Vitamin A affects the development of SIGNR1⁺ marginal zone macrophages in the spleen


Department of Molecular Cell Biology and Immunology, VU University Medical Center, P.O. Box 7057, Amsterdam, The Netherlands
Abstract
Marginal zone macrophages (MZMs), strategically located in the marginal zone of the spleen, are crucial for the initiation of immune responses against encapsulated bacteria. They are characterized by the expression of macrophage receptor with collagenous structure (MARCO), and most MZMs also express the polysaccharide receptor specific ICAM3 grabbing nonintegrin-related 1 (SIGNR1). Marginal zone B cells (MZ B cells) are located in close vicinity to the MZMs and the presence of both cell types is thought to be mutually dependent. Using mouse models for vitamin A deficiency and retinoic acid supplementation, we show that vitamin A is required for the development of MZ B cells. In contrast to MZ B cells, the development of MZMs is negatively regulated by vitamin A. In vitamin A deficient mice, a more prominent MZM population was found, whereas in mice kept on a diet containing high levels of the active vitamin A metabolite, retinoic acid, we observed a reduction in MARCO\textsuperscript{+}SIGNR1\textsuperscript{+} macrophages and a decreased uptake of the polysaccharide FITC-dextran. Together, these results demonstrate a differential role for vitamin A in the generation of MZ B cells and MZMs and shed a new light on the mutual dependency between MZ B cells and MZMs that was previously reported. In addition, our results suggest that vitamin A deficiency can affect immune responses towards encapsulated bacteria.
**Introduction**

Fast and efficient recognition of pathogens by cells of the immune system is essential for the protection against harmful pathogens. Secondary lymphoid organs such as the spleen play a crucial role in this process, because they facilitate the uptake and elimination of pathogens by immune cells. Blood enters the spleen via the splenic artery; this artery branches into the central arterioles. The layer of lymphoid tissue surrounding the splenic arterioles is known as the white pulp and contains T and B cell areas. The marginal zone of the spleen is an area that forms the border between the white and the red pulp. Here, specific macrophage subsets and B cells can be found that are specialized in the recognition of blood-borne pathogens. The marginal zone is characterized by the presence of MAdCAM1+ cells that line the marginal sinus. In this area, the blood flow decreases and pathogens can be captured from the blood 1,2.

Two subsets of macrophages are present in the marginal zone of the spleen. A layer of CD169+ macrophages is located directly beneath the MAdCAM1+ sinus lining cells and borders the B cell follicles. More towards the red pulp, marginal zone macrophages (MZMs) reside together with marginal zone B cells (MZ B cells) 1,2. MZMs are identified by the expression of macrophage receptor with collagenous structure (MARCO) and specific ICAM3 grabbing nonintegrin-related 1 (SIGNR1). MARCO is expressed by all MZMs while SIGNR1 is expressed by a fraction 3,4. Colony stimulating factor-1 (CSF-1 or M-CSF) regulates the survival and development of SIGNR1 expressing macrophages in the spleen. In osteopetrotic (op-) mutant mice that lack functional M-CSF, no SIGNR1 expression was observed, while MARCO was present 3,5.

MZMs are involved in the recognition and clearance of infections with polysaccharide encapsulated bacteria such as *Streptococcus pneumoniae* and *Neisseria meningitidis* 6,7. The expression of polysaccharide receptor SIGNR1 is crucial for this. It has been shown that SIGNR1 knockout mice are more susceptible to *S pneumoniae* infection, because they fail to clear the bacteria 8. Production of IgM by MZ B cells and activation of C3 complement pathway by binding to C1q are diminished in SIGNR1 knockout mice 6,9. In addition to the immune response towards encapsulated bacteria, CD169+ and SIGNR1+ macrophages in the marginal zone are required for the clearance of vesicular stomatitis virus (VSV), as was shown in op- mice that lack both CD169+ and SIGNR1+ macrophages 10. Also trapping of particulate antigens such as *Listeria monocytogenes* was severely impaired in the absence of CD169+ and SIGNR1+ macrophages while antigen presentation and T cell activation remained intact 11. Furthermore, CD169+ and SIGNR1+ macrophages are essential for the clearance of *Lymphocytic choriomeningitis virus* (LCMV) particles. In the absence of both macrophage populations, LCMV spreads from the spleen into peripheral organs, resulting in overwhelming viral replication 12. Taken together, MZMs are important for clearance of both bacterial and viral pathogens and thereby are crucial for maintaining immune homeostasis.

MZ B cells are characterized as IgMhighIgDlowCD21highCD23 lowCD1dhigh cells. They are closely related to follicular B cells that are IgMlowIgDhighCD21medCD23+CD1dflow 13.
Interactions with various cell types stimulate signaling pathways that are required for the development of MZ B cells. MZ B cells are absent in mice lacking RBP-J (a key mediator for Notch signaling) \(^1\). B cell specific deletion of Notch2 shows that the development of MZ B cells requires Notch2 while other B cell populations remain present \(^15\). Additionally, the balance between CXCL13 expression by follicular dendritic cells (DCs) and S1PR1 expressed by MZ B cells is crucial for the localization of MZ B cells in the marginal zone. MZ B cells are continuously attracted into the B cell follicle by CXCL13 and S1PR1 expression is required to overcome this attraction \(^16\).

Numerous studies have shown that MZ B cells are required for the presence of SIGNR1\(^+\) MZMs in the spleen. In CD19\(^-\) mice, in which all B cells are absent, MARCO\(^-\)SIGNR1\(^+\) macrophages are lacking, while the MARCO\(^-\)SIGNR1\(^-\) macrophages remain present \(^4,9,17,18\). A similar phenotype was seen in Notch2 deficient mice in which MZ B cells are missing: MARCO\(^-\)SIGNR1\(^+\) macrophages are absent, while the MARCO\(^-\)SIGNR1\(^-\) macrophages remain \(^4\). This suggests that the SIGNR1\(^+\) MZM population depends on signals provided by the MZ B cells. Vice versa, MZ B cells have been described to depend on SIGNR1\(^+\) macrophages, which indicate a mutual dependency \(^6,19\).

Vitamin A is crucial for various processes in the immune system. Especially the effects of vitamin A on the mucosal immune system have been extensively studied. For example, both the expression of gut homing molecules CCR9 and α4β7 by T and B cells and the generation of IgA secreting B cells were demonstrated to be vitamin A dependent \(^20,21\). Furthermore, retinoic acid (RA) producing macrophages are present in the lamina propria of the small intestine. Via the production of IL10 and RA, the active metabolite of vitamin A, these macrophages induce the generation of regulatory T cells \(^22\).

Vitamin A metabolizing DCs and macrophages in the gut mucosa hereby initiate and regulate mucosal adaptive immune responses and homeostasis. More recently, effects of vitamin A on the development of immune cells in the spleen have been described, whereby vitamin A was not only required for the development of MZ B cells \(^23\), but also turned out to be crucial for the development of Notch2 dependent DCs. Since both cell types are located in the marginal zone of the spleen, this prompted us to study the role of vitamin A in the presence and functionality of marginal zone macrophages.

Here, we report that vitamin A regulates the development of SIGNR1\(^+\) macrophages in spleen. This was not dependent on its effect on MZ B cells: while vitamin A positively regulated the development of MZ B cells, it inhibited the generation of MARCO\(^-\)SIGNR1\(^+\) macrophages. The expression of homing molecules MadCAM1, ICAM1 and VCAM1 or growth factors M-CSF and IL34 was not regulated by vitamin A. Increased uptake of RA, the active metabolite of vitamin A, resulted in a reduction of MARCO\(^-\)SIGNR1\(^+\) macrophages and decreased uptake of FITC-dextran in the spleen. Taken together, our studies show that vitamin A levels affects the generation of SIGNR1\(^+\) macrophages and SIGNR1 mediated uptake of dextran in the spleen.
**Materials and Methods**

**Mice**
Female C57BL/6 mice (Charles River Laboratories, Maastricht, The Netherlands) were fed a vitamin A deficient (VAD) (0 IU vitamin A/kg) or control (VAC) (4000 IU vitamin A/kg) AIN-93M diet produced with vitamin free casein (MP Biomedical, Solon, Ohio, USA or Research Diets Services, Wijk bij Duurstede, The Netherlands) from day 7.5-10.5 of pregnancy. Pups were weaned at week 5 and kept on their specific diet and under SPF conditions until they were sacrificed at week 9-14. For generation of retinoic acid high (RAH) mice, mice were fed with global 16% protein rodent diet (Harlan, Horst, The Netherlands) that contained 4.5 μg RA per g dry food supplemented with 100 μg RA per g dry food or vehicle control for 8-14 days (Sigma-Aldrich, Zwijndrecht, the Netherlands). To reduce degradation of RA by light, food was refreshed twice a day. Where indicated, mice were intravenously injected with 100 μg FITC-dextran (2000 kDa) (Sigma-Aldrich, Zwijndrecht, the Netherlands) 30 minutes prior to sacrificing. Spleens were embedded in O.C.T. TM medium (Tissue-Tek, Sakura, Alphen aan den Rijn, The Netherlands) immediately after sacrificing and snap frozen in liquid nitrogen vapor. The ethical committee of the VU University Medical Center approved all animal experiments.

**RNA isolation, cDNA synthesis and real time PCR**
Total RNA was isolated from total spleen homogenates using TRIzol reagent (Invitrogen Life Technologies, Breda, The Netherlands) and precipitation with isopropanol. cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Vilnius, Lithuania) according to manufacturers’ protocol. Real time PCR was performed using SYBR Green Mastermix (PE Applied Biosystems, Foster City, CA) on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems Foster City, CA). A standard curve was generated using pooled lymph node tissue to correct for primer efficiency. mRNA quantities were normalized to HPRT. Primer sequences are depicted in table 1.

**Immunofluorescence**
Spleens were harvested, embedded in O.C.T. TM medium (Tissue-Tek, Sakura, Alphen aan den Rijn, The Netherlands) and frozen at -80°C until further use. Cryosections (5 μm) were collected on gelatine coated glass slides. Upon fixation in acetone, slides were air-dried and blocked with 5% mouse serum in PBS prior to antibody staining. Sections were embedded in a mixture of polyvinylalcohol and glucose supplemented with DAPI (Invitrogen) and analyzed on a Leica DM6000 fluorescence microscope (Leica Microsystems, Netherland b.v., Rijswijk, The Netherlands).

**Spleen digestion and flow cytometry**
Spleens were digested with 1 WU/ml liberase TL (Roche Diagnostics GmbH, Mannheim, Germany) in 4 μg/ml lidocaïne at 37°C for 5-15 minutes while continuous stirring. Cells were incubated for an additional 10 minutes at 4°C with RPMI HE medium containing
10% FCS, 2% penicillin-streptomycin, 2% L-glutamine, 10 mM EDTA and 20 mM Hepes while stirring. Red blood cells were lysed by incubation with 1 ml ACK lysis buffer (0.15 M \( \text{NH}_4\text{Cl}, 10 \text{ mM} \text{ KHCO}_3, 0.1 \text{ mM} \text{ Na}_2\text{EDTA} \text{ at pH 7.2–7.4} \) ) for 1 minute at room temperature. Non-digested fragments were removed by filtration after which the single cell suspension was used for staining. Cells were analyzed with a Cyan ADP flow cytometer (Beckman Coulter, Woerden, The Netherlands). Data analysis was performed using FlowJo 9.2 (Tree Star).

### Antibodies

Antibodies against the following markers were used: IgM-bio (clone II/41) and IgD-PE (AMS 9.1) were purchased at BD Pharmingen. VCAM1 (clone 429), ICAM1 (clone YN1/1.7.4), Tim4 (clone RMT4-54), B220 (clone RA3-6B2), CD21/35 (clone eBio8D9) and CD23 (clone B3B4) were obtained from eBioscience and anti-rat IgG and streptavidin-555 from Invitrogen.

### Statistical analysis

Statistical significance was tested using GraphPad Prism 4 (La Jolla, California, USA) by performing a two-tailed Student’s T-test or ANOVA with Bonferroni’s correction as indicated. \( P<0.05 \) was considered to be significant.

### Results

**Vitamin A stimulates the development of marginal zone B cells**

C57BL/6 mice were fed a vitamin A deficient (VAD) (0 IU vitamin A/kg) or control (VAC) (4000 IU vitamin A/kg) diet from day 7.5-10.5 of gestation. Vitamin A deficiency was confirmed
by determining serum retinol levels (VAC: 1.164 +/- 0.081 μM, VAD: 0.019 +/- 0.001 μM). The presence of MZ B cells was assessed by immunohistochemistry. Marginal zone B cells (MZ B cells), defined as B220<sup>+</sup> cells that are located outside the MAdCAM1<sup>+</sup> ring of sinus lining cells, were virtually absent in spleens of vitamin A deficient mice (figure 1A). This is in line with previous reports that show a reduction in MZ B cells in spleens of VAD mice<sup>23</sup>.

The conversion of vitamin A into its biologically active metabolite retinoic acid (RA) is tightly regulated. To determine whether increased levels of RA enhance MZ B cell development, we fed mice with 100 μg RA/g dry food (RAH) or vehicle control (RAC) for 8-14 days. Upon administration of high levels of RA, the rim of B220 expressing cells surrounding the MAdCAM1<sup>+</sup> sinus lining cells was more extensive (figure 1A). MZ B cells are characterized by high IgM and low IgD expression, while follicular B cells show an opposite expression pattern<sup>13</sup>. In mice that received extra RA, a strong increase in IgM<sup>+</sup>IgD<sup>-</sup> cells in the marginal zone was detected, indicating an expansion of MZ B cells upon RA supplementation (figure 1B). Furthermore, flow cytometric analysis of MZ B cells by gating for B220<sup>+</sup>CD21<sup>high</sup>CD23<sup>low</sup> cells showed a significantly increased MZ B cell population in spleens of RAH mice (figure 1C). Together, these data indicate that vitamin A stimulates and is required for the development of MZ B cells in the spleen.

**Splenic S1PR1 expression is regulated by vitamin A**
MZ B cells express the CXCL13 receptor CXCR5 and S1P receptors. Follicular dendritic cells located in B cell follicles express CXCL13 and thereby continuously attract MZ B cells. The expression of S1PR1 and S1P3 by MZ B cells is required to overcome this attraction and the high levels of S1P in the marginal zone retain MZ B cells in the marginal zone<sup>16,24</sup>. As vitamin A has been shown to regulate CXCL13 expression during lymph node development<sup>25</sup>, we determined CXCL13 mRNA levels in total spleen homogenates of VAD and RAH mice by quantitative PCR. However, CXCL13 expression in the spleen was not affected by vitamin A deficiency or RA supplementation (figure 1D). In contrast, a significant reduction of S1PR1 expression was observed in spleens of VAD mice, while a significant increase of S1PR1 expression was seen in spleens of RAH mice, which correlates with the number of MZ B cells present in these spleens (figure 1D).

**Vitamin A regulates the presence of MARCO<sup>+</sup>SIGNR1<sup>+</sup> macrophages in the spleen**
Marginal zone macrophages (MZMs) are located in the outer region of the marginal zone, close to the red pulp border. They are a heterogeneous population, consisting of MARCO<sup>+</sup>SIGNR1<sup>+</sup> and MARCO<sup>+</sup>SIGNR1<sup>+</sup> cells<sup>3,26</sup>. The presence of MZMs has been shown to depend on the presence of MZ B cells<sup>1,4,14</sup>. As our studies indicated that vitamin A regulates the development of MZ B cells in the spleen, we set out to determine whether vitamin A also regulates the presence of MARCO<sup>+</sup>SIGNR1<sup>+</sup> and MARCO<sup>+</sup>SIGNR1<sup>+</sup> macrophages in spleens of VAD and RAH mice by immunofluorescence microscopy. In spleens of control mice (VAC and RAC), both MARCO<sup>+</sup>SIGNR1<sup>+</sup> and MARCO<sup>+</sup>SIGNR1<sup>+</sup> macrophages were found (figure 2A). Interestingly, in mice raised on a vitamin A deficient (VAD) diet,
Figure 1. Vitamin A stimulates the development of marginal zone B cells. Legend next page.
an increase in MARCO−SIGNR1+ macrophages was observed. Quantitative PCR on total spleen homogenates of VAD mice confirmed these findings. A significant increase in both SIGNR1 and MARCO transcripts was seen in the spleen of VAD mice (figure 2B). In contrast, when mice were fed with increased levels of RA (RAH), a strong reduction of MARCO−SIGNR1+ macrophages was observed (figure 2A). However, the decreased SIGNR1 expression was not reflected by the SIGNR1 and MARCO mRNA expression (figure 2B). CD169+ macrophages that are also located in the marginal zone and share developmental requirements with MZMs were not affected by vitamin A deficiency or supplementation of retinoic acid (chapter 6 and data not shown). Finally, MARCO−SIGNR1+ and MARCO−SIGNR1− macrophages were quantified in spleens of RAC and RAH mice using flow cytometry. Live cells expressing Tim4 (T cell immunoglobulin- and mucin-domain-containing molecule), a recently defined macrophage marker, were analyzed for MARCO and SIGNR1 expression. Significantly less MARCO−SIGNR1+ macrophages were found in spleens of RAH mice while MARCO−SIGNR1− macrophages remained unchanged (figure 2C).

Previous reports indicated that the presence of MZMs is directly dependent on the presence of MZ B cells in the spleen. However, here we show that although vitamin A stimulates the development of MZ B cells, it negatively regulates the development of MARCO−SIGNR1+ macrophages in the spleen.

**Vitamin A does not affect the expression of cell adhesion molecules MAdCAM1, ICAM1 and VCAM1**

Adhesion molecules ICAM1 (which interacts with αLβ2 and αMβ2 integrins) and VCAM1 (which interact with α4β1 and α4β7 integrins) are thought to play a role in the retention
Figure 2. Vitamin A influences MARCO<sup>+</sup>SIGNR1<sup>+</sup> macrophages in the marginal zone. (A) Spleen sections of VAC, VAD, RAC or RAH mice were stained for MARCO (red) and SIGNR1 (blue). Representative pictures were selected of 3-5 mice analyzed per group. (B) Relative SIGNR1 and MARCO expression were determined in by quantitative PCR total spleen homogenates of VAC or VAD and RAC or RAH mice. Expression was normalized to the expression of housekeeping gene HPRT. (C) Total number of MARCO<sup>+</sup>SIGNR1<sup>+</sup> and MARCO<sup>+</sup>SIGNR1<sup>-</sup> macrophages were determined in RAC and RAH mice. Live Tim4<sup>+</sup> cells were gated and MARCO<sup>+</sup>SIGNR1<sup>+</sup> and MARCO<sup>+</sup>SIGNR1<sup>-</sup> macrophages were shown as percentage of total live cell population. Both macrophage populations were gated for high MARCO expression as indicated. Data are shown as mean + SEM and are obtained from 1 or 2 experiments with 3-8 mice per group. *P<0.05, **P<0.01, ****<0.0001 student T-test.
Figure 3. Dietary vitamin A does not affect the expression of cell adhesion molecules. (A) Relative MAdCAM1, ICAM1, and VCAM1 expression were determined by quantitative PCR on total spleen homogenates of VAC and VAD mice. Expression was normalized to the expression of housekeeping gene HPRT. (B) Spleen sections of VAC and VAD mice were stained for ICAM1 (red) and VCAM1 (blue). Representative pictures were selected of 3 mice analyzed per group. (C) Relative MAdCAM1, ICAM1, and VCAM1 expression was determined by quantitative PCR on total spleen homogenates of RAC and RAH mice. Expression was normalized to the expression of housekeeping gene HPRT. (D) Spleen sections of RAC and RAH mice were stained for ICAM1 (red) and VCAM1 (blue). Representative pictures were selected of 5 mice analyzed per group. Data are shown as mean + SEM and are obtained from 1 experiment with 3-5 mice per group. *P<0.05 student T-test.
of MZ B cells in the marginal zone. Furthermore, MZMs and MZ B cells are located in close vicinity to MAdCAM1 expressing sinus-lining cells that can bind to α4β7 expressing cells. RA has been shown to stimulate ICAM1 expression in multiple cell types, while VCAM1 expression is described to be negatively affected by RA. To determine a potential mechanism via which vitamin A stimulates MZ B cell and MZM development, we tested whether vitamin A influences the expression of MAdCAM1, ICAM1 and VCAM1 in the spleen. The expression of MAdCAM1 and ICAM1 was not affected in total spleen homogenates of VAD or RAH mice. Strikingly, the expression of VCAM1 was increased in both VAD and RAH mice (figure 3A and 3C). However, immunohistochemical analysis did not show clear alterations in the expression of MAdCAM1, ICAM1 and VCAM1 (figure 1A, 3B and 3D). These results suggest that the positioning of MZ B cells and the development of MARCO⁺SIGNR1⁺ macrophages by vitamin A is not regulated via the expression of cell adhesion molecules MAdCAM1, ICAM1 and VCAM1.

Expression of macrophage growth factors M-CSF and IL34 is not vitamin A dependent

Growth factors are indispensable for the development and maintenance of macrophages in the spleen. In op⁻/⁻ mice, a spontaneous mutation in the macrophage colony-stimulating factor (M-CSF) gene results in a strong reduction in both CD169⁺ macrophages and SIGNR1⁺ macrophages, which indicates their dependence on this growth factor. However, MARCO expressing MZMs has been reported to still be present in op⁻/⁻ mice that lack functional M-CSF. This would indicate that SIGNR1 expression is stimulated by M-CSF. Recently, it was shown that M-CSF receptor (M-CSFR) also binds IL34, although they are not identical in biological activity. To determine whether the expression of M-CSF and IL34 are vitamin A dependent, expression of both growth factors in total spleen homogenates of VAD and RAH mice was determined by quantitative PCR. No significant differences were observed in the expression of M-CSF and IL34 in the spleens of mice that were either supplemented with retinoic acid or that were vitamin A deficient (figure 4). This suggests that the vitamin A does not regulate the development of MARCO⁺SIGNR1⁺ macrophages via the expression of growth factors M-CSF or IL34.

Figure 4. Dietary vitamin A levels do not affect the expression of M-CSF and IL34. Relative M-CSF and IL34 expression were determined by quantitative PCR on total spleen homogenates of VAC and VAD (A) or RAC and RAH (B) mice. Expression was normalized to the expression of housekeeping gene HPRT. Data are shown as mean + SEM and are obtained from 1 experiment with 3-5 mice per group.
Uptake of FITC-dextran by MARCO^+SIGNR1^+ macrophages is dependent on vitamin A

SIGNR1 is a polysaccharide receptor that mediates the uptake of polysaccharides and encapsulated bacteria such as *Streptococcus pneumoniae*[^38][^39]. To determine whether the polysaccharide dextran is differentially taken up by MARCO^+SIGNR1^+ and MARCO^+SIGNR1^- macrophages in the spleen, we injected mice with FITC-labeled dextran. FITC-dextran uptake was detected in 12.2 +/- 1.3% of the total live cell population (figure 5A and 5B). MARCO^+SIGNR1^+ macrophages bound significantly more FITC-dextran when compared to MARCO^+SIGNR1^- macrophages (figure 5A and 5D), which is in line with previous reports that show that SIGNR1 expression by MZMs is necessary for their ability to take up dextran[^39][^40].

![Graphs showing FITC-dextran uptake](image)

**Figure 5. Uptake of FITC-dextran is decreased in RAH mice.** (A) Representative histograms showing FITC-dextran uptake by total live cells (left), MARCO^+SIGNR1^- (middle) and MARCO^+SIGNR1^+ cells. (B) Total number of FITC-dextranhigh cells represented as percentage of total live cell population (left) and mean FITC-dextran fluorescence in FITC-dextranhigh live cell population (right). (C-D) Representative histograms and quantification showing FITC-dextran uptake by MARCO^+SIGNR1^- and MARCO^+SIGNR1^+ cells obtained from RAC mice (grey line histogram) and RAH mice (dark line histogram). (E) Spleen sections of RAC and RAH mice were stained for MARCO (red) and SIGNR1 (blue). Representative pictures were selected of 4 mice analyzed per group. Data shown are representative of 1 experiment with 4 mice per group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 student T-test (B) or ANOVA with Bonferroni’s correction (D).
Because a reduction of MARCO⁺SIGNR1⁺ macrophages was observed in RAH mice, we determined whether this also affected the uptake of FITC-dextran. A significant decrease in FITC-dextran^{high} cells was detected in mice supplemented with RA (figure 5B). Also the mean FITC-dextran uptake was significantly reduced upon RA supplementation. When we evaluated the uptake by the MARCO⁺SIGNR1⁺ and MARCO⁺SIGNR1⁻ macrophage subsets, RA supplementation did not affect FITC-dextran uptake in MARCO⁺SIGNR1⁻ cells. Although the MARCO⁺SIGNR1⁺ macrophage population was significantly reduced in RAH mice (figure 2C) and although this resulted in significant reduced overall uptake of FITC-dextran, the remaining MARCO⁺SIGNR1⁺ macrophages exhibited an increase in FITC-dextran uptake per cell (figure 5C and 5D). This suggests that the remaining MARCO⁺SIGNR1⁺ macrophages are still functional and that the higher ligand availability result in higher uptake. Together, these results indicate that vitamin A signaling negatively regulate the number of MARCO⁺SIGNR1⁺ macrophages in the spleen and thereby the overall uptake of ligands of SIGNR1.

Discussion
Vitamin A is crucial for the development and functioning of the immune system. Dendritic cells (DCs) and macrophages in the lamina propria of the small intestine convert vitamin A into its biologically active metabolite retinoic acid (RA) 41. Also in the spleen, vitamin A is crucial for immune homeostasis and activation. We recently showed that the development of Notch2 dependent DCs in the spleen requires vitamin A 42. Furthermore, we and others 23 showed that vitamin A is essential for the development and positioning of marginal zone B cells. Here, we show that even though vitamin A stimulates the development of MZ B cells, vitamin A negatively regulates the development of marginal zone macrophages (MZMs) in the spleen.

Association between MZ B cells and MZMs
The marginal zone of the spleen comprises various different cell types, including MZ B cells, CD8⁻ DCs, CD169⁺ macrophages, and marginal zone macrophage (MZMs). The development of MZ B cells is a complex process that requires various signals provided by immune cells in the marginal zone. Previous studies showed that MZ B cells require vitamin A for their development 23,42. Here, we confirm this finding and show an increased S1PR1 expression in spleens of mice fed on a diet containing high levels of retinoic acid. This correlates with the increased MZ B cell population that was found in these mice. If vitamin A regulates the balance between S1PR1 and CXCL13 expression, this potentially prevents MZ B cell migration into the follicle thereby resulting in accumulation of B cells in the marginal zone. However, it remains to be determined whether vitamin A directly regulates S1PR1.

In this study we report an inverse correlation between the presence of MZ B cells and MZMs in the splenic marginal zone. Previously, a strict dependency of the two cell types has been described in situations where a complete absence of one of the two
Development of SIGNR1+ MZMs

cell types had been induced. In the absence of SIGNR1+ MZMs a reduction in MZ B cells was observed and the remaining MZ B cells were not able to bind pathogens and to develop early IgM responses upon pathogen encounter. Specific disruption of SHIP (SH2-containing inositol-5-phosphatase 1) in myeloid cells resulted in migration of MZMs to the red pulp and loss of retention of MZ B cells in the marginal zone. Similarly, MZMs require MZ B cells as a loss in SIGNR1 expression was observed in CD19−/− and B cell specific Notch2−/− mice. This suggests a mutual dependency between MZ B cells and MZMs. However, our results indicate that vitamin A has opposite effects on MZ B cells and MZMs. In the situation described here, varying levels of retinoic acid differentially affect MZ B cells and MZMs, but the reduction of one or the other is probably not substantial enough to affect the presence of the other cell type. How this is regulated at the cellular level is not clear and further studies on RAR and RXR expression in the two cell types will be important.

**Is there a precursor relationship between MARCO+SIGNR1+ and MARCO+SIGNR1− macrophages?**

The developmental pathway of distinct macrophage populations in the splenic marginal zone is largely unknown and the relation between MARCO+SIGNR1+ and MARCO+SIGNR1− macrophages has not been clarified. MARCO+SIGNR1+ and MARCO+SIGNR1− macrophages may represent different cell lineages or activation states. Our data suggest that vitamin A negatively regulates the development of MARCO+SIGNR1+ macrophages, while MARCO+SIGNR1− macrophages seem to be less affected by vitamin A levels. Besides SIGNR1, no unique markers are available to distinguish MARCO+SIGNR1+ from MARCO+SIGNR1− macrophages. Therefore, it remains to be elucidated whether vitamin A regulates the development of MARCO+SIGNR1+ macrophages, or whether SIGNR1 expression is vitamin A dependent.

**Is there a role for LXR signaling in vitamin A dependent reduction of MZMs?**

Retinoic acid (RA) exerts extensive transcriptional regulation via binding to RAR-RXR heterodimers that subsequently bind to retinoic acid response elements (RAREs) in the promoter region of various genes. Multiple RAR and RXR genes have been identified, but their specific roles have yet not been identified. In addition to RARs, RXRs bind to other partners such as thyroid hormone receptor (TR), vitamin D receptor (VDR), peroxisome proliferator activated receptor (PPAR) and liver X receptor (LXR). Recently it was shown that LXRα is essential for the differentiation of CD169+ macrophages and MZMs in the spleen. In LXRα deficient mice, no CD169+, MARCO+ or SIGNR1+ cells were found in the spleen, while these populations remained unaffected in LXRβ deficient mice. Heterodimers between RXRs and LXRs bind LXR response elements (LXREs) in the promoter region of LXR target genes. Binding of RA to RXR has been shown to initiate a conformational change in LXR that allows binding of a co-activator, which affects gene transcription.
Here, we showed that enhanced uptake of RA results in a reduction in MZMs, while vitamin A deficiency results in an expansion of MZMs. Increased uptake of RA might result in competition of RARs with LXRs for RXR binding. Alternatively, increased RA binding to RXRs could influence gene transcription by LXR-RXR heterodimers. However, since LXRα has been shown to be essential for the differentiation of both CD169+ macrophages and MZMs and we only observed an effect of RA on MZMs, this postulated effect of RA on LXR mediated gene expression should be limited to MZMs.

To summarize, here we show that RA is required for MZ B cells development. Surprisingly, RA negatively regulates MZM development. No alterations in the expression of adhesion molecules (MAdCAM1, ICAM1, VCAM1) and macrophage growth factors (M-CSF and IL34) were detected. RA appeared to specifically affect MARCO+SIGNR1+ macrophages, while MARCO+SIGNR1− macrophages were less affected. As MZMs are important for the uptake of encapsulated bacterial pathogens, it is expected that vitamin A regulates the immune response towards these pathogens. In case of vitamin A deficiency, an expansion of MZMs potentially causes a faster clearance of encapsulated bacteria from the circulation. However, since MZ B cells are significantly decreased in vitamin A deficiency, this is expected to affect pneumococcal B cell responses. Indeed, vitamin A deficiency has been shown to result in decreased immune responses towards pneumococcal bacteria in animal models and vitamin A supplementation has been evaluated for its effect in preventing pneumococcal infections.

References


