Flippin' lipids

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B cell generation is disturbed in four newly identified mouse mutants bearing X-linked mutations in the gene encoding the ATPase ATP11C. These findings suggest that the distribution of membrane phospholipids confers a yet-to-be delineated developmental signal.

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The mechanisms of B lymphopoiesis are often described in terms of interrelated signaling and transcriptional networks that enforce cell fate 'decisions'[1–3]. Knowledge of these mechanisms is considerable in part because they are amenable to delineation by biochemical and genetic means. In contrast, little is known about how the structure and dynamics of cellular membranes contribute to development in the bone marrow. Two papers in this issue of *Nature Immunology* provide compelling evidence that a molecule linked to control of the distribution and turnover of aminophospholipids in membranes has an indispensable and very specific role in B lymphopoiesis[4,5].

Two independent laboratories have used *N*-ethyl-*N*-nitrosurea–mutagenesis to identify four separate mutant mouse strains that manifest profound peripheral B lymphopenia with X-linked recessive inheritance. Positional cloning and deep exon sequencing demonstrates that all four strains are associated with mutations in the gene encoding the putative P4 ATP phospholipid translocase ('flippase') ATP11C. Each strain is associated with a different point mutation predicted to alter the ATP11C protein, yet all four strains manifest a very similar B cell phenotype with severe and progressive deficiencies in the pro-B cell and pre-B cell compartments.

ATP11C is a member of a large (14 mammalian members) family of ubiquitously expressed transporters that flip aminophospholipids from the outer membrane leaflet into the cytosolic leaflet of plasma and endosomal membranes[6,7] (**Fig. 1a**). These ten-transmembrane integral proteins use ATP to transfer the polar head of the aminophospholipid through the hydrophobic center of the lipid bilayer against concentration gradients. The main substrates for the P4 ATP flippases seem to be phosphatidylserine (PS) and, to a lesser extent, phosphatidylethanolamine[8]. There are also ATP-dependent 'floppases' that move substrates such as cholesterol, sphingolipid and phosphatidylcholine into the outer leaflet of membranes, as well as bidirectional, ATP-independent 'scramblases'. Together, flippases and floppases serve to establish and maintain the asymmetrical distribution of cholesterol and phospholipids in biological membranes.

Most studies of flippases have used gene targeting in yeast[7]. In mammals, mutation of the gene encoding one family member, ATP8B1, is associated with heritable intrahepatic cholestasis[9]. Indeed, ATP11C is also important for liver homeostasis, as mice with mutant ATP11C develop hepatocellular carcinoma. However, beyond their role in the liver, there is very limited understanding of the *in vivo* functions of the P4 ATP flippases in complex organisms.

The importance of the usual flippase substrate, PS, is well described[8] (**Fig. 1b**). Normally, PS is almost exclusively found on the inner leaflet of the plasma membrane and on the cytosolic surfaces of early endocytic vesicles. Redistribution of PS to the plasma membrane external leaflet, as occurs in programmed cell death, leads to the rapid clearance of these apoptotic cells. In healthy cells, the concentration of PS in lipid rafts helps determine their composition of both protein and cholesterol. PS can also directly recruit signaling molecules. Members of the protein kinase C family have a C2 domain that binds PS when sufficient intracellular calcium is present. Signaling molecules that contain polycationic regions closely adjacent to hydrophobic side chains, such as farnesyl or fatty acyl moieties, can also be recruited to PS; examples of these include Ras, Rho and Src. In terms of biological functions in cells, PS can serve to determine the local curvature of membranes and recruit effector molecules to intracellular vesicles. Through these and other mechanisms, PS has a vital role in many cellular functions, including endocytosis, vesicle biogenesis and exocytosis.

The defects in B lymphopoiesis in mice with mutant P4 ATP are striking. Aberrations are observed in the earliest committed B cell progenitors (pro-B cells) and are the most severe in the subsequent pre-B cell and immature B cell compartments; the last compartment is about 50-fold smaller in the mutant mice. Furthermore, proliferation of both pro-B cells and pre-B cells is diminished, and the pre-B cell compartment fails to upregulate CD25, a marker dependent on the precursor B cell antigen receptor (pre-BCR). Detailed comparison with *Cd79a*$^{-/-}$ mice, which do not express a pre-BCR, indicates that initial expression of the intracellular immunoglobulin heavy chain is compromised at a stage before pre-BCR-mediated selection[4].

How could a molecule involved in moving aminophospholipids around in membranes induce such a profound and specific developmental block? Consistent with a defect in signaling through the interleukin 7 receptor (IL-7R), mutant mice are completely unresponsive to IL-7 overexpression *in vivo* even though this induces robust expansion of some B cell progenitor populations in wild-type mice. There are corresponding *in vitro* defects in proliferation in response to high concentrations of IL-7, and the *in vitro* differentiation

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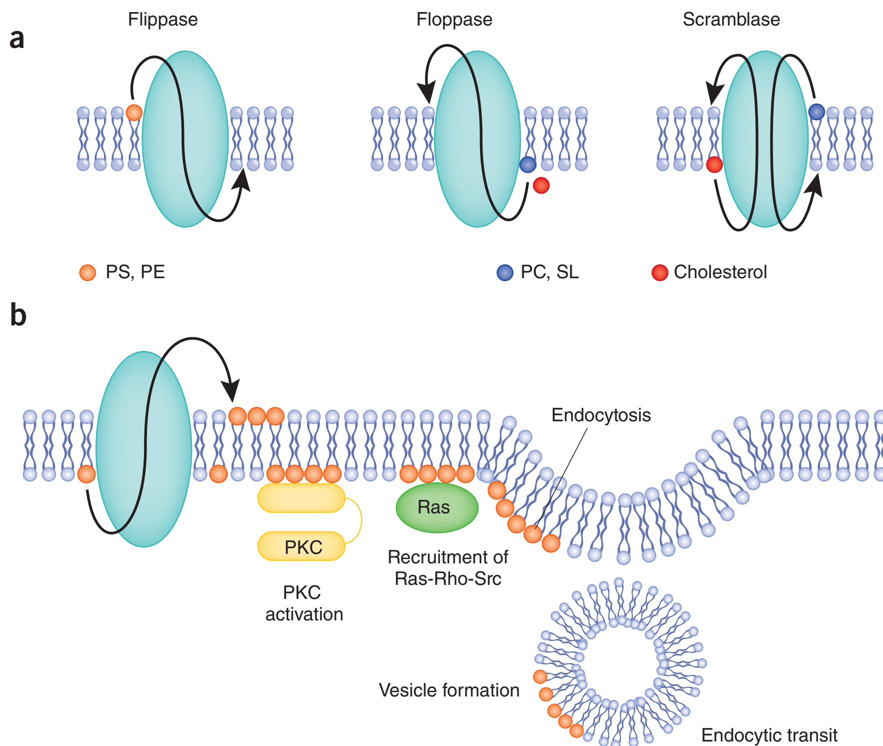

Figure 1 Flippases, floppases and PS. (**a**) Comparison of the functions of flippases, floppases and scramblases in the plasma membrane. Flippases (left) use ATP to move the aminophospholipids PS and, to a lesser extent, phosphatidylethanolamine (PE), from the outer leaflet to the inner leaflet of the PM against a concentration gradient. Floppases (middle) use ATP to transport substrates such as phosphatidylcholine (PC), sphingolipid (SL) and cholesterol against concentration gradients in the opposite direction. Scramblases (right) are ATP independent and less substrate specific and facilitate the movement of lipids along concentration gradients. (**b**) Some PS functions in cells. When cells undergo apoptosis (left), the activation of scramblases allows the rapid appearance of PS on the outer leaflet of the plasma membrane, where it provides an 'eat me' signal. On the PM inner leaflet (middle), PS helps organize lipid rafts and can serve to recruit members of the protein kinase C (PKC) family through their C2 domain, as well as signaling molecules containing hydrophobic side chains, such as Ras, Rho and Src. PS can induce local membrane curvature (right) and recruit specific effector complexes that facilitate receptor endocytosis, vesicle formation and endocytic trafficking.

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of pro-B cells into immature B cells is diminished. However, the magnitude of these *in vitro* defects seems to be less than that of the corresponding *in vivo* aberrations, which suggests that mutations in the gene encoding ATP11C induce more than a simple cell-intrinsic unresponsiveness to IL-7.

Indeed, only bone marrow lymphopoiesis is defective in mice with mutant ATP11C. They show normal fetal liver lymphopoiesis, and marginal zone B cells, which are not dependent on bone marrow lymphopoiesis because they self-renewing, are present in normal numbers. Subsequent studies demonstrate that B cell progenitors derived from ATP11C-deficient fetal liver do not support lymphopoiesis in the bone marrow[5]. These data demonstrate a cell-intrinsic but context-dependent requirement for ATP11C.

There are several possible reasons for the finding that the requirements for IL-7 responsiveness in the bone marrow are different from those in the fetal liver. It is likely that positioning of B cell progenitors relative to IL-7-defined niches is more complex in the bone marrow than in the liver[10]. There also might be more rigorous requirements for one or more ATP11C-dependent IL-7R signaling arms in the bone marrow than in the liver. Finally, the duration of IL-7R signaling might be important for the regulation of some targets, and it is very possible that ATP11C contributes to intrinsic mechanisms that regulate IL-7 responsiveness.

A compelling case can be made for aberrant IL7-R function in ATP11C mice. However, that might not be the whole story. Mice with mutant ATP11C have diminished expression of both immunoglobulin μ-chain and the transcription factor EBF, and this might reflect diminished signaling by the transcription factor STAT5 downstream of IL-7R[11,12]. However, published studies have indicated that STAT5 is permissive for development at the pro-B cell stage and that it does not directly induce differentiation programs in such cells[12]. Aberrant recombination of immunoglobulin genes also does not fully explain the phenotype of the mice with mutant ATP11C. Bypassing variable-diversity-joining recombination by providing recombined immunoglobulin heavy and light chains only partially reconstitutes B lymphopoiesis. Likewise, enforced expression of the prosurvival factor Bcl-2 only partially restores the size of the various B cell progenitor compartments. Together these observations suggest that ATP11C may have a role in enabling several processes needed for the population expansion and differentiation of pro-B cells and pre-B cells.

Pro-B cells with mutant ATP11C clear a PS analog from the external leaflet of the plasma membrane (flipped the lipid onto the internal leaflet) more slowly than wild-type cells do[4]. However, steady-state PS concentrations in the external leaflet are similar in wild-type and ATP11C-mutant cells. This defect in rate is specific for pro-B cells and is not detected either earlier or later in development. Interestingly, similar defects in PS turnover are detected in $CD4^+CD8^+$ and $CD4^-CD8^-$ thymocytes without corresponding aberrancies in development. As PS is the main known substrate for the P4 ATP flippase family, these data suggest that the kinetics of PS distribution are important in early B lymphopoiesis. However, the particular substrate specificity of ATP11C in unknown, and therefore it is possible that some other substrate or activity of ATP11C is required.

These two papers suggest that the distribution of phospholipids in membranes and the molecules that regulate this enable B lymphopoiesis. This new idea raises a whole host of questions. What exactly is ATP11C doing in pro-B cells? How does it enhance IL-7 responses and what other processes does it contribute to? Why does mutation of the gene encoding one ubiquitously expressed molecule induce such a specific defect? Why is ATP11C dispensable for T lymphopoiesis and why are there 14 mammalian P4 ATP flippases? These and other questions will need to be addressed in further studies.

COMPETING FINANCIAL INTERESTS
The author declares no competing financial interests.

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An unexpected role for MHC class II

Ghada S Hassan & Walid Mourad

In addition to their classical function in antigen presentation and their lesser known ability to act as signal transducers, major histocompatibility complex class II molecules are now shown to promote Toll-like receptor signaling. This intriguing role requires intracellular association with the kinase Btk and the costimulatory molecule CD40.

The innate immune response has evolved as a first line of defense against invading pathogens. Innate immunity operates through the recognition of pathogen-associated molecular patterns via specialized pattern-recognition receptors, including Toll-like receptors (TLRs), Nod-like receptors and RIG-I-like receptors. TLRs can be located intracellularly or on the plasma membrane. After engagement of the TLR, a cascade of intracellular events leads to inflammatory cytokine production, higher expression of costimulatory molecules on antigen-presenting cells and, subsequently, greater efficiency of antigen presentation by major histocompatibility complex (MHC) class II and class I molecules. In addition to their classical role in antigen presentation, MHC class II molecules have been linked to various other cellular processes ranging from proliferation, maturation and cytokine production to apoptosis. In this issue of *Nature Immunology,* Liu *et al.* demonstrate a nonclassical role for MHC class II molecules as important adaptor proteins in TLR-mediated signaling[1].

A role for MHC class II molecules in TLR signaling was first suggested by studies describing the defective inflammatory response of lipopolysaccharide-stimulated macrophages with no or low expression of MHC class II[2]. Subsequently, the crosstalk of MHC class II with TLRs was further demonstrated by experiments showing that coexpression of both molecules in human embryonic kidney HEK293 cells upregulates the responsiveness of TLRs, particularly TLR2 and TLR4, to their ligands[3]. In addition, MHC class II molecules have been shown to colocalize and physically associate with TLR2, a process that facilitates the synergy observed between these adaptive and innate elements[3]. Liu *et al.*, in contrast, do not observe any direct physical association between TLRs and MHC class II molecules[1]. However, they do report association of MHC class II with the costimulatory molecule CD40 and the tyrosine kinase Btk in the endosomes of TLR-stimulated macrophages and suggest that this association underlies the enhancing effect of MHC class II molecules on TLR-induced signaling. This TLR signal–enhancing role adds to a growing list of nonclassical functions for MHC class II molecules[4].

Indeed, studies have demonstrated a role for MHC class II dimerization in the production of proinflammatory molecules and chemokines by inflammatory cells[5]. Such dimerization, therefore, has the collective effect of recruiting leukocytes and amplifying the immune response. These functions of MHC class II have been shown to engage various intracellular signaling events, including activation of the signaling protein PLC, the kinases Src, Syk and PKC, and the mitogen activated kinases p38 and Erk[4]. As the MHC class II molecule lacks any known signaling motifs in its short cytoplasmic tail, it has been proposed that it might use associated signaling molecules at the membrane or intracellularly to initiate signaling, with the latter process being demonstrated here by Liu *et al.*[1]. Cell receptors such as CD20, CD79, CD23 and members of the immunoglobulin family (such as CD19), tetraspanin family (such as CD81 and CD82) and tumor necrosis factor receptor family (such as CD40) have all been shown to associate with MHC class II molecules and mediate their signaling[4]. Together such findings reinforce the idea of nonclassical MHC class II functions. However, whether these signaling functions link MHC class II molecules to signaling directly or only indirectly via the association of MHC class II with adaptor proteins has remained a mystery. Indeed, this question has remained largely unvisited for the better part of a decade, and it was not until this work by Liu *et al.* demonstrating the intersection of MHC class II with the TLR pathway that new light has been shed on the mechanism of MHC class II signaling.

Given the new evidence, it can be proposed that TLRs themselves could also enhance MHC class II–mediated responses. Thus, TLRs could act as adaptor receptors, influencing the responses induced by MHC class II molecules. Evidence for such a TLR-dependent MHC class II response has been provided by several lines of data. First, signaling mediated via TLRs or MHC class II molecules involves the activation of many shared intracellular effectors, such as protein tyrosine kinases. Second, TLR- and MHC-mediated responses both engage MyD88, an important adaptor molecule[6]. Third, stimulation with lipopolysaccharide can enhance autophagy-dependent antigen presentation by MHC class II molecules. Moreover, TLR signaling enhances association of the TLR adaptor proteins MyD88 and TRIF with beclin-1, a key regulator of autophagy and antigen uptake, and thus facilitates the access of antigens to MHC class II molecules[7]. Such a TLR-dependent MHC class II response could therefore explain certain aspects of intracellular signaling.

An additional link between MHC class II and TLR signaling is the finding by Liu *et al.* that CD40 is a key component in the crosstalk between the two pathways[1]. In this context, there is a distinct precedent for CD40–MHC class II interactions. Indeed, MHC class II can be associated with CD40 both physically and functionally[8,9]. For example, engagement of MHC class II with specific monoclonal antibodies enhances CD40-mediated activation of B cells. CD40 stimulation is able to provide the signal needed for MHC class II–mediated induction of tumor necrosis factor and interleukin 1β in the absence of MHC class II dimerization[8]. Furthermore, MHC class II molecules constitutively show stable

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