The nuclear receptor LXRα controls the functional specialization of splenic macrophages

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Macrophages are professional phagocytic cells that orchestrate innate immune responses and have considerable phenotypic diversity at different anatomical locations. However, the mechanisms that control the heterogeneity of tissue macrophages are not well characterized. Here we found that the nuclear receptor LXRα was essential for the differentiation of macrophages in the marginal zone (MZ) of the spleen. LXR-deficient mice were defective in the generation of MZ and metallophilic macrophages, which resulted in abnormal responses to blood-borne antigens. Myeloid-specific expression of LXRα or adoptive transfer of wild-type monocytes restored the MZ microenvironment in LXRα-deficient mice. Our results demonstrate that signaling via LXRα in myeloid cells is crucial for the generation of splenic MZ macrophages and identify an unprecedented role for a nuclear receptor in the generation of specialized macrophage subsets.

Macrophages have long been recognized as key components of cellular immunity. As sentinels of the immune system distributed throughout the body, macrophages ensure tissue integrity by scavenging altered host components, foreign materials and organisms1. Macrophages direct antimicrobial actions through the production of cytotoxic species, cytokines and chemokines that are required for the initiation and persistence of acute and chronic inflammation. Macrophages also contribute to immunosuppression and self-tolerance by removing apoptotic cells under noninflammatory conditions3.

Tissue macrophages have been traditionally categorized as part of the mononuclear phagocyte system and have been generally considered to be derived from circulating monocytes or myeloid progenitor cells4. Monocytes and resident macrophages are largely dependent on macrophage colony-stimulating factor-1 and its receptor for their differentiation and/or survival5. Tissue macrophages show a high degree of heterogeneity, a feature that reflects the individual functional specialization of specialized macrophage populations at different anatomical locations4,5. The functional diversity of macrophage populations is presumably determined by local signals present only under specific developmental, homeostatic or pathological tissue conditions4,5. However, the molecular programs that control macrophage specialization and function in different tissues remain poorly defined.

The spleen is the largest secondary lymphoid organ in the body and is important for the generation of immune responses to blood-borne antigens and for filtering the blood of senescent cells or potentially noxious material7. Those functions are linked to the phagocytic ability of specific macrophage subpopulations that are compartmentalized into different splenic domains, including the red pulp (RP), the marginal zone (MZ) and the white pulp (WP)1,7,8. RP macrophages, defined by expression of the markers F4/80 and CD68, are critical for the phagocytosis of effete red blood cells and contribute to iron recycling5. In contrast, tingible body macrophages in the WP of the spleen have lower expression of F4/80 but abundant expression of CD68 and MFG-E8 (an opsonin that binds to phosphatidylserine on apoptotic cells) and are involved in the engulfment of apoptotic lymphocytes generated during immunological reactions in the germinal center9. The MZ is located at the interface of the WP and the RP where most of the arterial blood that enters the spleen can be screened by several resident cell types, including two subsets of macrophages and a population of MZ B cells8,10. Macrophages in this area form two concentric rings and present a special combination of surface receptors: MZ macrophages are characterized by the expression of SIGN-R1 and MARCO, whereas metallophilic macrophages express CD169. Given their strategic position and their unique expression

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of pattern-recognition receptors, MZ macrophages are believed to participate in many aspects of host immunity. The molecular and transcriptional regulators responsible for the generation of MZ macrophages remain undefined.

The liver X receptors (LXRs) LXRα (encoded by Nr1h3) and LXRβ (encoded by Nr1h2) are members of the nuclear receptor superfamily of transcription factors with key roles in the control of sterol homeostasis. LXRβ has ubiquitous expression, and LXRα has high expression in the liver, adipose tissue, intestine and differentiated macrophages. In macrophages, which have high expression of both LXRα and LXRβ, the two receptors transactivate a similar gene program, except for the LXRα-specific target Cds1. LXRs also regulate gene expression through a process known as ‘transrepression’ and are important for the transcriptional responses of macrophages during the engulfment of apoptotic cells. Loss of LXRα and LXRβ in mice results in peripheral lipid accumulation and signs of overreaction of the immune system that become aggravated over time and culminate in systemic autoimmunity. As both LXRs activate and repress similar genetic programs in macrophages, it has been assumed that their functions in macrophages are largely redundant. Here we found that expression of LXRα, but not of LXRβ, in myeloid cells was crucial for the development of macrophages in the MZ of the spleen. Our results identify an unexpected role for a nuclear receptor in the genesis of tissue macrophage diversity.

RESULTS

Absence of MZ macrophages in LXR-deficient mice

To investigate a possible role for LXRs in the development of resident macrophages, we obtained tissue sections from wild-type mouse and mice lacking both LXRα and LXRβ (Nr1h3−/− Nr1h2−/−; collectively called ‘LXR-deficient’ here) and screened them by immunohistochemical analysis with monoclonal antibodies that recognize macrophage surface antigens. Expression of the markers F4/80 and CD68 was similar in wild-type and LXR-deficient tissues (Supplementary Fig. 1). Unexpectedly, we found a complete absence of CD169+ macrophages in the spleens of LXR-deficient mice (Supplementary Fig. 1). In contrast, we detected macrophages with CD169 expression in several other tissues of LXR-deficient mice, such as lymph nodes, Peyer’s patches, liver and intestine (Supplementary Figs. 1 and 2a). To further evaluate the effect of LXR expression on the development of the splenic macrophage repertoire, we used a combination of flow cytometry, immunofluorescence and immunohistochemical approaches. Expression of the surface markers F4/80, CD11b and CD68 by splenic macrophages and the microanatomical location of these cells were similar in wild-type and LXR-deficient mice (Fig. 1a,b), which suggested that LXR signaling was not involved in the development of RP or WP macrophages under steady-state conditions. However, the distinctive microenvironment of the splenic MZ was altered considerably in LXR-deficient mice (Fig. 1a,c). Indeed, expression of CD169, MARCO, SIGN-R1 and TIM-4 (a glycoprotein that binds phosphatidylserine in apoptotic cells) was selectively lost in LXR-deficient spleens (Fig. 1a−c).

Such dysregulation of those phagocytic markers could reflect the fact that their expression might be transcriptionally controlled by LXR signaling or that the cells expressing those proteins could be absent from LXR-deficient spleens. Expression of mRNA encoding MARCO, SIGN-R1 and CD169 was similar in LXR-deficient and wild-type (control) peritoneal macrophages (Fig. 2a). In addition, the MZ space between the RP (containing cells positive for F4/80 or the endothelial ligand VCAM-1) and the border of the WP (delineated by cells positive for laminin) was altered considerably in LXR-deficient mice (Fig. 2b).
Mature and function of both cell types in the spleen. MZ B cells and capture of blood-borne antigens suggested that the lack of MZ macrophages in LXR-deficient mice. Next we generated hematopoietic chimeras by transplanting bone marrow from wild-type or LXR-deficient donor mice into irradiated recipients. These findings suggested that the lack of MZ macrophages in LXR-deficient mice resulted from an intrinsic hematopoietic defect.

MZ B cells and capture of blood-borne antigens

Published work has suggested that crosstalk between MZ macrophages and a population of non-recruiting MZ B cells is important for the retention and function of both cell types in the spleen. MZ B cells are characterized by their location and CD1d+IgMloCD21hiCD23loIgDlo expression pattern. To evaluate the effect of the lack of MZ macrophages on the localization and frequency of B cells in the MZ, we compared wild-type and LXR-deficient spleen sections and cell suspensions by immunofluorescence, immunohistochemistry and flow cytometry. Unexpectedly, the B220+CD1d+IgMhi B cell population (which normally resides outside the WP) was confined to the inner border of the follicles in LXR-deficient spleens. LXR-deficient spleens had a smaller fraction of B220+CD1d+IgMhi MZ B cells than wild-type (control) spleens had. As MZ B cells have been functionally linked to immune responses to thymus-independent antigens, we evaluated the response of wild-type and LXR-deficient mice to trinitrophenyl (TNP)-conjugated Ficoll, a type 2 thymus-independent antigen. LXR-deficient mice had significantly lower titers of immunoglobulin M (IgM) in response to immunization with TNP-Ficoll than their wild-type (control) counterparts. These results indicated that LXR signaling was important for the retention of MZ B cells in their anatomical splenic compartment and also participated in early IgM responses to thymus-independent antigens.

Because rapid clearance of blood-borne antigens by MZ macrophages is important in preventing the dissemination of potentially harmful agents, we investigated the ability of LXR-deficient mice to recognize and clear blood antigens in the absence of MZ macrophages. We found that a panel of intravenously injected bacterial or yeast components, inactivated microorganisms and opsonized particles were efficiently captured by splenic macrophages and generally localized together with MZ macrophages in wild-type mice. In contrast, circulating antigens aberrantly ‘percolated’ into the RP of LXR-deficient mice and seemed to be widely dispersed and associated with F4/80+ macrophages. These data showed that intact LXR signaling was required for the generation of an efficient network of scavenging macrophages in the spleen.
Development of MZ macrophages depends on LXRα signaling

To determine if the defect in MZ macrophages observed in LXR-deficient mice was dependent on one of the two LXRs, we analyzed sections from wild-type, LXRα-deficient (Nr1h3−/−), LXRβ-deficient (Nr1h2−/−) and LXR-deficient spleens by a combination of immunofluorescence and immunohistochemistry. Unexpectedly, LXRα-deficient mice showed selective ablation of MZ macrophages that was almost completely identical to that observed in LXR-deficient mice (Fig. 5a, top). Moreover, immunohistochemical and immunofluorescence analysis showed that the establishment of the MZ space between the WP and RP areas was also LXRα dependent (Fig. 5a, middle and bottom, and Supplementary Fig. 5). Consistent with those observations, transplantation of LXRβ-deficient bone marrow into LXR-deficient recipients led to recovery of the MZ space and MZ macrophages, whereas LXRα-deficient donor bone marrow did not restore the MZ microarchitecture (Fig. 5b, top and middle). Moreover, transplantation of LXRβ-deficient bone marrow into LXR-deficient mice restored the efficient capture of blood-borne antigens and the induction of IgM responses, coincident with the replenishment of MZ macrophages, but transplantation of LXRα-deficient bone marrow did not (Supplementary Fig. 6). Notably, reconstitution with either LXRα-deficient bone marrow or LXRβ-deficient bone marrow resulted in less accumulation of neutral lipid in LXR-deficient spleens (Fig. 5b, bottom), which indicated that whereas LXRα was uniquely required for the development of MZ macrophages, regulation of cholesterol metabolism in the spleen was controlled by either LXRα or LXRβ.

To confirm the importance of hematopoietic signaling LXRα in the generation of MZ macrophages, we created a mouse line with selective ablation of LXRα in hematopoietic cells (Supplementary Fig. 7). We crossed mice homozygous for loxP-flanked Nr1h3 alleles (Nr1h3fl/fl) with mice with transgenic expression of Cre recombinase under the control of the hematopoietic compartment–specific Vav1 promoter (Vav-Cre)24. The efficiency of deletion of Nr1h3 in Nr1h3fl/flVav-Cre+ mice (the offspring with hematopoietic LXRα deficiency), assessed as mRNA, was over 95% in bone marrow and spleen (Supplementary Fig. 7b). We assessed expression of the LXRα-specific target Cds225 to confirm the loss of LXRα activity in the spleen and bone marrow of Nr1h3fl/flVav-Cre+ mice (Supplementary Fig. 7b). Immunoblot analysis with an LXRα-specific antibody26 (Supplementary Fig. 8) confirmed substantial absence of LXRα from macrophages and the spleen (but only a minimal effect in the liver) of Nr1h3fl/flVav-Cre+ mice (Fig. 6a, top, and Supplementary Fig. 7b). The development of F4/80+ RP macrophages was unaffected in Nr1h3fl/flVav-Cre+ mice, but there was complete lack of MZ macrophages (Fig. 6a, bottom).

To gain insight into the cellular mechanisms controlled by LXRα signaling that lead to the development of MZ macrophages, we overexpressed LXRα in hematopoietic stem cells (HSCs) through the use of LXRα transgenic mice. While LXRα expression in HSCs was impaired by spleen transplantation, similar to wild-type spleen transplantation, transplantation of LXRα transgenic bone marrow into LXRα-deficient recipients led to recovery of the MZ space and MZ macrophages (Supplementary Fig. 8).
Figure 5 MZ macrophage differentiation is specifically controlled by LXRα. (a) Immunofluorescence analysis (top) of wild-type, LXRα-deficient (LXRα-KO), LXRβ-deficient (LXRβ-KO) and LXR-deficient spleen sections stained with anti-CD169, anti-SIGN-R1 and anti-F4/80, and immunohistochemical analysis of F4/80+ RP macrophages (brown) and laminin-positive endothelial cells (blue; middle) and of MARCO+ cells (blue) and CD169+ cells (brown; bottom) in consecutive sections. (b) Immunohistochemical localization (top and middle) of MZ macrophages (MARCO+ blue and CD169+ brown), and F4/80+ (brown) of bone marrow (above: donor→recipient). Bottom, staining of spleen sections from the mice above with oil red O. Scale bars, 50 μm. Data are representative of four independent experiments with three to five mice per group (a) or two independent experiments with six mice per group (b).

of a myeloid-specific lentiviral system27,28. For this, we enriched bone marrow for HSCs, transduced the HSCs with myeloid cell–specific lentivirus encoding green fluorescent protein (GFP) and transplanted the transduced HSCs into recipient mice. We found that at 8–12 weeks after transplantation, around 40% of blood monocytes obtained from the recipient mice were GFP+ (Fig. 6b, top, and data not shown). Next we used lentivirus expressing either GFP or LXRα to transduce HSC-enriched bone marrow obtained from LXRα-deficient donor mice and reconstituted LXRα-deficient recipient mice with that bone marrow. Analysis of spleen sections showed partial recovery of cells that expressed CD169, MARCO and TIM-4 in the MZ compartment in mice given transplantation of cells transduced with LXRα-expressing lentiviruses (Fig. 6b). These results indicated that LXRα expression in hematopoietic cells was important for the generation of MZ macrophages.

Monocytes replenish MZ macrophages in LXRα-deficient mice

Although it is generally assumed that bone marrow–derived monocytes give rise to macrophages in vivo4, a direct link between emigrated monocytes and the differentiation of splenic MZ macrophages under steady-state conditions has not yet been established5. We evaluated whether the frequency of the classical CD115+Ly6Chi and nonclassical CD115+Ly6Clo monocyte subsets might be altered in LXRα-deficient mice. Analysis by flow cytometry demonstrated that CD115+Ly6Chi and CD115+Ly6Clo populations were present at similar frequencies in the bone marrow and blood of wild-type and LXRα-deficient mice (Fig. 7a). Unexpectedly, LXRα-deficient mice had more splenic monocytes than did wild-type mice, an effect that was restricted to the nonclassical CD115+Ly6Clo subset (Fig. 7a). Thus, an absence of monocytic precursor cells could not account for the absence of MZ macrophages in LXRα-deficient mice.

Next we assessed the potential of mature monocytes to drive the generation of MZ macrophages under steady-state conditions. We first analyzed the ability of intravenously transferred bone marrow...
Figure 7 Adaptive transfer of monocytes reconstitutes the splenic MZ microenvironment in LXRα-deficient mice. (a) Flow cytometry (top) of monocyte subsets of wild-type and LXRα-deficient cells from bone marrow (BM), blood and spleen, detecting classical (CD115+Ly6C<sup>hi</sup>) and nonclassical (CD115+Ly6C<sup>lo</sup>) monocytes by the expression of Ly6C and CD62L by CD115<sup>+</sup> cells in bone marrow and blood and by the expression of Ly6C by CD115<sup>+</sup> F4/80<sup>+</sup> cells in spleen. Below, frequency of various subsets of monocytes (keys) in bone marrow, blood and spleen, measured by flow cytometry. (b) Immunofluorescence analysis of consecutive spleen sections from nonirradiated LXRα-deficient mice without reconstitution (control; left) or reconstituted with monocytes (mo) from wild-type spleen (middle) or bone marrow (right). Arrows indicate RP-WP borders. Scale bars, 50 μm. Data are representative of four independent experiments (a; mean ± s.e.m. of seven mice) or three independent experiments with five to six mice per group (b).

Because Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> monocytes have been proposed to serve distinct roles in inflammation<sup>a</sup>, we used our LXR<sup>α</sup>-deficient model to investigate whether these monocyte subsets had a difference in their ability to generate MZ macrophages. We found that CD115<sup>+</sup>CX3CR1<sup>hi</sup> and CD115<sup>+</sup>CX3CR1<sup>lo</sup> monocytes from the bone marrow (corresponding to Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> subsets, respectively) promoted the development of MZ macrophages with similar efficacy (Supplementary Fig. 9a). Furthermore, purified Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> monocytes from the spleen had equivalent expression of mRNA encoding LXRα, LXRβ and the transporter ABCA1 (but not of mRNA encoding the chemokine receptor CCR2, used as a control to distinguish Ly6C<sup>hi</sup> versus Ly6C<sup>lo</sup> monocytes; Supplementary Fig. 9b). These results suggested that classical and nonclassical monocytes may have redundant roles in the differentiation of certain monocyte subsets under steady-state conditions. However, as Ly6C<sup>hi</sup> cells have been shown to generate Ly6C<sup>lo</sup> monocytes<sup>29,30</sup>, we cannot exclude the possibility that Ly6C<sup>lo</sup> monocytes were the immediate precursors of MZ macrophages in LXRα-deficient mice in our experimental model.

Activation of LXRα accelerates MZ macrophage development

Scattered macrophages can be detected during the late phases of the embryonic development of mouse spleen, but the organization and specialization of separate splenic macrophage compartments occurs during the first weeks after birth<sup>7,8</sup>. We studied the expression of macrophage markers in wild-type and LXR-deficient mice during the postnatal transition of the spleen until completion of its full mature appearance. Examination of spleen sections obtained from mice 1–4 weeks of age showed that despite the equal abundance of F4/80<sup>+</sup> and CD68<sup>+</sup> cells in wild-type and LXR-deficient mice, MZ macrophages did not appear during spleen development in LXR-deficient mice (Fig. 8a and data not shown). Thus, LXR signaling was selectively required for the differentiation of MZ macrophages during maturation of the spleen. Notably, the postnatal development of MZ macrophage subpopulations correlated with a 10- to 12-fold increase in the expression of mRNA encoding LXRα but no change in the expression of mRNA encoding LXRβ (Fig. 8b).

Finally, we assessed the effect of genetic or pharmacological activation of LXRα on the development and function of MZ macrophages. Analysis of spleen samples from 3-week-old mice with transgenic expression of constitutively active LXRα (with the transcription-activation domain VP16 linked to LXRα expressed from the macrophage- and adipose-specific aP2 promoter (aP2-VP16-LXRα<sup>31</sup>) in macrophages and adipocytes showed accelerated formation of the MZ microenvironment, with more MZ macrophages in the mature position than in their wild-type control counterparts (Fig. 9a). Consistent with these histological changes, the spleens of aP2-VP16-LXRα mice had higher expression of SIGN-R1 and CD169 as well as of the established LXR target ABCA1 (Fig. 9a). As aP2 expression in monocytes is restricted to Ly6C<sup>lo</sup> monocytes<sup>32</sup>, it was possible that VP16-LXRα activity in the Ly6C<sup>lo</sup> subset contributed to accelerated formation of MZ macrophages in this model.

We also evaluated whether LXR activity is important for the functions of the MZ compartment through use of the synthetic LXR agonist
GW3965 in vivo in three different assays: the phagocytosis of dextran linked to fluorescein isothiocyanate; the IgM response to TNP-Ficoll; and the generation of MZ B cells. Analysis of those activities showed no difference between wild-type mice treated with GW3965 and their control counterparts treated with the vehicle dimethyl sulfoxide (data not shown), which indicated that supraphysiological activation of LXR did not alter MZ macrophage function in adult mice. We also investigated whether pharmacological activation of LXRα altered the replenishment of splenic macrophages after experimental depletion of tissue macrophages through the use of clodronate-containing liposomes. Such acute depletion of phagocytes provides a model with which to analyze the progressive renewal of splenic macrophages in adult mice, a process that required 6–8 weeks for full restoration of all subtypes (data not shown). We then used the same model combined with administration of GW3965 to LXRα-deficient or LXRβ-deficient mice to activate exclusively LXRβ or LXRα, respectively.

**Figure 8** LXR signaling is necessary for the postnatal development of splenic MZ macrophages. (a) Immunohistochemical analysis of RP macrophages (F4/80+) and MZ macrophages (MARCO+) in spleen sections obtained from wild-type and LXR-deficient mice 1–4 weeks after birth (time, above images). Scale bars, 50 µm. (b) Expression of mRNA encoding LXRA, LXRB, CD169 and MARCO in spleens from wild-type and LXRA-deficient mice 1–28 d after birth (horizontal axes; results normalized as in Fig. 2a and presented relative to those of wild-type spleens). Data are representative of three independent experiments with three to four mice per genotype (a) or two independent experiments (b; mean and s.e.m. of six mice per genotype).

**Figure 9** Activation of LXRA accelerates the development and renewal of MZ macrophages. (a) Immunofluorescence analysis (left) of spleen sections from 3-week-old wild-type and aP2-VP16-LXRA mice, stained with anti-MARCO, anti–SIGN-R1 (MZ macrophages), anti-CD169 (MZ metallophilic macrophages) and anti-laminin (endothelial cells). Scale bars, 25 µm. Right, abundance of mRNA encoding ABCA1, CD169, SIGN-R1 and MARCO in spleen samples from 3-week-old wild-type and aP2-VP16-LXRA mice (n = 5 per genotype; results normalized as in Fig. 2a and presented relative to those of wild-type spleens). (b) Immunohistochemical analysis of CD169, MARCO and F4/80 in spleen sections from LXRβ-deficient and LXRA-deficient mice left macrophage sufficient and then left untreated (PBS Ctrl) or depleted of macrophages through the use of clodronate-containing liposomes (Clo-lip) and assessed 48 h later without further treatment (Clo-lip 48 h) or, 2 weeks later, treated for 1 week with dimethyl sulfoxide (Clo-lip 3 weeks + DMSO) or GW3965 (Clo-lip 3 weeks + GW3965). Scale bars, 150 µm. Data are representative of two independent experiments with three to five mice per group (a; mean and s.e.m.) or five to six mice per group (b).
Mice treated intravenously with clodronate-containing liposomes showed general depletion of splenic macrophages 48 h after treatment (Fig. 9b), as expected. We allowed separate cohorts of LXRα-deficient or LXRβ-deficient mice to recover for two weeks after injection of clodronate-containing liposomes and then treated them for one additional week with GW3965 or vehicle control. Activation of LXRβ was unable to promote the development of MZ macrophages in the absence of LXRα (Fig. 9b). In contrast, activation of LXRα resulted in a greater frequency of CD169+ and MARCO+ MZ macrophages than that in control mice treated with vehicle (Fig. 9b). Replenishment of F4/80+ macrophages was not altered by the activation of LXRα or LXRβ under these conditions (Fig. 9b). Notably, because activation of LXRα did not promote the expression of MZ markers in other splenic subsets (Fig. 9b), our results suggested that specific signals at the interface of the WP and RP were also required for the generation of MZ macrophages. Collectively, our results established that LXRα was crucial for the generation of a specific subset of splenic phagocytes under developmental or homeostatic conditions.

DISCUSSION

Tissue macrophages are a heterogeneous group of cell subsets found in all organs that have distinct surface markers and specialized functions depending on the local microenvironment. Several transcription factors, including PU.1, C/EBPα and KLF4, have important roles in the differentiation of the monocye-macrophage lineage in vitro and in vivo. However, models of genetic deficiency in those factors often show profound effects on multiple cell types. Thus, the transcriptional programs that control the generation of particular resident macrophage subsets remain largely unexplored. We have shown here that myeloid LXRα signaling was directly involved in the development of specific macrophage subsets important for the efficient capture of circulating antigens. To our knowledge, LXRα is the first molecule shown to be selectively required for the development of all MZ macrophages in the steady state. Our data have identified an additional physiological role for LXRα in myeloid cells driving the differentiation of splenic MZ macrophages. LXRα signaling in monocytes or myeloid progenitors was required for the production of MZ macrophages not only during the neonatal development of the spleen but also throughout the continuous renewal of these cell subsets in adult life.

Loss of MZ macrophages in LXRα-deficient mice had many consequences for spleen architecture and physiology. First, the much smaller space between the RP and WP in the MZ of LXRα-deficient mice affected the retention of MZ B cells that unexpectedly relocalized to the inner part of the follicle. Although reciprocal crosstalk between myeloid cells and B cells is reported to maintain MZ integrity, our results showed MZ B cells in LXRα-deficient mice would constitute a permissive environment for many circulating pathogens.

Consistent with inadequate recognition of circulating antigens, the early IgM response to TNP-Ficoll was impaired in LXRα-deficient mice. Because the expression of markers of the marginal sinus endothelium was normal in LXR-deficient mice, we conclude that the vascular network developed properly in these mice. As a consequence, blood arriving from the central arterioles drained directly into the RP and possibly reached other tissues with uninspected material. It is likely that the deficiency in MZ macrophages described here contributes to the susceptibility of LXRα-deficient mice to Listeria monocytogenes or Mycobacterium tuberculosis. The published studies and our results here, we propose that signaling via LXRα represents an integral component of the systemic antimicrobial immunity, at least in part, through regulation of the development of splenic MZ macrophages.

Although the relevance of the diversity of tissue macrophages has been recognized for decades, the origin of mononuclear phagocyte cells has recently received considerable attention. Whether tissue-resident macrophages derive from common hematopoietic precursors or from yolk sac-derived precursors is a matter of intense research. There is also evidence that macrophages can self-renew locally independently of their bloodstream precursors. Although similar questions remain about the origin and renewal of mouse splenic macrophages, the many different subsets that reside in the spleen and the fact that the spleen continues to function as a hematopoietic organ in adult rodents add further complexity to this issue. Our results provide the first demonstration to our knowledge that a single nuclear receptor controls the differentiation of the splenic MZ macrophage population through myeloid-restricted signaling.

Several lines of evidence suggested that the lack of MZ macrophages in LXRα-deficient mice resulted from a defective sublineage developmental step. Studies of bone marrow transplantation or conditional hematopoietic deletion of LXRα indicated that LXRα-expressing myeloid cells gave rise to MZ macrophages. A complementary gain-of-function approach with a macrophage-specific lentivirus confirmed that LXRα expression in myeloid cells was needed to reprogram target cells with the appropriate MZ-differentiating potential in vivo. These data also indicated that the development and organization of RP and WP macrophages in the spleen took place independently of the formation of the MZ and of LXRα signaling. Notably, published studies have demonstrated impaired differentiation of F4/80+ macrophages but normal development of MZ macrophages in mice deficient in the transcription factor Spi-C. We interpret the correlation between LXRα expression and splenic macrophage compartmentalization as evidence that supports the proposal of a separate pathway that controls the generation of MZ macrophages.

Although monocytes contribute to the greater abundance of tissue macrophages in several pathological settings, the monocytic origin of resident macrophages in the steady state has not been clearly established. We have shown that adoptively transferred wild-type monocytes partially reconstituted the MZ microenvironment in LXRα-deficient mice. Whereas classical and nonclassical monocytes have distinct roles in inflammation, partial replenishment of the vacant MZ microenvironment in LXRα-deficient mice by monocytes under homeostatic conditions was accomplished by both Ly6Chigh monocytes and Ly6Clow monocytes. Our data indicated that both circulating monocytes and local splenic monocytes were able to function as precursors of MZ macrophages under conditions of LXRα deficiency. Our findings do not exclude the possibility that MZ macrophages in wild-type mice may be derived from embryonic precursors under homeostasis without contributions from circulating monocytes, as demonstrated for other tissue-resident populations.

Finally, we found that enhancement of LXRα activity during macrophage renewal in mature mice led to an accelerated MZ macrophage-differentiation program. Thus, intrasplenic induction and activation of LXRα in myeloid cells is crucial for the generation and maintenance of MZ macrophages both during development and in adult life. We hypothesize that the instructive signals that drive the full maturation of monocytes into MZ macrophages and upregulate LXRα expression in the spleen should be released at the neonatal stage.
and remain present throughout adult life. As oxyestrogens have been shown to influence various functions of cells of the immune system in vivo, including proliferation and migration\(^{45–47}\), we are tempted to speculate that spleen-specific sterol derivatives have a role in activating LXR\(\alpha\)- and LXR\(\beta\)-deficient macrophages during differentiation into splenic macrophages.

**METHODS**

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

N.A.-G., J.A.G., G.G. and M.D. designed and did experiments and analyzed data; J.V.d.L.R., I.H.H., M.C.-A., F.L., C.T. and S.B. did experiments; C.H., P.G.L., M.A., S.A., T.M., S.L., A.L.C., P.T. and A.H. provided reagents and intellectual input and analyzed or interpreted data; N.A.-G. and A.H. contributed to the writing of the manuscript; and A.C. supervised the project, designed and did experiments, analyzed data and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Mice were maintained under pathogen-free conditions in a temperature-controlled room and a 12-hour light-dark cycle. Chow and water were available ad libitum. LXRα- and LXRβ-sufficient wild-type (Nr1h3+/+; Nr1h2+/+) mice, LXRα-deficient (Nr1h3−/−) mice, LXRβ-deficient (Nr1h2−/−) mice and LXR-deficient (Nr1h2+/−;Nr1h3−/−) mice on a mixed Sn129/C57Bl/6 and C57Bl/6 background (backcrossed more than ten generations) were originally obtained from D. Mangelsdorf46. Nr1h3+/− mice on the C57Bl/6 background were obtained from Institut Clinique de la Souris (Illkirch, France); C57Bl/6 mice with transgenic expression of Yav-Cre were obtained from D. Kiousis24 through a collaboration with Susana Gonzalez (CNIC, Spain); ap2–VP16LRα mice (with transgenic expression of constitutively active VP16-LXRα in macrophages and adipocytes) have been described31; mice with sequence encoding GFP or DsRed under the control of the Actb promoter (encoding β-actin) were from the Jackson Laboratory. All animal studies were conducted in accordance with the Animal Research Committees of the Consejo Superior de Investigaciones Científicas and Universidad de Las Palmas de Gran Canaria.

Reagents and antibodies. The synthetic ligands of LXR (GW3965) were from the Jackson Laboratory. All animal studies were conducted in a temperature-controlled room and a 12-hour light-dark cycle. Chow and water were available ad libitum. LXRα- and LXRβ-sufficient wild-type (Nr1h3+/+; Nr1h2+/+) mice, LXRα-deficient (Nr1h3−/−) mice, LXRβ-deficient (Nr1h2−/−) mice and LXR-deficient (Nr1h2+/−;Nr1h3−/−) mice on a mixed Sn129/C57Bl/6 and C57Bl/6 background (backcrossed more than ten generations) were originally obtained from D. Mangelsdorf46. Nr1h3+/− mice on the C57Bl/6 background were obtained from Institut Clinique de la Souris (Illkirch, France); C57Bl/6 mice with transgenic expression of Yav-Cre were obtained from D. Kiousis24 through a collaboration with Susana Gonzalez (CNIC, Spain); ap2–VP16LRα mice (with transgenic expression of constitutively active VP16-LXRα in macrophages and adipocytes) have been described31; mice with sequence encoding GFP or DsRed under the control of the Actb promoter (encoding β-actin) were from the Jackson Laboratory. All animal studies were conducted in accordance with the Animal Research Committees of the Consejo Superior de Investigaciones Científicas and Universidad de Las Palmas de Gran Canaria.

Flow cytometry and progenitor assays. Bone marrow cells were obtained from femurs and tibias by flushing with culture medium. Blood cells were obtained by collection of EDTA-treated blood samples. Spleen cells were obtained with a GentleMACS dissociator and a mouse spleen–dissociation kit according to the manufacturer’s instructions (Miltenyi Biotec). In all cases, single-cell suspensions were obtained and erythrocytes were lysed with a hypotonic buffer. Cells were washed and resuspended in PBS with 0.1% BSA and 0.1% sodium azide. Single-cell suspensions were stained for 30 min at 4 °C with the appropriate antibodies. Cells were then analyzed on Beckman Coulter Epics XL, BD FACSCan/Canto II, BD FACS Vantage or BD FACSaria with FlowJo software (TreeStar). Monocytes were identified as CD11b+CD11c−F4/80+ and Ly6C+ or Ly6C-. Splenic phagocytes were detected by the expression of major histocompatibility complex class II, F4/80, CD68 and TIM-4. Macrophage DC progenitors were identified as lineage-negative CD11b+CD11c+ cells. Blood cells were counted with an Abacus automated hematological counter (Diatran). Colony-forming units in culture (CFU-C) were measured in blood and bone marrow samples. Blood and bone marrow were collected and diluted in lymphocyte solution (Cedarlane Labs) and were centrifuged to obtain mononuclear cells. Cells were cultured in semisolid media containing 1.25% methylcellulose (Sigma-Aldrich), 30% FBS (StemCell Technologies), 1% BSA, 0.1 mM 2-mercaptoethanol and conditioned medium (12.7% vol/vol) from WEHI-3 cells (a mouse leukemia cell line that expresses interleukin 3 (IL-3)), BKH–HM-5 cells (a baby hamster kidney cell line that produces the cytokine GM-CSF) and BHK-MKL cells (a baby hamster kidney–human neuroendocrine skin carcinoma cell line stably transfected to produce the ligand for the stem cell factor receptor c-KIT). Cultures were plated in duplicate in 35-mm culture dishes, and CFU-C was assessed on day 6 or 7 with an inverted microscope.

Histology. Tissues were collected and fixed in 4% buffered formalin, dehydrated in successive alcohol solutions, embedded in paraffin wax, and sectioned for staining with hematoxylin and eosin. Alternatively, tissues were directly collected from mice, embedded in optimum cutting temperature compound (Tissue-Tek) and snap-frozen in liquid nitrogen and isopentane. Frozen sections 4 µm in thickness were air-dried and fixed with cold acetone, nonspecific binding was blocked with 6% BSA and 2% preimmune serum in PBS, and sections were stained with fluorescence-conjugated antibodies diluted in blocking solution; nuclei were stained with DAPI (4,6-diamidino-2-phenylindole); Vectashield mounting medium fluorescence with DAPI; Vector). Primary purified antibodies were also detected by Alexa Fluor–conjugated secondary antibodies (Molecular Probes). For non–fluorophore-conjugated antibodies, biotinylated secondary antibodies were visualized by the streptavidin–biotin-peroxidase method (Vectorstain ABC Kit; Vector Laboratories) and were stained with cromogen DAB (3,3′-diaminobenzidine tetrahydrochloride) or alkaline phosphatase (DAB HRP or Vector Blue Substrate kits; Vector Laboratories). Nuclei were counterstained with hematoxylin or methyl green (Sigma). Sections were observed with an LSM 5 PASCAL laser-scanning microscope (Carl Zeiss) or an Eclipse 90i (Nikon).

For transmission electron microscopy, wild-type and LXR-deficient mice were killed and perfused with fixation solution (2% glutaraldehyde). Spleens were fixed with 2% OsO4, dehydrated and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and were observed with an electron microscope at the electron microscopy core facility of Universidad de Las Palmas de Gran Canaria.

In vivo treatment. For capture of circulating antigens, the following reagents were used: labeled high–molecular-weight dextran, inactivated S. aureus and zymosan (all from Life Technologies); GFP-labeled E. coli (which have been described15); and platelets purified by centrifugation of whole blood in citrate-phosphate-dextrose solution. Platelet-rich plasma was collected and washed with PBS. Platelets were resuspended with 5 µg anti–mouse CD41. Antigens were injected intravenously into wild-type and LXR-deficient mice for a 30-minute period. Spleens were collected and tissues were prepared for immunofluorescence analysis as described above.

For liposome-mediated depletion of splenic macrophages, mice were injected intravenously with liposomes loaded with clodronate (Cl2MBP; dichloromethylene-bisphosphate) or with liposomes loaded with PBS (Encapsula Nanosciences) in 200 µl suspension solution. Mice were killed at the appropriate times and tissues were collected for histological analysis.

For treatment with LXR ligands in vivo after depletion of macrophages with clodronate liposomes, a stock solution of GW3965 was diluted in PBS and injected intraperitoneally (at a dose of 20 mg per kg body weight) every 2 days for 1 week. Mice were killed and total RNA was prepared 2 h after the final injection of ligand.

Bone marrow transplantation and adoptive transfer. For bone marrow transplantation, wild-type and LXR-deficient recipient mice (6–8 weeks of age) were lethally irradiated with 900 rads and were given transplantation of 3 x 10^6 bone marrow cells from 6- to 8-week-old wild-type, LXRα-deficient, LXRβ-deficient or LXR-deficient donor mice by retro-orbital injection. Recipient mice were killed 8–12 weeks after transplantation and spleen sections were then analyzed for the presence of macrophages as described above.

For adoptive transfer, total monocytes from bone marrow or spleens were sorted on a FACS Vantage or FACS Aria (BD Biosciences) as CD11b+CD11c+ to a purity of >90%. Cells were injected intravenously (2 x 10^6 to 3 x 10^6 cells).
into nonirradiated LXRα-deficient mice. Recipient mice were killed 8–12 weeks after that injection and spleens were processed as described above. For homing experiments, unfractioned splenocytes or bone marrow cells from mice with transgenic expression of GFP or Ds-Red were injected intravenously together into wild-type recipient mice. Target tissues were processed, and the homing of GFP+ and Ds-Red+ monocytes was analyzed by flow cytometry 24 h after that injection. In another set of experiments, monocytes were purified from bone marrow or spleens of Cx3cr1GFP/+ mice. Classical CD115+CX3CR1lo and nonclassical CD115+CX3CR1hi monocytes (1 × 106 to 2 × 106 cells) were transferred into LXRα-deficient mice. Spleens were isolated and analyzed by immunofluorescence as described above.

Lentivirus production and infection of HSC-enriched bone marrow. A published lentiviral system with a macrophage-specific synthetic promoter was used27. Lentivirus expressing GFP alone or both GFP and LXRα, plus the plasmids pMDlg-pRRE, pRSV/Rev and pMD2G, were transfected into HEK293 human embryonic kidney cells through the use of FuGENE-HD (Promega). After 48 h, supernatants were collected and viral particles were centrifuged at 86,000 g on a SW41Ti swinging-bucket rotor (Beckman). Pellets were resuspended in StemPro 34 SFM medium plus 1% l-glutamine and 1% penicillin-streptomycin, and the concentration of lentiviral particles was determined by flow cytometry of transduced RAW264.7 mouse macrophage cells transduced with serially diluted lentiviral particles expressing GFP alone. For the preparation of cells for lentiviral transduction, total bone marrow was enriched for HSCs with an EasySep Hematopoietic progenitor enrichment kit (StemCell Technologies) or by treatment of donor mice for 4 d with 5-fluorouracil. The resultant enriched bone marrow cells were resuspended for 18 h in StemPro-34 medium supplemented with 10 ng/ml of IL-3, IL-6, IL-1α and mouse stem-cell factor. Cells were then infected for 24 h with protamine sulfate–treated α-particles of lentivirus expressing GFP alone or both GFP and LXRα. At 8 h before transplantation, recipient mice were irradiated as described above. Transduced HSC cells were resuspended in PBS and were injected intravenously (2 × 105) into LXRα-deficient recipient mice together with a fraction of LXRα-deficient total bone marrow cells (5 × 105). After 8–12 weeks mice were killed and blood and spleens were collected and tissues were processed for histology as described above.

Immunization and analysis of IgM by enzyme-linked immunosorbent assay. For analysis of thymus-independent IgM responses, wild-type and LXR-deficient mice were immunized by intravenous injection of 100 µg TNP-Ficoll (Biosearch Technologies). Serum samples were obtained before injection and on days 4 and 7 after immunization with TNP-Ficoll. Then, 96-well flat-bottomed plates (Nunc MAXISORP) were precoated for 1 h at 37 °C with poly-l-lysine (Sigma) in a humidified chamber and were incubated for 18 h at 4 °C with a solution containing TNP-BSA as capture antigen (25 µg/ml). After blockade of nonspecific sites for 1 h at 37 °C with PBS containing 2% FBS and 1% BSA, serial dilutions of serum samples were added, followed by incubation for 2 h at 20–25 °C. Wells were then washed with 0.05% Tween-20 in PBS and were incubated with horseradish peroxidase–conjugated goat antibody to mouse IgM (diluted 1:500). Wells were developed with TMB substrate solution (3,3′,5,5′-tetramethylbenzidine). After incubation for 5 min at 20–25 °C, the reaction was stopped with 50 µl of 3 M H2SO4 and absorbance was measured at 450 nm with an enzyme-linked immunosorbent assay plate reader (Bio-Rad).

Analysis of DNA, RNA and protein. Total RNA was collected with TRIzol reagent (Invitrogen). RNA was reverse-transcribed with iScript reverse-transcription kit (Bio-Rad). An Applied Biosystems 7900 sequencer detector was used for real-time quantitative PCR (SYBRgreen) as described15 (primer sequences, Supplementary Table 1). Expression was normalized to that of mRNA encoding the ribosomal protein 36B4. Genomic DNA for genotyping was obtained from mouse tails with a PureGene mouse tail kit from QiaGen. Primers and PCR specifications for Nr1h330 or alleles encoding Yav-Cre were obtained from Institut Clinique de la Souris and S. Gonzalez, respectively (Supplementary Table 1). For immunoblot analysis, peritoneal macrophages and RAW264.7 or HeLa human cervical carcinoma cells stably overexpressing LXRα were cultured as described15. Whole-cell lysates obtained with radioimmunoprecipitation buffer were boiled and then were separated by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Amersham), nonspecific binding was blocked with 0.1% Tween-20 and 5% milk in TBS, and membranes were incubated with the appropriate antibodies. Monoclonal antibody to LXRα and polyclonal antibody to ABCA1 were used at a dilution of 1:1,000 and 1:3,000, respectively. Blots were washed in 0.5% Tween-20 in TBS and were visualized with ECL-Plus (Amersham Biosciences) and the Chemi-Doc imaging system (Bio-Rad).

Statistical analysis. Student’s t-test was used for statistical analysis of two samples. For multiple comparisons, data were evaluated by one-way analysis of variance (ANOVA). Values of P < 0.05 were considered significant.

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