVA de Lisboa	RRID:CVCL_IJ12
Experimental models: Organisms/strains		
Mouse: C57BL/6	IGC (Oeiras, Portugal)	N/A
Mouse: C57BL/6: Thy1.1	IGC (Oeiras, Portugal)	N/A
Mouse: C57BL/6: Ly5.1	IGC (Oeiras, Portugal)	N/A
Mouse: OT2: $RAG2^{-/-}$	Luís Graça Lab (iMM, ULisboa; IGC, Portugal)	N/A
Oligonucleotides		
Aldh1a2 sense 5′-ATCAAGGAGGCTGGCTTTCC-3′	This paper	N/A
Aldh1a2 antisense 5′-ATGCCGATGTGAGAAGCGAT-3′	This paper	N/A
Actin b sense 5′-CCAACCGTGAAAAGATGACC-3′	This paper	N/A
Actin b antisense 5′-ACCAGAGGCATACAGGGACA-3′	This paper	N/A
Software and algorithms		
FlowJo v10	TreeStar	N/A
GraphPad Prism Software 5.03	GraphPad Software	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice and animal procedures

C57BL/6, C57BL/6.Thy1.1 and C57BL/6.Ly5.1 mice were purchased from IGC (Lisbon, Portugal). OT2.$RAG2^{-/-}$ mice were bred and provided by LGraça Lab (IMM, ULisboa; IGC, Portugal). The mice were maintained at FMV's animal facility (FMV, ULisboa) under specific pathogen-free conditions, in cages with sawdust as bedding, in a room with controlled temperature (22-25°C) with a 12 hours-light/dark cycle and were fed standard laboratory diet and water *ad libitum*. The mice were used when they were between eight and twenty-four weeks old. In *in vivo* experiments, cell donors were either females or males and recipient mice were males.

All animal-involving procedures were conducted following Portuguese national guidelines (in *Artigo* 31° of *Decreto-Lei* 113/2013) and European Commission recommendations (Directive no. 2010/63/EU) for the use of animals in scientific research. The protocols were approved

by the FMV, ULisboa "Ethics and Animal Welfare Committee" (ORBEA, FMV, ULisboa) and licensed by the Portuguese National Authority for Animal Health (DGAV License number 0421/000/000/2020).

METHOD DETAILS

In vivo DC expansion

DCs were expanded *in vivo* by engraftment of murine melanoma cells B16-Flt3l (kindly provided by Paula Videira, NOVA School of Science and Technology, Universidade NOVA de Lisboa). The cell line was maintained in complete DMEM medium (Gibco, #61965-059) with 10% FBS (Gibco, #A5256701) and 100 IU/mL penicillin + 100 μg/mL streptomycin (Invitrogen, #15140-122) at 37°C in 5% CO_2. Before inoculation, the cells were dissociated with 0.05% trypsin-EDTA (Gibco, #25300-054), washed twice and resuspended in sterile PBS (Gibco, #14190-169). Between 5 – 8 x 10^6 B16-Flt3l cells were inoculated subcutaneously in the flank of C57BL/6 mice. Tumor growth was monitored for 7 – 12 days and after this time mice were euthanized for spleen collection.

DC isolation

Spleens were washed in sterile PBS at the flow cabinet, cut in pieces (1 mm) with scissors in 6 cm petri dishes and digested in 10 mL of warmed (37°C) RPMI medium (ThermoFisher, #11875093) added of DNAse I (Roche, #11284932001; 1 μg/mL) and Collagenase IV (Gibco, #17104-019, 200 U/mL) and incubated at 37°C for 30 minutes with occasional stir. Splenocytes were then collected by grinding the spleen pieces through sterile 70 μm strainers (Corning, #431751) with a plunge of a sterile 2 mL syringe. Filtered splenocytes were centrifuged (300 *g*, 6 min), resuspended in red cell lysis buffer (1X) (Invitrogen, #00-4333-57), 5 mL per spleen, and incubated at room temperature for 5 min. The cells were then washed twice with MACS buffer (1X PBS + 2% FCS + 2 mM EDTA). Dendritic cell isolation, either with or without previous *in vivo* expansion with B16-Flt3l, was achieved by magnetic negative selection using the MojoSort Mouse Pan Dendritic Cell Isolation Kit (Biolegend, #480097) following manufacturer instructions.

In vitro DC stimulation

All PAMPs used as stimuli (Invivogen: P_3CSK_4, #tlrl-pms; MPLA, #tlrl-mpls; LPS, #tlrl-pb5lps) were diluted in X-Vivo 15 medium (Lonza, #LONZBE02-060F). Negatively selected DCs were cultured unstimulated or stimulated with the TLR agonists in the indicated concentrations, for 24 hours in the indicated culture medium (serum-free medium XVivo15 or RPMI medium with or without low-endotoxin (Gibco, Premium Plus, #A4766801) or regular FCS (Gibco, #A5256701)). After this, the cells and supernatants were collected and centrifuged at 300 *g* for 8 min. Supernatants were aliquoted for storage at -80°C for later cytokine evaluation and pelleted DCs were either directly resuspended in lysis buffer for RNA extraction and stored at -80°C until further processing, or were washed twice with PBS and used for Aldefluor assay or for coculture with $CD4^+$ T cells.

ALDH activity

Aldefluor Assay (Stemcell Technology, #01700) was used to quantify the ALDH enzyme activity by DCs stimulated by different TLR ligands. The protocol followed manufacturers' recommendations with slight modifications: pelleted cells (1 x 10^5 cells) were washed in Aldefluor Assay Buffer and resuspended in 200 μL of the same solution containing Aldefluor Reagent. An inhibited control was prepared for each sample and, here, cells were resuspended in Aldefluor Assay Buffer with diethylaminobenzaldehyde (DEAB) inhibitor and Aldefluor Reagent. All samples and controls were then incubated for 40 mins at 37°C, then washed, stained with the necessary antibodies for immunophenotyping analysis by flow cytometry, and finally resuspended in 0.5 mL of Aldefluor Assay Buffer.

$CD4^+$ isolation for DC co-cultures

Spleens from OT2.$Rag2^{-/-}$ mice were washed in sterile 1X PBS and directly mashed with a sterile syringe plunge over a moisten 70 μm strainer, coupled to a 50 mL centrifuge tube (Corning, #734-1812). After red blood cell lysis, 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 manufacture recommendations.

DC-T cell co-cultures

Stimulated DCs were pulsed with MHC class II $Ova_{323-339}$ peptide (Invivogen, #vac-isq; 100 mM), during 2 hours at 5% CO_2 37°C, after which they were washed in PBS and co-cultured with naïve $CD4^+$ from OT2.$Rag2^{-/-}$, in the presence of freshly added retinol (Sigma-Aldrich, #R7632) at the indicated concentrations. Co-cultures were incubated for the indicated number of days, in X-Vivo 15 medium, at 37°C with 5% CO_2. In optimization experiments presented in Figure 1, the same culture medium conditions used for DC stimulation described above were used. At the longer periods of incubation (7 and 9 days) the half of the culture medium was replaced by fresh medium at day 5.

Flow cytometry

Single-cell suspensions were obtained from the DC stimulations for the Aldefluor Assay and from the co-cultures for the determination of α4β7 and CCR9 gut-homing markers. Briefly, cells were surface stained for 30 mins at 4°C (dendritic cells) or 15 mins at 37°C plus 15 mins at

room temperature (staining of homing receptors on OT-II cells) in PBS with 2% FBS (Gibco, Premium Plus, #A4766801). After washing, cells were fixed (Foxp3/Transcription Factor Staining Buffer Set, eBioscience, #00-5523-00). Surfaces staining was performed in MACS buffer with anti-Thy1.1 (Clone HIS51, eBiosciences), anti-Thy1.2 (Clone 53-2.1, eBioscience), anti–CD11c (Clone N418, eBioscience), anti-MHCII (I-A/I-E) (Clone M5/114.15.2, eBioscience), anti-CD4 (Clone RM4-5, eBioscience), anti-CD44 (Clone IM7, Biolegend), anti-CD62L (Clone MEL-14, eBioscience), anti-α4β7 integrin (Clone DATK32, Invitrogen), anti-CCR9 (Clone eBioCW-1.2, Invitrogen), anti-TCR Vβ5.1,5.2 (Clone MR9-4, BD Pharmingen), anti-CD45.2 (Clone 104, Invitrogen), anti-CD40 (Clone 1C10, Biolegend), anti-CD80 (Clone 16-10A1, Biolegend), anti-CD86 (Clone GL-1, Biolegend). Cell viability was assessed with a Live/Dead Fixable Dead Cell Stain Kit (Invitrogen, #L34965 or #L34975) for dead-cell exclusion. For some flow cytometry analysis, the cells were stimulated for 4 h in culture medium containing 25 ng/ml PMA (Invivogen, #tlrl-pma), 250 ng/ml ionomycin (Invivogen, #inh-ion-3), 10 μg/ml brefeldin A (Invivogen, #inh-bfa) and monensin (Biolegend, #420701) 2 μM. In addition to the surface staining with the mentioned monoclonal antibodies, intracellular staining was performed after fixation and permeabilization with the Foxp3/Transcription Factor Staining Buffer Set Foxp3 Staining Set (eBioscience, #00-5523-00), according to the manufacturer's instructions. Antibodies used for intracellular staining were: anti-Foxp3 (Clone FJK-16s, eBioscience) and IFN-γ (Clone XMG1.2, eBiosciences). Flow cytometry was performed in Attune NxT cytometer (ThermoFisher) and data was analysed with FlowJo v10 software (TreeStar).

Cytokine quantification

The assessment of cytokine levels on culture supernatants was performed by ELISA using distinct mouse Ready-Set-Go kits (eBiosciences, #88-7064-88; #88-7105-88; #88-7121-88; #88-7324-88; #88-7314-88) to individually estimate the levels of IL-6, IL-10, IL-12(p70), TNF-α and IFN-γ, or by the cytometry-based multiplex assay Legendplex (Biolegend) to measure cytokines associated with Th1 (IFN-γ), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17A) and Treg (IL-10) in the same sample. In both cases we followed the manufacturer's instructions.

Isolation of total RNA and qPCR analysis

Isolation of RNA from dendritic cells was performed using E.Z.N.A. Total RNA kit I (Omega Bio-Tek Inc, #R6834-02) following the manufacturer's instructions. Cell pellets were re-suspended in 350 μL of TRK Lysis Buffer supplemented with 2-mercaptoethanol and homogenized using syringe and needle (21 G). Genomic DNA contamination was eliminated by on-column digestion with RNase-free DNase Set I (Omega Bio-Tek Inc, #E1091-02). RNA was eluted in 40 μL of Nuclease-free water and quantified in a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). Complementary DNA was synthesized with the SOLIScript kit (Solis BioDyne, #06-33-00050). Quantitative PCR was performed using the SensiFAST SYBR Hi-ROX kit (Meridian Bioscience, #BIO-92020) with ROX as reference dye, on a StepOnePlus Real-Time PCR System (Applied Biosystems, #4376600). Each sample was tested in duplicate for the expression of *Aldh1a2* (5′-ATCAAGGA GGCTGGCTTTCC-3′ and 5′-ATGCCGATGTGAGAAGCGAT-3′) and *Actin b* (5′-CCAACCGTGAAAAGATGACC-3′ and 5′-ACCAGAGGCAT ACAGGGACA-3′). For a relative comparison of the gene expression, $2^{-\Delta CT}$ values were first determined in reference to the *Actin b* expression of the same sample and then normalized to the average $2^{-\Delta CT}$ values of the P3C stimulated samples.

Homing index assay

DC-T cell co-cultures were stablished as described above. After 3 days, half the volume of the culture medium was replaced and at day 5 the cells were collected, washed, and stained during 20 minutes at 37 °C. The cells from the cultures with DCs stimulated with MPLA alone were stained with CFSE (CellTrace CFSE Cell Proliferation Kit, for flow cytometry; Invitrogen, #C34554) and the cells from MPLA+P3C activated cultures were stained with CTV (CellTrace Violet Cell Proliferation Kit, for flow cytometry; Invitrogen, #C34557). After washing, CFSE and CTV stained cells were counted, adjusted to a proportion 1:1, and inoculated intravenously in recipient mice (5×10^6 cells of each type). After 20h, blood, skin draining lymph nodes (inguinal lymph nodes) and gut-associated secondary lymphoid organs (Peyer's patches and mesenteric lymph nodes) were collected and processed for flow cytometry. The relative loads of stained cells in each organ were normalized to the stained cells found in the blood.

Immunization experiment

OT-II cells were isolated from spleens of OT2.Rag2$^{-/-}$ mice using CD4 (L3T4) MicroBeads (Miltenyi Biotec, #130-049-201) and 2.5×10^6 cells were transferred intravenously to Ly5.1 (CD45.1) mice. Recipient mice were immunized subcutaneously in the right flank with 50 μg of ovalbumin (OVA EndoFit, Invivogen, #vac-pova) plus 12.5 μg MPLA or with the same mixture added with 12.5 μg P3C. Between day 3 and day 7 after immunization, the mice were treated daily with FTY720 (1 mg/kg; Sigma-Aldrich, #SML0700) to inhibit the exit of lymphocytes from lymph nodes. One day after the last treatment, the mice were euthanized and the right inguinal lymph node (draining the immunization site), the left inguinal lymph node (contralateral skin draining lymph node) and gut-associated secondary lymphoid organs (mesenteric lymph nodes and Peyer's patches) were collected. The cells were processed for flow cytometry to evaluate the presence of OVA-specific OT-II cells (CD45.2+TCRVβ5.1,5.2+) and were restimulated *ex vivo* (1×10^6 cells/mL in X-VIVO 15 medium, Lonza) with ovalbumin (OVA EndoFit, Invivogen) at 50 μg/mL during 72 h. The secretion of IFN-γ in the culture supernatants was tested by ELISA (IFN gamma Mouse Uncoated ELISA Kit, Invitrogen), following the manufacturer's instructions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical evaluations were performed using GraphPad Prism Software 5.03. Data are presented as the mean ± standard error of the mean (s.e.m.) of three to four replicates. Significant differences between each single stimulation group and the double stimulation group were determined by unpaired two-tailed Student's t-test and between single and double adjuvanted immunization with two-way ANOVA with Bonferroni post-tests and were ranked: ns – not significant, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.