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cytokine IL-18.[4,5] Because IL-18 is an interferon γ–driving cytokine, this allowed for the retention of a similar pathogenic model centered on interferon γ; however, instead of perforin defects being the proximal mediator, the upstream event was overproduction of IL-18, either by genetic (*NLRC4*, *XIAP*, *CDC42*) causes or for as yet unknown reasons.

Although these 2 means to an interferon γ cytokine storm have been largely considered separately in studies, Tsoukas et al now show that these 2 pathways synergize to produce an even more fulminant storm. Perforin deficiency is typically insufficient to produce spontaneous HLH, requiring an infection to set off the disease. Similarly, transgenic overproduction of IL-18 in mice does not produce MAS. However, when a mouse is generated that has both defects, spontaneous HLH occurs, driven by interferon γ and CD8 T cells. Perhaps most striking, even mice that are only haploinsufficient for perforin, but also overproduce IL-18, develop hyperinflammatory disease.

It has been increasingly recognized that patients with unexplained HLH/MAS may have an increased burden of heterozygous genetic defects in cellular cytoxicity genes. However, such defects are reasonably common in healthy populations and thus insufficient to explain the development of HLH by themselves. The work of Tsoukas et al now provides a model that can add clarity to this apparent conundrum, suggesting that in the setting of heterozygous defects in perforin, excess IL-18 can tip the precarious balance of the immune system from homeostasis toward a cytokine storm. IL-18 is a clinically measurable cytokine; therefore, this information could become important diagnostically to determine HLH/MAS status and may have therapeutic implications, because interferon γ blockade is already available, and IL-18 blockade has been described in single patients[6] and has an ongoing clinical trial (registered at www.clinicaltrials.gov as #NCT03113760).

It is interesting to speculate that there may be other cytokines or defects that can combine in the same synergistic manner to contribute to total HLH/MAS risk. Other IL-1 family members have been implicated, including IL-1β[7] and IL-33.[8] Perhaps heretofore unrecognized immune pathways also play a role in contributing. Continuing to add to the model of independently small insults that can synergize to produce large pathologic effects in a cytokine storm will be an important area of investigation in driving both diagnostic and therapeutic improvements in the care of HLH/MAS.

Conflict-of-interest disclosure: E.M.B. is principal investigator of a clinical trial sponsored by AB2Bio for tadekinig alfa in NLRC4 and XIAP. ■

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DOI 10.1182/blood.2020007469

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PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

Comment on Margraf et al, page 2200

Implicating ILK in inflammation

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In this issue of *Blood*, Margraf et al selectively delete integrin linked kinase (ILK) in myeloid cells of mice to show that this integrin-binding protein suppresses chemokine-induced neutrophil extravasation and ischemia-induced reperfusion injury.[1]

The mechanism underlying these blunted responses in the ILK-deficient animals is a consequence of failure of the cells to activate the leukocyte integrin lymphocyte function-associated antigen-1 (LFA-1) ($\alpha_L\beta_2$, CD11a/CD18), such that it can recognize ICAM-1 on endothelial cells. ILK localizes in adherent complexes on the plasma membrane and is well-recognized to be a key regulator of integrin function. Indeed, ILK was first identified as an integrin β_1 subunit binding partner in a yeast 2 hybrid screen.[2] ILK associates tightly with 2 other proteins—particularly interesting new cysteine-histidine rich protein (PINCH) and parvin—to form the ILK-PINCH-parvin (IPP) complex, which serves as an adaptor from many binding partners and provides a linkage between integrins and the actin cytoskeleton.[3] Global knockout of ILK is lethal in mice, fish, and flies,[3,4] and tissue-specific knockouts of ILK have been associated with a wide range of organ-specific defects.

In the study by Margraf et al, the central role of ILK relates to its regulation of LFA-1 ($\alpha_L\beta_2$, CD11a/CD18), 1 of the 4 β_2 leukocyte integrins that is composed of a non-covalent heterodimer of the transmembrane α_L and β_2 subunits (see figure). On unstimulated leukocytes, LFA-1 exists in a quiescent state in which it exhibits low capacity to bind ICAM-1. Upon cytokine stimulation of leukocytes, LFA-1 transforms

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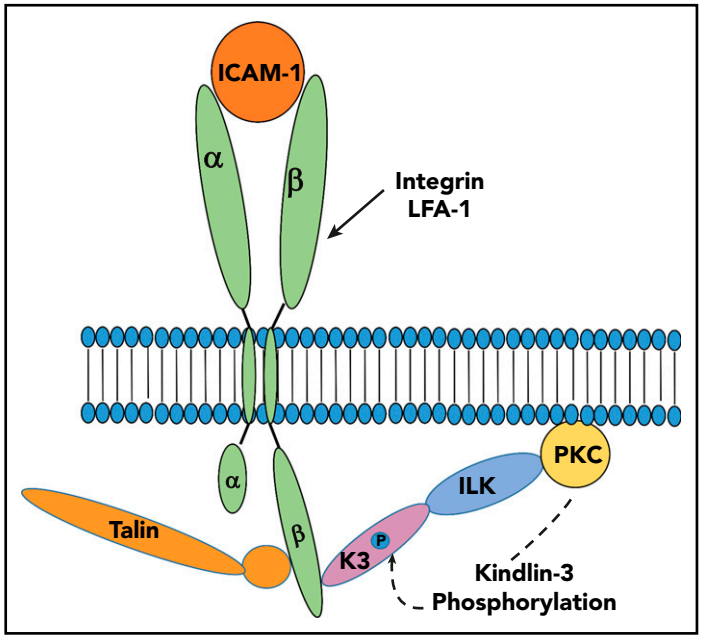

A new ILK-dependent pathway of LFA-1 activation on neutrophils. ILK facilitates recruitment of PCK-α to the cell membrane where it supports phosphorylation of a specific residue in kindlin-3. This modification enhances the capacity of kindlin-3 to cooperate with talin-1 to activate integrin LFA-1 and its recognition of ICAM-1. Illustration by Katarzyna Bialkowska.

to a high-affinity and high-avidity state in which it can bind to ICAM-1 expressed from endothelial cells. This interaction can lead to arrest and extravasation of leukocytes, including neutrophils. This transition of the integrin, referred to as activation, is not only necessary for LFA-1 to efficiently recognize ICAM-1 but is also necessary for productive engagement of many ligands by many different integrins. Integrin activation is induced by association of the cytoplasmic tail of the β subunit with specific binding partners. These interactions trigger an inside-out signal that traverses the transmembrane segments and ultimately optimizes the relative positioning of the ligand-binding motifs within the extracellular domains of the α and β subunits to accommodate ligands such as ICAM-1. Talins, which are large cytoskeletal proteins, and kindlins, a 3-member family of cytoplasmic proteins, are both critical for integrin activation.[5,6] Both talin and the kindlins contain a 4.1-ezrin-radixin-moesin (FERM) domain that regulates integrin activation by binding to the cytoplasmic tail of the integrin β subunit, although they bind to different sites in the β subunit. In leukocytes, it is kindlin-3 that binds to integrin β subunits and regulates activation of LFA-1.[5,6]

The study by Margraf et al establishes a pivotal role of ILK in activating LFA-1 and its recognition of ICAM-1 for neutrophil recruitment into tissues and also defines a mechanism for the ILK–LFA-1 collaboration in mounting an inflammatory response. The authors show that ILK is required for phosphorylation of kindlin-3 on a single serine residue, and this post-translational modification enhances its ability to promote activation of LFA-1. Such phosphorylation of kindlin-3 has been shown to enhance activation of β_3 integrins.[7] If ILK were a kinase, the pathway would be simple and direct because ILK and kindlins bind to one another.[8] However, although still somewhat controversial, the preponderance of recent evidence suggests that ILK is not a true kinase but is instead a pseudokinase.[3] Therefore, it is likely that the ability of ILK to support kindlin-3 phosphorylation is indirect. Margraf et al demonstrate that kindlin-3 phosphorylation depends on the capacity of ILK to recruit PKC-α to the cell membrane, where its kinase activity leads (directly or indirectly) to kindlin-3 phosphorylation. Thus, by using a PKC-α inhibitor or bone marrow chimera deficient in PKC-α, LFA-1–dependent leukocyte arrest is significantly blunted, and a mutant kindlin-3 that could not be phosphorylated reduced LFA-1–mediated interaction with ICAM-1.

To summarize, the Margraf et al study suggests a pathway leading to LFA-1 activation as depicted in the figure. Cytokine stimulation supports recruitment and extravasation of neutrophils during inflammation by activation of LFA-1. This activation is dependent upon ILK, which recruits PKC-α to the cell membrane where it encourages the phosphorylation of kindlin-3, which enhances LFA-1 activation in cooperation with talin. Thus, optimal alignment of talin, kindlin, and PKC-α by their interaction with membrane lipids is likely to be critical for maximal integrin activation.

One interesting facet of the Margraf et al study is that although ILK is involved in ICAM-1 recognition by LFA-1, fibrinogen recognition by a second leukocyte integrin, Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) (which contains the same β_2 subunit and therefore the same binding sites for ILK, kindlin-3, and talin-1), does not show the same dependence on ILK for its activation. There are several possible explanations. One interesting possibility is that the mechanism of activation may be distinct for the 2 integrins. Intracellular signaling induced by fibrinogen binding to Mac-1 is particularly dependent on integrin clustering[9] whereas LFA-1 activation is more dependent on conformational changes in the integrin.[10] Both LFA-1 and Mac-1 interact with ICAM-1, but distinct sites in ICAM-1 are recognized by the 2 integrins, and the induction of different downstream signaling events raises the possibility of biased agonism/antagonism to control specific inflammatory responses mediated by each integrin.

Conflict-of-interest disclosure: The authors declare no competing financial interests. ■

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DOI 10.1182/blood.2020008316

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RED CELLS, IRON, AND ERYTHROPOIESIS

Comment on Sangkhae et al, page 2206

Maternal hepcidin: the only player on the field?

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In this issue of *Blood*, Sangkhae et al[1] use murine model systems and hepcidin analogs to offer valuable new insights into the central role of maternal hepcidin in determining fetal iron status, the deleterious consequences of its elevation during pregnancy, and its potential relevance in the setting of inflammation. Iron deficiency during pregnancy is prevalent and associated with negative outcomes for both mother and fetus. Inflammation during pregnancy is also common, often subclinical, and associated with adverse outcomes. Profound changes in iron homeostasis occur with systemic inflammation in other settings, many of these being consequent to the increased production of the hormone hepcidin. Investigating the effects of inflammation on iron homeostasis during pregnancy has been challenged by the complex interplay between maternal, placental, and fetal regulatory processes.

Pregnancy is normally a very low hepcidin state in both mother and fetus, thereby optimizing plasma iron availability for expanding fetal needs. The investigators used mice in which hepcidin was knocked out in either the mother or the fetus and determined that maternal rather than fetal or placental hepcidin determines fetal iron status. Loss of maternal hepcidin increased iron delivery to placenta and fetus; loss of fetal hepcidin had no significant effect on measured iron parameters in mother or fetus. The mechanisms by which pregnancy suppresses maternal hepcidin remain to be determined but are of great interest as elucidation may uncover new molecules and/or pathways that can be used therapeutically. The basis for the very low expression of liver fetal hepcidin (compared with postnatal timeframes) is likewise unknown. Plausibly, the hypoxic environment and consequent high erythropoietin levels contribute. Erythroferrone, an erythroid suppressor of hepcidin,[2] is upregulated by erythropoietin. Because liver is the primary site of fetal erythropoiesis, local production of erythroferrone may be important. A fetal regulatory circuit presumably exists that allows the fetus to convey increased iron needs in settings of excessive fetal erythropoiesis or multiparous pregnancies.

The investigators next examine whether maternal hepcidin is in excess in pregnancies complicated by inflammation. They determine that systemic inflammation (lipopolysaccharide injection) overcomes the factors that suppress maternal hepcidin. To specifically parse out the effect of hepcidin on maternal-fetal iron metabolism, they administered minihepcidins to uninflamed pregnant dams (see figure). In a high-dose regimen, minihepcidin treatment decreased maternal serum and hepatic iron, increased splenic iron, and caused anemia. At the highest-dose regimen, the effect was severe enough to decrease placental iron and weight, despite compensatory "selfish placenta"[3] increases in transferrin receptor 1 (increasing iron uptake from the mother), and decreases in ferroportin (withholding iron transfer to the fetus). The consequences to the fetus in this setting were severe, leading to demise. A shorter-duration regimen did not decrease placental iron and led to decreased fetal serum, liver, and brain iron concentrations and fetal anemia. Similar changes of smaller magnitude were seen at a lower-dose regimen that did not significantly affect maternal iron parameters or hemoglobin. If relevant to the human situation, such observations suggest that the fetus might be sensitive to maternal elevations in hepcidin not apparent in routine screening tests for maternal iron status. Because inflammation during pregnancy is often subclinical, it is important in studies on iron metabolism during pregnancy to assess maternal hepcidin-mediated effects.[4] Moreover, because ferritin is an acute phase protein, using maternal or cord ferritin levels in assessing iron status might be confounded despite the absence of clinical inflammation.

It is important to emphasize that hepcidin excess is not the only mediator of the changes in iron metabolism with inflammation. Ceruloplasmin, transferrin, and ferritin are each regulated by inflammatory signals.[5] Inflammation also suppresses erythropoiesis, and thus iron utilization, by mechanisms that are not entirely hepcidin dependent. As such, the minihepcidin experimental systems used by Sangkhae et al would not be expected to completely model iron metabolism during inflammation. They do, nonetheless, raise the possibility that elevated maternal hepcidin contributes to negative outcomes with inflammation during pregnancy.

The investigators' studies clearly show the deleterious consequences of elevated maternal hepcidin on fetal iron status. Do elevations in fetal hepcidin have any contribution? As pointed out by the authors, increases in fetal hepcidin have been shown to affect fetal iron status in certain experimental systems, including inflammation. Administration of lipopolysaccharide to pregnant mice increased fetal liver hepcidin production, in association with decreased fetal serum iron.[6] Intraamniotic injection of lipopolysaccharide in rhesus macaques had similar effects, and without increased maternal cytokines or maternal hepcidin.[6] Suggesting that these observations are

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