autoimmune or autoaggressive disease. The new climax of research on Perforin, as reported here, may soon be followed by the development of novel therapeutics targeted to enhance or diminish Perforin cytotoxicity, to enhance our fitness and survival.

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Lymphoid Organs for Peritoneal Cavity Immune Response: Milky Spots

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Milky spots are located in the omentum of the peritoneal cavity and their classification as lymphoid organs has been debated. In this issue of *Immunity*, Rangel-Moreno et al. (2009) provide compelling data to consider them as unique secondary lymphoid organs.

The omentum is formed by a double layer of mesothelial cells that connects the stomach, pancreas, spleen, and colon (Williams and White, 1986). Embedded within the omentum are opaque structures, which are clusters of leukocytes, called milky spots. Milky spots are mainly composed of macrophages and B1 cells, resembling the cellular composition found in the peritoneal cavity. B1 cells form a unique subset of B cells that can be distinguished from conventional B (B2) cells by expression of distinct cell-surface markers and antigen receptors that can bind common bacterial epitopes, as well as by their recognized potential to produce natural antibodies that provide a first protection to bacterial infections. B1 cells are localized in distinct anatomical locations, such as the peritoneal and pleural cavities, but are also present in the spleen. For their localization to these body cavities, but not the spleen, the chemokine CXCL13 is of great importance because in *Cxcl13*$^{-/-}$ mice, B1 cells as well as milky spots are absent from the pleural and peritoneal cavities (Ansel et al., 2002). As a consequence, *Cxcl13*$^{-/-}$ mice, as well as mice deficient for the CXCL13 receptor, CXCR5, have strongly reduced titers of natural antibodies (Ansel et al., 2002; Hopken et al., 2004). Therefore, milky spots are an important source of natural antibodies.

Because milky spots mainly consist of macrophages and B1 cells, and are described to lack dendritic cells as well as follicular dendritic cells, controversy exists as to whether to mark these milky spots as secondary lymphoid organs. In this issue of *Immunity*, Rangel-Moreno et al. (2009) addressed the immunological potential of milky spots in the absence of lymph nodes, spleen, and Peyer's patches. Here, they used splenectomized lymphotoxin-alpha (LTα)-deficient mice (*Lta*$^{-/-}$), which as a result of their deficiency already lacked lymph nodes and Peyer's patches. These animals, devoid of secondary lymphoid organs, were reconstituted with wild-type bone marrow (SLP mice) and compared to irradiated C57BL/6 mice that were similarly reconstituted with wild-type bone marrow. Antigens injected into the peritoneal cavity of SLP mice were shown to collect in the milky spots, resulting in the generation of antigen-specific antibodies. Furthermore, germinal center B cell responses, supporting isotype switching, somatic hypermutation, and some affinity maturation, as well as proliferation of T cells in response to intraperitoneally injected antigens, could be observed in the milky spots. Not only are these milky spots occupied by locally activated lymphocytes, but also lymphocytes that encounter antigens elsewhere were shown to recirculate through the milky spots. These experiments clearly identified the milky spots as part of the general surveillance route of antigen-experienced lymphocytes in search for their antigen.

What does it take to enter and leave the milky spots? Milky spots contain high endothelial venules (HEVs) that express the peripheral lymph node addressin (PNAd) as well as the mucosal addressin (MAdCAM-1), permitting entry of lymphocytes from the bloodstream into the milky

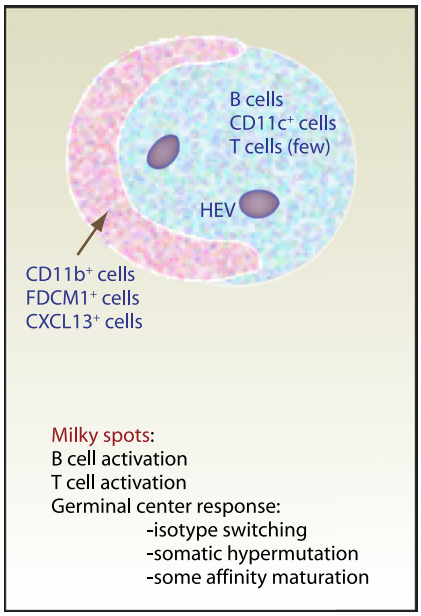

Figure 1. Schematic Overview of Milky Spot Function, Architecture, and Formation
Milky spots are formed by clustered B cells, with high endothelial venules, $CD11c^+$ cells, and some T cells within the B cell areas. CXCL13-producing cells, which colocalize with $CD11b^+$ and $FDCM1^+$ cells, are positioned in a cup-like structure around the B cell clusters. In these milky spots, B and T cell activation can take place, and germinal centers, supporting isotype switching, somatic hypermutation, and some affinity maturation, can also be found.

spots (Berberich et al., 2008; Rangel-Moreno et al., 2009). In fact, milky spots were proposed to be the port of entry for B1 cells that migrate from the bloodstream toward the peritoneal cavity (Ansel et al., 2002). For the entry of B2 cells into the milky spots, only $\alpha4\beta7$-MAdCAM-1 interaction was shown to be essential, whereas L-selectin was not needed (Berberich et al., 2008). However, the entry from the bloodstream into the milky spots must have additional requirements, given that the cellular composition of the milky spots is very different from lymph nodes and Peyer's patches, which express the same addressins on HEVs. In addition, Rangel-Moreno et al. (2009) showed that a flow from the peritoneal cavity to the milky spots allows entry of antigens as well as lymphocytes into the milky spots. For lymphocytes, this process requires receptors that are sensitive to pertussis toxin treatment. Altogether, it seems that cells enter the milky spots from the bloodstream, whereas they may thereafter move freely from the milky spots to the peritoneal cavity and back to the milky spots.

The overall architecture of the milky spots is unique because the B cell clusters do not appear to contain centrally positioned networks of follicular dendritic cells (FDCs). FDCs in conventional lymphoid organs are located in the center of B cell follicles and express CD35 and CD21, as well as FDCM1 and FDCM2, while they produce CXCL13. In milky spots, however, only some expression of CD35 could be detected in the center of the B cell clusters, whereas expression of CXCL13 was very uncharacteristically located on the outside of these clusters. Expression of CXCL13 colocalized with $FDCM1^+$ and $CD11b^+$ cells. The lack of conventional FDC networks may account for the reduced ability to support affinity maturation in mice that only contain milky spots (Rangel-Moreno et al., 2009).

Not only are milky spots different from lymph nodes and Peyer's patches in their cellular composition and morphology, but also their formation seems to follow distinct cues. In man, the formation of milky spots starts at 20 weeks of gestation, when clusters of monocytes and macrophages can be observed, and true milky spots are apparent at week 35. This development is clearly much later than that of human lymph nodes, which can be dissected out starting at week 13 of gestation (Cupedo et al., 2009). For the formation of lymph nodes and Peyer's patches, it is now well established that lymphoid tissue inducer cells (LTi cells), expressing $LT\alpha\beta$, trigger lymphoid tissue stromal organizer cells that express the lymphotoxin-β receptor ($LT\beta R$). This interaction, which leads to the production of chemokines, expression of adhesion molecules, and synthesis of cytokines, is required for the formation of LNs and PPs (Vondenhoff et al., 2009). For milky spot formation, Rangel-Moreno et al. (2009) show that LTi cells are dispensable and thus that milky spots follow a distinct developmental program. Although milky spots are strongly reduced in absence of $LT\alpha$, $LT\alpha$ is not crucial to induce essential chemokines because CXCL13 was normally expressed in the omentum of $Lta^{-/-}$ mice. The function of $LT\alpha$ is more likely to be related to regulating the function of HEVs, which are absent in milky spots of $Lta^{-/-}$ mice. The importance of $LT\beta R$ signaling for HEV function and maintenance has been reported for lymph nodes (Browning et al., 2005). It is tempting to speculate that HEVs are in fact essential for the maintenance of milky spots.

Absolutely required for milky spots formation is the chemokine CXCL13, and in analogy with the development of lymph nodes and Peyer's patches, the cells that make CXCL13 could be denoted as "organizer cells." Rangel-Moreno et al. (2009) show that CXCL13 expression colocalizes in $CD11b^+$ cells, presumably macrophages, and stromal cells in an atypical cup-like structure around the B cell areas (Figure 1). In line with this is the reported expression of CXCL13 by peritoneal macrophages as well as radio-resistant cells in the omentum (Ansel et al., 2002). Therefore, these CXCL13-producing stromal cells and macrophages can be considered as the milky-spot-organizing cells. The macrophages in the omentum certainly fulfill the requirement of organizer cells because in parabiosed mice (mice with joined blood circulation), only 10% of these cells were shown to be replaced by cells from the partner over a period of 8 weeks (Ansel et al., 2002) and are thus sessile cells that could organize a structure.

The inducing signal for CXCL13 expression is, unlike in LN and PP development, not delivered by LTi cells, B cells, $LT\alpha$, $LT\beta$, or $TNF\alpha$ (Ansel et al., 2002; Rangel-Moreno et al., 2009). One can envision that activation of macrophages and perhaps even stromal cells, i.e., by bacterial encounter or particulate antigens, may lead to the induction of CXCL13 within the milky spots. Consistent with this model is the observation that germ-free BALB/c mice have strikingly reduced titers of natural antibodies to phosphorylcholine, a cell-surface component of several bacteria (Gearhart et al., 1975). This is similar to $Cxcl13^{-/-}$ mice, which lack milky spots. Perhaps also CXCL13 expression in the omentum is strongly reduced in germ-free mice, consequently leading to reduction or absence of milky spots. Indeed, germ-free rats were reported to have strongly reduced numbers of milky spots (Beelen et al., 1980). The localization of the $CD11b^+$ cells in the omentum seems to be independent of CXCL13 because these cells were present in distinct areas of the omentum in $Cxcl13^{-/-}$ mice and it is therefore likely that additional signals that determine the position of $CD11b^+$ cells are involved in milky spot formation. It remains to be seen whether their positioning is determined

during a certain time window in development. Future studies will also have to resolve whether indeed bacterial components and perhaps other stimuli evoke the expression of CXCL13 in the peritoneal cavity.

Now that we know that activation of T lymphocytes can take place in the milky spots it remains to be seen whether these cells will subsequently leave the milky spots and if so, to which part of the body they preferentially migrate. Analysis of homing receptors expressed on lymphocytes activated in milky spots should give a first indication. Furthermore, the association of the omentum with the gastrointestinal tract makes one wonder whether lymphocyte subsets that are characteristically associated with the mucosal immune system, such as Treg cells, Th17 cells, and IgA-producing B cells, are also found, and perhaps generated, in milky spots. Future studies are needed to address these questions.

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