Cross-talk between iNKT cells and CD8+ T cells in the spleen requires the IL-4/CCL17 axis for the generation of short-lived effector cells

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Mounting an effective immune response relies critically on the coordinated interactions between adaptive and innate compartments. How and where immune cells from these different compartments interact is still poorly understood. Here, we demonstrate that the cross-talk between invariant natural killer T cells (iNKT) and CD8+ T cells in the spleen, essential for initiating productive immune responses, is biphasic and occurs at 2 distinct sites. Codeployment of antigen and adjuvant to antigen-presenting cells results in: 1) short-lived interactions (0 to 6 h), between CD8+ T cells, dendritic cells (DCs), and iNKT cells recruited outside the white pulp; 2) followed by long-lasting contacts (12 to 24 h) between iNKT cells, DCs, and CD8+ T cells occurring in a 3-way interaction profile within the white pulp. Both CXCR3 and CCR4 are essential to orchestrate this highly dynamic process and play nonredundant in T cell memory generation. While CXCR3 promotes memory T cells, CCR4 supports short-lived effector T cell generation. We believe our work provides insights into the initiation of T cell responses in the spleen and their consequences for T cell differentiation.

iNKT cells | CD8 T cell priming | CCL17 | CD8 T cell memory | spatiotemporal kinetics

The CD8+ T cells are essential for tumor eradication by recognizing peptide–MHC-I complexes at the cell surface. In the lymph node (LN), T cell priming occurs in 3 distinct phases (1). Briefly, at early stages T cells undergo multiple transient encounters with dendritic cells (DCs), followed by long-lasting contacts concomitant with cytokine production. Then, T cells resume their rapid migration and start proliferating. The establishment of extensive contacts with DCs appears to be essential for mounting high-quality CD8+ T cell responses, in particular regarding memory T cell generation (2, 3). CD8+ T cell priming depends on efficient cross-presentation, allowing exogenous antigens to be processed into peptides and subsequently presented by MHC-I molecules. Among conventional DCs (cDC), cDC1 express XCR1 and CLEC9a surface markers, and are considered to be the major antigen-presenting cell (APC) involved in this process in vivo (4). The apparent superior ability of cDC1 depends on an optimal intracellular machinery adapted for this pathway (5, 6). Furthermore, their critical positioning within secondary lymphoid organs to get direct access to antigens (7, 8), combined with their ability to produce high amounts of IL-12 (9), explain why cDC1 are so efficient in cross-presentation. In addition, splenic CD169+ metallocophilic macrophages (MM) or their equivalent in the LN have been proposed to fulfill this role as well (10, 11).

Next to DCs, helper cells are also important to promote effective cellular immunity by enhancing CD8+ T cell clonal expansion, differentiation, and survival (12). Besides CD4+ T cells, invariant natural killer T (iNKT) cells can achieve this task. iNKT cells represent a specialized subset of innate immune cells characterized by the expression of a restricted Vβ T cell antigen receptor (TCR) repertoire composed of the canonical variable α-region 14/joining α-region 18 (Vα14-Jα18) chain in association with the Vβ2, Vβ7, or Vβ8 chain in mice (13). iNKT cells specifically recognize lipid antigens such as α-galactosylceramide (α-GaLCer) presented by CD1d molecules expressed by DCs, macrophages, and B cells. Upon activation, they rapidly secrete large amounts of cytokines and induce the subsequent activation of different cell types, including DCs, NK cells, and conventional T cells. In the spleen, iNKT are mainly localized in the red pulp (RP) and, upon immunization with α-GaLCer, they are recruited to the marginal zone (MZ), where they get activated by MZ macrophages (MZY) and DCs (14, 15). Previous studies, including our own work (16, 17), have pointed out that efficient iNKT help requires antigen presentation to both cell types from the same APC. However, it is still unclear where and when iNKT cells and CD8+ T cells meet, and whether this process occurs concomitantly or sequentially.

In this study, we aimed at elucidating the spatiotemporal kinetics of the cross-talk between iNKT cells and CD8+ T cells in the spleen. We exploited nanovaccines that can efficiently code-liver antigen and adjuvant to APCs. With different molecular, the spleen requires the IL-4/CCL17 axis for the generation of short-lived effector cells.

Significance

Efficient iNKT help require antigen presentation to both the CD8+ T cell and the helper cell interacting with the same DCs. However, it remains unclear where and when iNKT cells, DCs, and CD8+ T cells meet, but it is rather accepted that these interactions should occur sequentially. Here, we show that iNKT cell help occurs in 2 spatiotemporal distinct phases and involves rather concomitant interactions. Indeed, at 24 h, iNKT cells are massively recruited in the white pulp, and the majority of activated CD8+ T cells are forming concomitant long-lasting interactions with iNKT cells and DCs. This study illustrates the importance of simultaneous delivery of antigen and adjuvant and should be considered in the design of new vaccines or immunotherapies.

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with minimal amounts of antigen and adjuvant and we have demonstrated that vaccination greatly relies on the codelivery, suggesting that efficient iNKT help requires antigen presentation to both CD8\(^+\) T cells and iNKT cells from the same APC (17). Importantly, we have previously shown that the vaccination efficiency depends solely on the help from iNKT cells and not from CD4\(^+\) T cells to generate potent OVA-specific CD8\(^+\) T cells (17). As very limited information is available on how 200-nm-sized PLGA-based nanovaccines (carrying both antigen and adjuvant) enter the spleen and which cells are the primary target, we first examined the capture of fluorescently labeled PLGA nanovaccines in the spleen at early (2 and 6 h) and late time points (24 h) following intravenous injection. The capture was rapid and transient, as we were able to detect nanovaccines in the spleen within 2 h (Fig. 1 A and B), whereas at 24 h, fluorescent nanovaccines were hardly visible (Fig. 1 A and B). This was also confirmed by flow cytometry (SI Appendix, Fig. S1); however, with this technique, CD169\(^+\) MM could not be assessed for the quantification. As expected, CD68\(^+\) macrophages and CD11c\(^+\) DCs formed the main subsets involved in the nanovaccine uptake (~70% and 30%, respectively, at 6 h) (Fig. 1 C and D). Interestingly, nanovaccines were excluded from the WP, but accumulated at the MZ (Fig. 1 A). In accordance, at 6 h about 50% of all nanovaccines detected in the spleen were taken up by CD169\(^+\) macrophages, followed by CLECs (cDC1)
and SIGNR1 macrophages (Fig. 1 C and D). Importantly, the nature of the adjuvant encapsulated in the PLGA nanovaccine did not affect capture by distinct APC subsets, since nanovaccines containing TLR-L (PolyIC + R-848) instead of α-Galcer exhibited the same uptake pattern (Fig. 1 D). Thus, nanovaccines are captured by both DCs and macrophages located in the MZ during the first hours after intravenous administration.

α-Galcer–Carrying Nanovaccines Induce CCL17 and CXCL9 Production by CD8 T Cells and DCs. We next evaluated whether the iNKT cell adjuvant α-Galcer could induce the production of specific sets of chemokines when compared to more common adjuvants such as TLR-L. To this end, mice were vaccinated and 6 h later different cell subsets were sorted by flow cytometry (CD69+ and CD69− OVA-specific CD8+ T cells [OT-I] and cDC subsets, XCR1+ DC [cDC1] and CD11b+ DC [cDC2]) (SI Appendix, Fig. S2). Subsequently, mRNA expression of multiple cytokines, chemokines, and their receptors was measured on sorted populations. Exploiting microarrays, we assessed the expression profile of chemokines and cytokines in OT-I CD8+ T cells, based on their activation state (CD69 expression) and the nature of adjuvants carried by the nanovaccine. Performing a nonsupervised clustering, we observed that OT-I CD8 T cells were clustered together, and depended more on the type of adjuvant used than on their activation status (Fig. 2 A). This was also true for DCs, whose cytokine/chemokine profiles demonstrated a clustering based on the type of adjuvant rather than on the cDC subset (Fig. 2B). A closer look at the data showed that different chemokine pathways were triggered by the different types of adjuvants (SI Appendix, Table S1). We thus decided to explore this phenomenon in more detail by performing qRT-PCR on the different cell subtypes. CCR4 ligands (CCL17 and CCL22) and CXCR3 ligands (CXCL9 and CXCL10) were specifically induced by α-Galcer nanovaccine (Fig. 2 C and D). In particular, the chemokines CCL17 and CXCL9 were up-regulated by α-Galcer in OT-I T cells and cDCs. In sharp contrast, CCL3 and CCL4 expression, both CCR5 ligands, were specifically induced in activated OT-I T cells of mice that received TLR-L–carrying nanovaccines (Fig. 2 C and D). Finally, IL-12 production, which is important for the initiation of cytotoxic responses,

![Fig. 2.](https://www.pnas.org/cgi/doi/10.1073/pnas.1913491116)
CCL17 protein expression was also induced 6 h after α-Galcer stimulation has a major impact on splenic chemokine networks by specific induction of CCL17 and CXCL9 expression in multiple immune cell types.

**CXCL9 and CCL17 Expression Patterns Are Dynamic over Time in the Different Spleen Compartments.** Since we found in various cell types that iNKT cells specifically induce the expression of CCL17 and CXCL9 at mRNA levels, we next sought their protein level expression networks by specific induction of CCL17 and CXCL9 expression in the RP and only start to be expressed in the WP at later stages. This may have important consequences for the migratory behavior of the cells.

**CD8+ T Cell Localization in the Spleen Is Biphasic during Early Stages of Activation.** Following the cues of T cell-attracting chemokines, we assessed whether T cells were following a similar path. The localization of antigen-specific OT-I CD8+ T cells was tracked over time by confocal microscopy. As expected, OT-I T cell behavior was also highly dynamic early after nanovaccine administration in accordance with the chemokine profiles (Fig. 4A). Interestingly, within the first 6 h, almost half of OT-I T cells accumulated at the MZ and in the RP where CXCL9 and CCL17 were detected exclusively (Figs. 3A–D and 4B). At this stage, T cells started to get activated, as evidenced by CD69 expression (Fig. 4C and D). In addition, we evaluated the distance between recruited OT-I T cells and DCs or CD169+ macrophages, and we found that they were closer to CD11c+ DC and CLEC9a+ DC than to CD169+ macrophages (SI Appendix, Fig. S4A). This suggests that CD8+ T cells were recruited toward DCs rather than to CD169+ macrophages during this time frame. Later, from 12 h on, when chemokines became expressed in the WP (Fig. 4A, B, and D), OT-I T cells were redirected and during this second phase were mainly found in the WP forming stable T–T clusters together with CD11c+ DC (Fig. 4E and F) and CLEC9a+ DC but not with CD169+ macrophages (SI Appendix, Fig. S4B). At this time, they also started producing IFN-γ and CD69 expression reached a level of ~90% at 24 h (Fig 4C–E). Finally, we questioned the origin of OT-I T cells accumulating outside the WP at 6 h, which can come either from the WP or from the RP. To do so, we compared the localization of OT-I T cells that have been injected 1 d before the vaccination or 3 h after (referred to as early migrating OT-I), since it has been shown that 3 h was enough for intravenously injected T cells to home to the WP (18). To accurately quantify this, we compared the ratio of “OT-I-early migrating OT-I” in the WP between control and vaccinated mice. As depicted in SI Appendix, Fig. S4C, we found that the main origin appears to be from the WP, suggesting that T cell trafficking is transiently affected upon nanovaccine administration.

To substantiate these findings, we next studied the migratory behavior of antigen-specific CD8+ T cells within various splenic compartments. Since intravital microscopy for the spleen is extremely challenging (19), we opted for an explanted organ approach using perfused thick sections of spleen for live imaging. During early stages after vaccine delivery (2 to 6 h), we observed that OT-I T cells kept their normal high-speed motility of around 7 μm/min in the WP as at the steady state (Fig. 5A and B and Movie S1). In the MZ and the RP, OT-I T cells exhibited a somewhat slower speed with a mean velocity of 5 μm/min (Fig. 5C and D and Movie S2). This slowing could result from repetitive short encounters with APCs. This notion was supported by the finding that in the absence of OVA antigen or with polyclonal CD8+ T cells, the velocity was slightly but significantly higher in those regions during this time frame (Fig. 5C and D). During the second phase (12 to 24 h), when T cells were redirected to the WP, OVA-primed T cells significantly slowed down their migratory behavior (Fig. 5A and B), reflecting the formation of stable conjugates together with DCs (Fig. 4E and F and Movie S3). Altogether, these results demonstrate that antigen-specific
CD8+ T cells exhibit a biphasic behavior, with a first transient accumulation at the MZ and the RP early after nanovaccine administration, where they interact shortly with DCs, and at later stages with the recruitment of CD8+ T cells in the WP, with long-lasting contacts involving multicellular clusters with DC.

**CCR4 and CXCR3 Are Essential in Early CD8 T Cell Activation.** Since our results point out that both CCL17 and CXCL9 are important chemokines in our vaccination strategy, we decided to evaluate the expression of their respective receptors, CCR4 and CXCR3, on OT-I T cells.

At the steady state, a fraction of naive OT-I T cells already harbors CXCR3 at the cell surface, thus rendering these cells responsive to CXCL9 at very early stages. In contrast, CCR4 could not be detected at the cell surface of naive cells and became only up-regulated after vaccination (Fig. 6A). In order to reveal which factors might induce its expression on the cell surface over time, we cultured in vitro purified OT-I T cells in the presence of IL-4 combined or not with OT-I–specific peptide (SIINFEKL) to trigger T cell activation and monitored CCR4 expression by flow cytometry. We found that only the combination of IL-4 and SIINFEKL induced a strong expression of CCR4, while cells exposed to either of them alone only induced a mild expression (SI Appendix, Fig. S5A). CCR4 expression was rapidly induced since it could already be detected from 6 h on, although still higher expression levels were seen at 24 h. Coming back to in vivo vaccination setting, we were able to detect surface expression of CXCR3 on OT-I T cells at early stages of vaccination (6 h). However, the levels of mRNA expression and protein were lower when compared to the control (Fig. 6A and SI Appendix, Fig. S5B). Nonetheless, the vast majority of detected CXCR3+ OT-I T cells expressed CD69, suggesting an important role of this chemokine receptor to recruit antigen-specific T cells in the early stages of activation. At 6 h, the expression of CCR4 was up-regulated at the mRNA level but was not detectable at the cell surface (Fig. 6A and SI Appendix, Fig. S3B). Perhaps engagement of the receptor by its ligand induces its internalization or prevents its recognition by the antibody when the epitope is shielded. At 24 h, the proportion of OT-I T cells expressing CXCR3 strongly increased and CCR4 expression was detected on a fraction of activated CD69+ OT-I T cells (Fig. 6A). We then evaluated the contribution of these chemokine receptors in vivo for early T cell activation and recruitment in the WP. Interestingly, we found that blocking each of them impacted early T cell activation monitored by CD69 expression 6 h after vaccination (Fig. 6B). Furthermore, we saw that the recruitment of OT-I T cells in the WP was negatively impacted by CXCR3 or CCR4 blockade, as depicted in Fig. 6C. Altogether, these results show that the expression of CXCR3 and CCR4 on antigen-specific T cells is triggered upon vaccination and participate in their activation and recruitment.

**iNKT Cross-Talk with CD8+ T Cells Involves Concomitant Interactions in 2 Distinct Phases.** Since CD8+ T cells were rapidly recruited to the MZ and the RP after nanovaccine administration, we hypothesized that they could encounter already activated iNKT cells in these areas during this first phase. Therefore, we first examined whether iNKT cells were activated within the first 6 h upon nanovaccine administration (SI Appendix, Fig. S6A). As expected, we found that most of the iNKT cells were indeed activated according to their CD69 expression.

We then attempted to directly visualize interactions between OT-I T cells and iNKT cells by confocal microscopy. Adoptive transfer of highly purified and fluorescently labeled iNKT cells into naive mice, followed by nanovaccine administration, allowed us to retrieve both cell types. Using a biotinylated anti-CD11c antibody, we were able to detect and quantify anti-CD11c+ DC clusters (2 OT-I cells in contact or more) and anti-CD11c+ iNKT cell clusters (2 iNKT cells in contact or more) at the different time points. Statistical analysis by 1-way ANOVA test: ***P < 0.001; ****P < 0.0001; mean ± SEM.
Fig. 5. OT-I T cells form long-lasting contacts with DC in the WP 24-h postvaccination. CD8+ OT-I yet i T cells were isolated, labeled with CCR dye, and adoptively transferred prior vaccination. The next day, nanovaccines containing OVA and α-Galcer were intravenously administered in mice. At different time points, mice were killed, spleens harvested, and embedded in a low-melting agarose gel. Thick sections of 500 μm were performed using vibratome and stained with anti-CD169 and anti-CD11c antibodies. Live imaging was performed using a spinning-disk microscope equipped with a thermostated chamber and perfused at a rate of 0.8 ml/min with medium bubbled with 95% O2 and 5% CO2. OT-I migration was evaluated on movies depicting tracks (n > 30) start from the same origin. Axes bars represent the scale in microns. (A and C) Quantification of the OT-I T cell mean velocity calculated in micrometers per minute. Results are derived from 4 independent experiments and involve at least 2 mice per condition and time point. Statistical analysis by 1-way ANOVA test: **P < 0.01; ***P < 0.0001; mean ± SEM.

Such an approach was unfortunately limited by the rapid down-regulation of TCR expression on iNKT cell surface triggered by their activation, rendering the quantification difficult after 6 h. To circumvent this drawback and to further study these interactions over prolonged periods of time, we exploited CXCR6-GFP mice, which have been already used in intravital microscopy studies to track NKT cells in the liver (20). As depicted in Fig. 7A, in these mice, CXCR6-GFPbright cells represent a homogenous population in the spleen and the vast majority of them are NKT cells (based on coexpression of CD3 and NK1.1). Based on CD1d-α-Galcer dextramer binding, 62% of this population can be designated as type I NKT cells or iNKT cells (CD1d-restricted NKT cells recognizing α-Galcer) and the remainder of the cells are most likely type II NKT cells, which do not bear a specific marker. Furthermore, upon vaccination, the CXCR6-GFPbright cell population was not increasing and remained representing NKT cells (SI Appendix, Fig. S7A). Assessment of NKT cell distribution in these mice revealed that their localization followed the same biphasic dynamics as OT-I T cells (Figs. 4B and 7B and C). During the first phase (6 h), they were localized to the MZ and RP where they formed NKT−NKT clusters (Fig. 7D), whereas during the second phase (24 h), most of them were found in the WP where NKT−NKT clusters could still be observed. By evaluating the interactions between NKT cells and OT-I T cells, we were able to reveal close contacts (less than 3 μm) between NKT cells and OT-I T cells together with DCs (Fig. 7E and F). Indeed, while ~20% of OT-I cells were found in close contact with NKT cells in the MZ and the RP in the first phase (6 h), almost half of OT-I cells were found in concomitant interactions in the WP in the second phase (24 h) (Fig. 7F).

To establish the identity of CXCR6-GFPbright cells, we confirmed that they were not CD8+ T cells by immunostaining, as illustrated in the SI Appendix, Fig. S7B. Of course, we cannot guarantee that the observed contacts are all with-type I NKT cells (iNKT cells), since they represent 60% of the CXCR6-GFPbright cell population (forming the main population), but we assume that in the majority of the quantified contacts, iNKT cells are involved. By live imaging, we quantified the duration of these contacts and found that these interactions were short and independent of the presence of the OVA antigen at the early stages (Fig. 7G and Movie S4). However, at the late stages, we observed long-lasting interactions with about half of the contacts that lasted at least 30 min (duration of the entire movie shown in Movie S5). To exclude that the observed interactions could only be attributed by the high density of both OT-I and NKT cells in the WP at the late stages, we randomly simulated the position of NKT cell in these regions. As shown in Fig. 7H, we found that the distance between OT-I and NKT cells was closer in reality than when distributed randomly, supporting unambiguously a true connection between NKT and OT-I T cells.

Finally, we addressed the role of CXCR3 and CCR4 in the recruitment of NKT cells in the WP at the late stages and found, surprisingly, that CXCR3 and CCR4 have a differential role in this process (Fig. 7I). Indeed, CXCR3 plays a positive role in this process while, in contrast to T cells, CCR4 negatively impacts this migration. Based on these findings, we demonstrate that NKT cells are first recruited in the MZ and in the RP, where they get activated and can interact shortly with OT-I T cells. In the second phase, they follow the behavior of OT-I T cells by being recruited toward the WP, where they form concomitant long-lasting interactions with DCs.

The IL-4/CCR17/CXCR4 Axis Regulates CD8+ T Cell Differentiation into SLECs. We next addressed the functional impact of CXCR3 and CCR4 on the generation of CD8+ T cell memory. In particular, we focused on CCR4 since the role of this chemokine receptor in SLECs.

First, we aimed to confirm in vivo the link between IL-4 and the CCR4 pathway, as we previously showed in vitro in SI Appendix, Fig. S5A. As expected, we found that IL-4 was only detected in the sera of animals administrated with nanovaccines containing an iNKT cell agonist (Fig. 8A). Maximal production was found in the early stages (6 h) when OT-I T cells accumulate outside the WP. Interestingly, we could detect IL-4 signaling in OT-I T cells by pSTAT6 staining only during this time frame and not in the late stages (Fig. 8B). We then evaluated the impact of IL-4 blockade in vivo on the expression of CCR4 on OT-I T cells and CCL17 production in the spleen. We found that IL-4 blockade strongly reduced CCR4 levels on OT-I T cells (Fig. 8C) and was sufficient to abolish the production of CCL17 in the spleen (Fig. 8D), thus confirming the key role of IL-4 in the induction of the CCR4 pathway.

We then studied the consequence of either CXCR3 blockade or IL-4/CCR17 blockade at the time of the vaccination on the generation of memory precursor cells (MPEC) and SLEC 10 d postnanovaccines administration. Interestingly, we found that blocking CXCR3 drastically reduced the numbers of antigen-specific T cells at day 10 postvaccination (Fig. 8E). This can mainly be explained by the strong reduction of the generation of MPEC in these conditions (Fig. 8F). As a consequence, a higher
proportion of SLEC was found. Conversely, blockade of either CCL17 or IL-4 did not negatively impact the numbers of antigen-specific T cells at day 10, with even a slight tendency to increase (Fig. 8G). Importantly, the blockade of each of these molecules reduced the generation of SLEC and increased the proportion of MPEC in a completely opposite fashion to CXCR3 (Fig. 8H).

Thus, CXCR3 and CCR4 have clear differential roles in the generation of MPEC or SLEC, respectively.

**Early Cross-Talk between iNKT and CD8+ T Cells Impacts the Generation of Both MPEC and SLEC.** Since the IL-4/CCL17/CXCR4 axis is crucial for the generation of SLEC and IL-4 is only produced during the early stages (phase 1), we hypothesized that early cross-talk between iNKT and CD8+ T cells might be important for the generation of SLEC.

To test that, we unsynchronized the delivery of antigen and the iNKT cell agonist by administrating nanovaccines (nanoparticles, NP) containing OVA at the same time, 10 h after (postphase 1) or 48 h after (postphases 1+2) the injection of NP containing α-Galcer, as illustrated in Fig. 9A. We first evaluated the expansion of antigen-specific T cells 10 d after antigen administration and we found that both phases are crucial in this process (Fig. 9B).

Then, we studied as previously the differentiation of antigen-specific T cells into MPEC or SLEC at day 10 (Fig. 9C). Interestingly, we found that both phases are important for the generation of MPEC when we looked at the relative numbers of MPEC OT-I T cells (Fig. 9D). More importantly, we saw that only the absence of the phase 1 is enough to nearly abolish the generation of SLEC (Fig. 9E) and that phase 2 did not give any additive effects. Thus, as predicted, the early cross-talk (phase 1) between iNKT and CD8+ T cells is crucial for the efficiency of the nanovaccine and the generation of SLECs.

To summarize, we propose a model in which iNKT-CD8+ T cell cross-talk occurs in 2 spatiotemporal phases and involves simultaneous interactions between 3 cell types (SI Appendix, Fig. S8). The first phase (2 to 6 h postvaccination) occurs in the MZ and the RP where recruited OT-I T cells interact shortly with activated iNKT cells and DC. During the second phase (12 to 24 h), OT-I T cells, iNKT cells, and DC establish long-lasting contacts forming a “ménage à trois” in the WP. In this process, CXCR3 and CCR4 play a differential role. The IFN-γ/CXCR3 pathway will be involved in the generation memory CD8+ T cells, while the IL-4/CCR4 pathway will regulate the generation of SLECs. We believe that our study highlights the importance of iNKT cells as helper cells to orchestrate the complex dynamic process of cross-priming in the spleen and its importance for proper memory T cell generation.

**Discussion**

Here we report that CD8+ iNKT cell cross-talk occurs in 2 spatiotemporal distinct phases in the spleen. During the first 6 h, following the capture of nanovaccines by DCs and CD169+
Macrophages at the MZ and in the RP, CD8+ T cells and iNKT cells accumulate in these regions. Recruited CD8+ T cells interact with APCs and concomitantly with iNKT cells. We found that CXCR3 and CCR4 are the key chemokine receptors involved in this complex choreography and these molecules, respectively, promote the generation of MPEC and SLEC.

As previously described, α-Galcer adjuvant exploits an alternative pathway for CD8+ T cell priming, by privileging CCR4 ligands instead of CCR5 ones, in contrast to other adjuvants, such as TLR-L (16). Our work confirms and extends this observation. Next to CCR4/CCL17, we identified the CXCL9/CXCR3 pathway as equally important for cross-priming in the context of α-Galcer. Although CXCL9 has been associated with early CD8+ T cell memory responses (21), our results pinpoint that, in the first place, a fraction of naive CD8+ T cells already

Fig. 7. NKT cells make long-lasting contacts together with OT-I T cells and DC at late stages of activation. (A) Single-cell suspension of splenocytes from CXCR6-GFP mice were stained with CD8, CD3, and NK1.1 antibodies, and Cd1d-α-Galcer dextramer and analyzed by flow cytometry. (B and F) CD8+ OT-I T cells were purified, labeled with CFR dye, and adoptively transferred in CXCR6-GFP mice prior to vaccination. One day later, nanovaccines containing OVA and α-Galcer were intravenously administered in CXCR6-GFP mice. At 0, 6, and 24 h, mice were killed, spleens harvested, and fixed in order to perform cryosections (20 μm) for subsequent immunofluorescent staining. Cryosections were stained with either anti-CD169 or anti-CD11c antibodies. (B) Representative images of the CD169 (red) and CXCR6-GFP (green) co-staining at the different time points. (Scale bar, 50 μm.) (C) Percentage of the CXCR6hi cell localized in the WP at the different time points. (D) Percentage of the clustering of CXCR6hi cells at the different time points. (E) Percentage of OT-I T cells in close contacts (distance inferior to 3 μm) with CXCR6hi cells in the MZ/RP or in the WP at the different time points. (F) Representative images of OT-I (white), CD11c (red), and CXCR6-GFP (green) co-staining at 24 h in the WP. (Scale bar, 10 μm.) (G) Percentage of OT-I T cells in close contacts (distance inferior to 3 μm) with CXCR6hi cells in the MZ/RP or in the WP at the different time points. (H) Percentage of OT-I T cells in close contacts (distance inferior to 3 μm) with CXCR6hi cells in the WP at the different time points. (I) Representative images of OT-I (white), CD11c (red), and CXCR6-GFP (green) co-staining at 24 h in the WP. (Scale bar, 10 μm.) (G) CD8+ OT-I T cells were purified, labeled with CFR dye and adoptively transferred in CXCR6-GFP mice prior to vaccination. Sixteen hours later, nanovaccines containing OVA and α-Galcer or only α-Galcer were intravenously administered in CXCR6-GFP mice. Six and 24 h later, mice were killed, spleens harvested, and embedded in a low-melting agarose gel. Thick sections of 500 μm were performed using a vibratome, and stained with anti-CD169 and anti-CD11c antibodies. Live imaging was performed using a spinning-disc microscope equipped with a thermostated chamber and perfused at a rate of 0.8 mL/min with medium bubbled with 95% O2 and 5% CO2. OT-I and NKT contacts were evaluated on movies lasting 30 min. (H) From analyzed images for the 24-h time point, the positions of NKT cells were extracted. For each picture, 20 runs of simulation were performed in order to randomly change the location of NKT cells. The closest distance between OT-I and NKT cells was measured for every OT-I T cell and the mean of the closest distance between these 2 cells was calculated and then compared to the 1 from the original picture. (I) One day prior to vaccination, anti-CXCR3 blocking antibodies or CCR4 antagonist was injected intraperitoneally into CXCR6-GFP mice. Then, nanovaccines containing OVA and α-Galcer were intravenously administered, and spleens were harvested 24 h after vaccination, and fixed in order to perform cryosections (20 μm) for subsequent immunofluorescent staining with anti-CD169 antibody. NKT cell density (cells/mm2) in the WP was calculated. Results involve at least 2 mice per condition and time point. Statistical analysis by 1-way ANOVA test for C, D, G, and I, and by t test for E and H: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; mean ± SEM.
vested and analyzed by flow cytometry. MPEC were defined as CD127hi
were intravenously administered in mice and 10 d later spleens were har-
traperitoneally. The next day, nanovaccines containing OVA and

One first issue to address is whether these cells directly cross-prime or transfer the antigen to
dC1. We were not able to address this question in our study since CD169+ macrophage isolation was not satisfactory. Indeed, as described by Gray et al. (33), CD169 molecules appear to be acquired by a vast array of cell types after organ digestion both in the LN (25, 34) and in the spleen (35), rendering the purification of pure CD169+ MM unrealizable. We have observed similar features, and thus we could not rely on the apparent CD169 expression in macrophages and DCs. This is an important open issue in particular because numerous studies have analyzed this population based on CD169 expression. Nonetheless, our results suggest that dC1 are primarily responsible for cross-
priming. This notion is supported by the finding that, in early stages, CD8+ T cells are recruited much closer to dC1 than CD169+ MM, and at later stages, long-lasting contacts involve DCs but not CD169+ MM in the WP. However, CD169+ MM might represent a nonnegligible source for chemokines, such as CXCR3 ligands.

Another important question is, how is cross-talk orchestrated between CD8+ T cells and iNKT cells? Does it involve direct contact between 3 different cell types (DCs, CD8+ T cells, and iNKT cells) or sequential spatiotemporal interactions? Our data support the idea that concomitant interactions can occur throughout the 2 stages moving from the RP toward the WP. Although we cannot formally exclude that sequential interactions may happen as well, we show that half of the OT-I T cells express the receptor CXCR3, and moreover, its expression can be increased overtime, as others have described (22). Second, its ligand CXCL9 can be strongly induced not only during recall responses, as has been described (21, 23, 24), but also during primary responses when α-Galcer is used as an adjuvant, as we currently show. Importantly, we report also that CCL17 and CXCL9 expression patterns are very dynamic and follow DC and CD8+ T cell redistribution. These 2 chemokines are produced outside the WP during the first phase and then partly relocalized into this compartment at later stages. This likely can be explained by DCs that produce CCL17 and CXCL9, while migrating from the RP and MZ to the WP, driven by CCR7 up-
regulation (25). In this study, we show that these 2 chemokine pathways play a differential role in promoting T cell memory. In particular, the role of CCR4 in the CD8+ T cell memory generation has never been really evaluated, to our knowledge. In this study, we found that CXCR3 promotes memory T cell generation, whereas CCR4 promote the T cell differentiation into SLECs. Importantly, we obtained similar results with IL-4 blockade, suggesting that CXCR4 might be important to recruit antigen-specific T cells close to IL-4-producing cells in order to promote the generation of SLECs. Conversely, CXCR3 might be important for the T cell recruitment close to IFN-γ-producing cells, promoting thus the generation of memory CD8+ T cells.

A remaining question is, which cells are responsible for the cross-priming? We saw that nanovaccines were rapidly captured at the MZ and in the RP, mainly by CD169+ macrophages and DCs present in these areas. This was not surprising since these cells have previously been shown to capture a wide array of antigen types (24, 26–29), dC1 are considered to be the main cell type involved in cross-priming in vivo (30, 31). However, under certain circumstances CD169+ MM have been shown to fulfill this role as well (10, 11). At this time, it is not clear whether these cells directly cross-prime or transfer the antigen to dC1 (32). We were not able to address this question in our study since CD169+ macrophage isolation was not satisfactory. Indeed, as described by Gray et al. (33), CD169 molecules appear to be acquired by a vast array of cell types after organ digestion both in the LN (25, 34) and in the spleen (35), rendering the purification of pure CD169+ MM unrealizable. We have observed similar features, and thus we could not rely on the apparent CD169 expression in macrophages and DCs. This is an important open issue in particular because numerous studies have analyzed this population based on CD169 expression. Nonetheless, our results suggest that dC1 are primarily responsible for cross-
priming. This notion is supported by the finding that, in early stages, CD8+ T cells are recruited much closer to dC1 than CD169+ MM, and at later stages, long-lasting contacts involve DCs but not CD169+ MM in the WP. However, CD169+ MM might represent a nonnegligible source for chemokines, such as CXCR3 ligands.

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Fig. 8. IL-4/CCL17 axis promotes the generation of short-lived effector cells. (A) Nanovaccines containing OVA and α-Galcer or TLR-L were intravenously administered in mice. At different time points, mice were killed, sera were collected, and ELISA for IL-4 was performed. (B) CD8+ OT-I T cells were iso-
lated, labeled with CFTR, and adoptively transferred prior to vaccination. The next day, nanovaccines containing OVA and α-Galcer were intravenously administered in mice. At 6 or 24 h, mice were killed, spleens harvested, stained, and analyzed by flow cytometry. Representative expression of pSTAT6 on OT-I T cells in control or vaccinated mice at 6 and 24 h. Results are representative of 2 independent experiments. (C and D) CD8+ OT-I T cells were isolated, labeled with CFTR, and adoptively transferred prior to vacci-
nation. One day prior to vaccination, isotype or anti-IL-4 blocking antibodies were injected intraperitoneally. The next day, nanovaccines containing OVA and α-Galcer were intravenously administered in mice and spleens from the 24-h time point were harvested. (C) Expression of CCR4 on OT-I T cells was evaluated by flow cytometry. (D) Expression of CCL17 in spleen cryosections (20 μm) was evaluated by confocal microscopy. (E–H) One-thousand CD8+ OT-I T cells were adoptively transferred prior to vacci-
nation into CD45.1 mice. One day prior to vaccination, isotype, anti-CXCR3 (E and F), or anti-CCL17 or IL-4 antibodies (G and H) were injected in-
traperitoneally. The next day, nanovaccines containing OVA and α-Galcer were intravenously administered in mice and 10 d later spleens were har-
vested and analyzed by flow cytometry. MPEC were defined as CD127hi
KLGR1+ cells and SLEC as CD127lo KLGR1+ cells. Pool from 2 independent experiments with at least 5 mice per condition. Statistical analysis by 1-way ANOVA test for A and t test for C–H. *P < 0.05, **P < 0.01, ***P < 0.001; mean ± SEM.
necessary for the efficiency of the nanovaccine and the generation of short-lived effector cells. It can be hypothesized that the CD8+ T cells in close contacts with iNKT cells might receive additional signals that will impact their subsequent differentiation into effector or memory cells. Indeed, local inflammation cues can clearly influence this process and skew it toward effector cell generation (37). In particular, it is tempting to speculate that additional signals will impact their subsequent differentiation program of neighboring cells.

In summary, our study sheds light on the role of iNKT cells in T cell priming and shows a biphasic process of iNKT cell generation (37). In particular, it is tempting to speculate that local inflammation cues can clearly influence this process and skew it toward effector cell generation (37). Additionally, we found that the IL-4/CCL17/CCR4 axis is necessary for the efficiency of the nanovaccine and the generation of short-lived effector cells. It can be hypothesized that the CD8+ T cells in close contacts with iNKT cells might receive additional signals that will impact their subsequent differentiation into effector or memory cells. Indeed, local inflammation cues can clearly influence this process and skew it toward effector cell generation (37).

**Experimental Procedures**

**Mice.** All animal work was done in accordance with Institutional Animal Care and Use Guidelines of the Central Animal Laboratory (Nijmegen, The Netherlands). All mice were housed in specific pathogen-free conditions before use. Wild-type C57/B6J mice were from the Charles River, OT-I yeti or Rag1-/- mice were bred in house. CXCR6-GFP mice were kindly provided by the Rachel Golub laboratory, Pasteur Institute, Paris, France, and were maintained as heterozygous at the animal facility of the Cochin Institute Paris, France.

**Antibodies and Reagents.** The following antibodies were used for flow cytometry: anti-CD8α (53-6.7), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD127 (A7R34), anti–KLGR-1 (MAFA), anti–mPDCA1 (129C1), anti–Vα2 (B20.1), anti–NK1.1 (PK136), anti–B220 (RA3-6B2), anti–CD11b (M1/70), anti–CD11c (N418), anti–F4/80 (BM8), anti–MHC-II (M5/114.15.2), anti–CCR4 (2G12), anti–CXCR3 (CXCR3-173), anti–XCR1(SET), anti–CD3 (145-2C11), anti–CD5 (53-7.3), and anti–TCRβ (H57-597), all from Biolegend; anti–Vj5.1/5.2 (M99-4) from eBioscience.

The following antibodies were used for microscopy: anti–CD169 (3D6.112), anti–CD11c (N418), anti–CD68 (FA-11), and anti–CXCL9 (MIG-2F5.5), all from Biolegend; anti–mCLEC9a (polyclonal sheep IgG) and anti–mCCL17 (polyclonal goat IgG), both from R&D systems; anti–CD3 (polyclonal rabbit) from Abcam; anti–SIGNR1 from AbD Serotech.

**CD1d–α-GalCer dextramer was obtained from Immudex.** Corresponding secondary antibodies were bought either from Life Technologies (donkey anti-goat) or from Jackson Immunoresearch Laboratories [Goat anti-hamster, Fab’ (2) donkey anti-rat, donkey anti-rabbit, donkey anti-sheep].

PLGA (Resomer RG 502 H, lactide/glycolide molar ratio 48:52 to 52:48) was purchased from Boehringer Ingelheim. Solvents for PLGA preparation (dichloromethane) were obtained from Merck. Polyvinyl alcohol (PVA) was obtained from Sigma. R848 was from Enzo Life Sciences, poly I:C from Sigma-Aldrich, and endotoxin-free OVA from Hyglos. α-GalCer was purchased from Funakoshi. DMSO 99.9%, Triton-X100, and Tween 20 was purchased from Boehringer Ingelheim.

**PLGA Synthesis and Characterization.** PLGA nanovaccines were manufactured and characterized as described previously (17).

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**Fig. 9.** Early cross-talk between iNKT and OT-I T cells is crucial for the generation of short-lived effector cells. CD8+ OT-I T cells were isolated and 1.000 cells were adoptively transferred prior to vaccination. The next day, nanovaccines containing α-GalCer were intravenously administered in mice. At the same time 0 h, 10 h later (+10 h), or 48 h later (+48 h) nanovaccines containing OVA were intravenously delivered. Ten days after NP OVA administration, spleens were harvested and analyzed by flow cytometry. MPEC were defined as CD127hi KLGR1 cells and SLEC as CD127lo KLGR1 cells. (A) Diagram of the experimental set up. (B) Evaluation of OT-I T cell counts relative to the CD8+ T cell population. (C) Representative staining of MPEC and SLEC by flow cytometry at day 10. (D and E) Percentage (Left) or relative counts (Right) of MPEC (D) or SLEC (E). Statistical analysis by t test: *P < 0.05; mean ± SEM.
Cell Suspension and Flow Cytometry. Spleens were harvested and digested with DNase I (ThermoFisher) and Collagenase III (Worthington) at 37 °C for 30 min. After digestion, a mechanical disruption and a passed over 100-μm cell strainers (Corning). Red blood cell lysis was performed with 2 mL of 1× ammonium chloride solution for 5 min at room temperature. Cell suspension was then stained with fluorescently labeled antibody in PBA (PBS 1% BSA 0.01% sodium azide) for 20 min at 4 °C in presence of CD16/CD32 blocking (24G2 BD Pharmedics). For NKt staining, cells were incubated 10 min with fluorescently labeled CD1d-iGaLCer dextramer at room temperature in PBA prior incubation with fluorescently labeled antibodies. Cells were analyzed with FACS verse, FACS LSRII, or FACS Fortessa (BD). Results were analyzed with Flowjo (Tree Star).

Adoptive Cell Transfer and Vaccination Experiments. OT-I CD8+ T cells were isolated from spleen and peripheral LNs by a negative selection kit (CDBi isolation kit mouse Miltenyi) with added biotinylated anti-CD44 antibodies according to Greter et al. (38). Purity was routinely superior to 95%. OT-I cells were labeled with either CTV (Life Technologies) or CFR (Life Technologies) with a dye concentration of 5 μM following the manufacturer's procedure. Next, 1 to 2 × 10^6 dye-labeled OT-I CD8+ T cells were adoptively transferred into congenic wild-type mice, followed by intravenous injection of nano-vaccine 1 d later with a dose corresponding to 1 μg OVA, 1.5 ng sGαLCer, 215 ng R848, and 105 ng poly I:C. For the blocking experiment, 500 μg anti-CXCR3 blocking antibodies, 300 μg of IL-4 or CCL17 antibodies, or their corresponding isotype (Bioxcell/R&D systems) were injected intraperitoneally 1 d prior to vaccination. Alternatively, 2.5 μg of CCR4 antagonist (CAS 864289-85-0 Cayman) or vehicle (0.05% DMSO) was injected intraperitoneally 1 h at 42 °C, then the reverse-transcriptase was heat-inactivated for 10 min at 70 °C. Finaly, amplified RNA was purified with RNAClean XP beads, resuspended in nuclease-free water, and stored at −80 °C prior use.

CTV of OT-I cells was produced by a reverse-transcriptase reaction using random hexamer amplification for 1 h at 42 °C and the enzyme was subsequently heat-inactivated 10 min at 70 °C. CDNA was then used for gene arrays (mouse Cytokines & Chemokines RT2 Profiler PCR Array for cdc or mouse Chemokines & Chemokine Receptors RT2 Profiler for OT-I T cells, Qiagen), or with manual qPCR with SYBR green reaction. Samples were read on Bio-Rad CFX96. Cell cluster analysis was performed with Qiagen analysis software.

Cytofluorometry and Confocal Microscopy. Spleens were fixed with 4% PFA for 2 h on ice. Successive baths of 10%, 20%, and 30% fructose were performed at 4 °C for 4 h. Organs were then embedded in OCT (Sakura), snap-frozen, and stored at −80 °C. Ten- to 20-μm cryosections were performed on a cryotome (Microm HM 500 Cryostat) at temperatures between 20 °C and −21 °C. For immunostainings, residual PFA was quenched with PBS 0.1M Glycine for 5 min at room temperature, sections were then stained overnight at 4 °C with primary antibodies in PBA 0.1% Triton-X100. After washing with PBA, sections were then stained in PBA with secondary antibodies 1 h at 4 °C, washed, and then counterstained with DAPI (Sigma) if needed. Sections were finally washed in PBS and then in milliQ and mounted with a coverslip and Prolong Antifade Diamond mounting medium (Life Technologies). Confocal microscopy was performed with Olympus FV1000 or Zeiss LSM880 with 20x or 40x objectives. Pictures were then analyzed with ImageJ. To analyze NP uptake or chemokine expression, a background threshold was applied to quantify the total fluorescence intensities. Concerning the calculation for fluorescence intensities per square micrometer, fluorescence background was subtracted and then pictures were analyzed. Percentage of cells per area was performed on the total of cells in the whole section. Cells with a minimal distance shorter than 3 μm were considered to be in close contact.

Seeds and Imaging. Spleens were embedded in a solution of 7.5% low gelling-temperature agarose (type VII-A; Sigma-Aldrich) prepared in PBS. Slides (500 μm) were cut with a vibratome (VT 1000S, Leica) in a bath of ice-cold PBS. Slices were then transferred to 0.4-μm organotypic culture inserts (Millicell; Millipore) in 35-mm Petri dishes containing 1 mL RPMI 1640 without Phenol red. Live vibratome sections were stained for 15 min at 37 °C with antibodies. All antibodies were diluted in RPMI without Phenol red and used at a concentration of 10 μg/mL. Tissue sections were imaged with a DMS500 upright microscope equipped with an upright spinning-disc confocal microscope (Leica) in a 37 °C thermostated chamber. For dynamic imaging, tumor slices were secured with a stainless steel slice anchor (Warner Instruments) and perfused at a rate of 0.8 μL/min with a solution of RPMI without Phenol red, bubbled with 95% O2 and 5% CO2. Next, 10x or 25x water-immersion objectives were used and 30-min movies of different areas were recorded. Velocity was calculated using the plugin Manual Tracking from ImageJ. For CD1d+iGaLCer staining, thick sections were stained in PBA for 30 min at room temperature, and then 2 h on ice with CD169 and CD11c antibodies. Thick sections were then fixed 20 min at room temperature with 4% PFA, washed with PBS, and then imaged by confocal microscopy.

In Vitro Culture. For in vitro culture, 50 × 10^6 purified OT-I CD8+ T cells were cultured in a 96U-well plate with a complete medium (RPMI 1640 supplemented with 10% FCS, nonessential amino acid [Gibco], L-glutamine [Gibco], anti-anti [Gibco], sodium pyruvate [Gibco], Heps, and β-mercaptoethanol)
at 37 °C for 5 or 24 h. Concentrations of SIINFEKL and IL-4 were, respectively, 1 μg/mL and 10 ng/mL.

**Statistical Analysis.** For statistical analysis, 1-way ANOVA or Mann–Whitney t test was used with Graphpad: *P* < 0.05, **P** < 0.01, ***P*** < 0.001, and ****P*** < 0.0001.

**Data Availability.** All data supporting the findings of this study have been deposited in Figshare.


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