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Previews

Alert from a Distant Neighbor: Spread of Antiviral Immunity through Anion Channels

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Cytosolic DNA detection via the DNA sensor cGAS initiates a major cell-intrinsic response to infection and malignancies. In this issue of *Immunity*, Zhou et al. (2020) report that the catalytic product of cGAS, cGAMP, can alert bystander cells over large distances through its cell-to-cell transmission via volume-regulated anion channels.

The discovery and characterization of the DNA sensor cyclic guanosine monophosphate (GMP)-AMP synthase (cGAS) has galvanized our understanding of cellular responses to viral and microbial infections, autoimmunity, cancer malignancies, and cell senescence. cGAS senses DNA of aberrant subcellular localization through interaction via its DNA-binding domain, an event that triggers the catalysis of the second messenger and small molecule cGAMP. cGAMP then induces a STING-TBK1-IRF3-dependent signaling cascade that culminates in the expression of IRF-3-induced genes and type I interferons (IFNs), which in turn elicit an antiviral state by transactivating multiple IFN-stimulated genes (Gao et al., 2013; Sun et al., 2013). In addition to its activity in the very same cell in which it has been synthesized, cGAMP can serve as an amplificator of innate immunity by spreading to bystander cells, in which stimulator of interferon genes (STING) is directly activated (Figure 1). Transfer of cGAMP to adjacent cells via gap junctions enables a fast and sustained antiviral response to modified vaccinia virus Ankara infection (Ablasser et al., 2013). Similarly, naturally occurring membrane fusion sites established in HIV-1-infected cultures, which are mediated by interaction of viral glycoproteins presented on the surface of infected cells and cellular receptor and coreceptor complexes expressed on bystander cells, allow the intercellular spread of cGAMP cells (Xu et al., 2016). Two studies proposed that cells might even exploit virus particles as cGAMP vehicles for cell-to-cell transmission, giving some interesting prospects for development of cGAMP-loaded vaccine vector particles (Bridgeman et al., 2015; Gentili et al., 2015). Recently, the folate-organic phosphate antiporter Slc19a1 was identified as a transporter of exogenous cyclic dinucleotides in human cells. In this issue of *Immunity*, Zhou et al. (2020) report on the ability of LRRC8 volume-regulated anion channels (VRACs) to mediate export of cGAMP to, and import of cGAMP from, the extracellular space, thus potentially enabling cGAMP-based intercellular communication over long distances and circumventing the need for close, physical contact between cells (Figure 1). By screening a limited number of pharmacological anion channel modulators in HSV-1-infected mouse embryonic fibroblasts, the authors identified that inhibition of VRACs resulted in increased cell death and enhanced virus spread. These observations correlated with suppression of the type I IFN response that was normally observed in HSV-1-infected cells. The contribution of VRACs at mounting the virus-induced immune response was corroborated by a genetic approach, during which the expression of *Lrrc8a*, the gene encoding an essential subunit of VRAC, was ablated. This genetic manipulation resulted in a blunted type I IFN response and enhanced virus spread. Importantly, these observations were recapitulated in several primary mouse cell types, including lung fibroblasts and bone-marrow-derived macrophages, suggesting that VRAC-mediated enhancement of antiviral responses is a rather ubiquitous phenomenon. Individual deletions of all possible VRAC subunits revealed that HSV-1 infection-induced type I IFN responses are specifically boosted by heteromeric VRACs composed of the two subunits LRRC8A and LRRC8E.

Interestingly, pharmacological and genetic interference with VRACs modulated neither the spread of the RNA vesicular stomatitis virus (VSV) nor the corresponding cellular type I IFN response. These results let the authors suspect that cGAS-STING-dependent signaling pathways, which are triggered by DNA replication intermediates and DNA genomic material that happen to escape the shielding viral capsid of several DNA viruses including HSV-1 but not RNA viruses, might be involved. TBK1, IRF3, and STAT1 phosphorylation was robustly activated both in HSV-1- and VSV-infected wild-type cells. However, ablation of *Lrrc8a* expression compromised these events in the context of HSV-1 but not VSV infection. Equally puzzling, when cGAMP was directly transfected into *Lrrc8a*-negative cells to directly activate STING, TBK1, IRF3, and STAT1 were efficiently phosphorylated.

VRACs' physiological functions include the transport of metabolites and drugs. Among other roles, they regulate cell volume by releasing anions and organic osmolytes upon cell swelling (Chen et al., 2019). Given their ability to mediate transport of anions, the negative charge of cGAMP, and the surprising observation that cGAMP was operative in *Lrrc8a*-negative cells only upon direct transfection, it was very tempting to test whether VRACs are involved in cGAMP uptake processes.

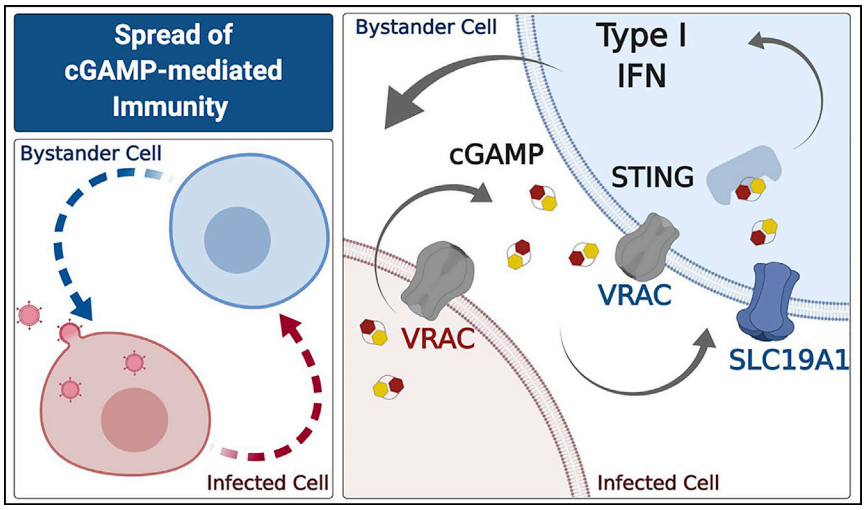

Figure 1. Spread of cGAS-Mediated Innate Immunity to Bystander Cells
Upon activation, the DNA sensor cGAS produces the cyclic dinucleotide 2′-3′-cGAMP. This second messenger does not only induce an autocrine signaling cascade but can also trigger a STING-dependent type I IFN response in bystander cells. The most recently discovered route of intercellular spread of cGAMP, discovered and characterized by Zhou et al. (2020), includes export and import via voltage-regulated anion channels (VRACs), allowing transfer of extracellular cGAMP over long distances and overcoming the need of direct physical contact of cells.

Strikingly, extracellularly added cGAMP provoked type I IFN responses in a largely *Lrrc8a*- and *Lrrc8e*-dependent manner, in line with the idea that VRACs are involved in cellular import of exogenous cGAMP. VRAC inhibitors and agonists reduced and promoted cGAMP uptake, respectively, confirming the emerging working hypothesis of VRAC-mediated uptake of cGAMP. Furthermore, cell swelling induced by VRAC activation through exposure to hypotonic medium enhanced uptake of extracellular cGAMP, in constrast to hypertonic medium conditions.

However, one question remained still unanswered: what is the source for extracellular cGAMP whose cellular uptake was shown to occur via VRACs? Upon HSV-1 infection of wild-type cells, the authors detected cGAMP in the supernatant of cell cultures. Viability of cells was preserved, arguing against the possibility that cGAMP was released by virus-producing cells that may be in the process of undergoing necrotic cell death. It rather seemed that cGAMP produced in response to virus infection was exported into the extracellular medium. Concentrations of cGAMP in the culture supernatant of *Lrrc8a*-deficient cells were reduced, indeed suggesting that VRACs mediate, in addition to cGAMP import, its export. Finally, whole-cell patch-clamp experiments provided direct demonstration of the ability of cGAMP to pass through VRAC pores.

Transwell experiments provided evidence that physical interaction of cells is not required for cGAMP exchange, supporting the concept of transfer of free cGAMP between cells. Because the majority of previous reports of intercellular transfer of cGAMP in the context of virus infection demonstrated the strict need of physical interaction between donor and recipient cells (Ablasser et al., 2013; Xu et al., 2016) or of fusogenic extracellular vesicles or virions serving as vehicles (Bridgeman et al., 2015; Gentili et al., 2015) for a functional cGAMP-mediated cellular response, the observation of apparently free and active cGAMP prone to cellular import comes as a surprise and might hint toward cell line, cell type, cell species, or culture condition specificities among the different studies.

In vivo, *Lrrc8e*$^{-/-}$ mice, in comparison to wild-type mice, displayed compromised responses to HSV-1 infection, higher HSV-1 gene expression levels, and higher HSV-1 titers in several tissues and organs, indicating that expression of the specific VRAC heteromer that serves as an immonotransporter is crucial to control viral infections. Although it can not be excluded that *Lrrc8e* deficiency results in other processes unrelated to cGAMP transport, absence of alteration of the course of VSV infection and its corresponding cellular response argues against this possibility.

Although the work presents convincing evidence for VRACs serving as a cGAMP conduit, future research to fully unravel this mechanism is warranted. Because VRAC-mediated import and export processes occur along a concentration gradient (Chen et al., 2019), it can be assumed that the directionality of cGAMP transport follows the same rule. Given the possibility of a dilution effect of cGAMP in the extracellular milieu, the distance that these cGAMP transport processes can overcome will be interesting to quantify, which may vary in different body compartments and tissues and depend on cell density and the flowrate of extracellular fluid. Furthermore, it will be important to identify the half-life of extracellular cGAMP that was shown to be susceptible to cleavage by phosphodiesterases (Carozza et al., 2020), depending on their abundance and activities in different tissues. Several DNA viruses encode for antagonists of the cGAS-STING pathway, which however only dampen but not fully block this cellular antiviral defense mechanism. It will thus be informative to test if, additionally, DNA viruses can specifically counteract the intercellular transfer of cGAMP, e.g., through direct inhibition of VRAC activity. Interestingly, virally encoded phosphodiesterases, which reduce the quantities of available cGAMP in the infected cell, have already been identified in poxviruses (Eaglesham et al., 2019). Along this line, intercellular transfer of cGAMP to bystander cells might contribute to its escape from degradation by these viral enzymes. Specifically, comparing the kinetics of virus replication to those of cGAMP production and subsequent intercellular transfer will yield important insights.

Overall, this study expands on the growing concept of the importance of intercellular communication in the context of virus infection and antiviral defense. It introduces the notion that cell-to-cell spread of innate immunity may occur, in a given tissue, over larger distances than previously anticipated. Future research will elucidate if and how VRAC-mediated intercellular transfer of cGAMP is counteracted by viral antagonistic strategies and to what extent it modulates

cGAMP-dependent processes beyond antiviral immunity.

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T Cells: Bridge-and-Channel Commute to the White Pulp

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In contrast to lymph nodes, the lymphoid regions of the spleen—the white pulp—are located deep within the organ, yielding the trafficking paths of T cells in the white pulp largely invisible. In an intravital microscopy tour de force reported in this issue of *Immunity*, Chauveau et al. show that T cells perform unidirectional, perivascular migration through the enigmatic marginal zone bridging channels.

Most of our knowledge on T cell trafficking in secondary lymphoid organs comes from studies in lymph nodes. Naive T cells of the blood circulation continuously move through lymph nodes by crossing through a specialized set of postcapillary venules, the high-endothelial venules (HEVs). T cells interact with HEVs in a well-defined sequence of molecular events, involving the presence of chemokines and adhesion receptors, before they transmigrate through the endothelial layer and enter the T cell cortex of the lymph node. After scanning the T cell parenchyma for antigens presented by antigen-presenting cells, T cells leave the lymph node by entering efferent lymphatics, allowing their return to the systemic blood circulation. In contrast to lymph nodes, which are plugged into the lymphatic system and filter interstitial fluid, the spleen is the immunological filter of the blood circulation. Due to its direct location in the circulatory system, the spleen is unique among all secondary lymphoid organs with a special splenic architecture that is grossly divided into the lymphocyte-rich white pulp (WP) and the erythrocyte-rich red pulp (RP). A region termed the marginal zone (MZ) forms the border between these two compartments in rodents. In the WP, T cells ensheath central arterioles that supply the spleen with arterial blood, while B cells organize as follicles at the outer parts. The MZ surrounds the B cell follicles and harbors special subsets of B cells, macrophages, dendritic cells, and stromal cells (Mebius and Kraal, 2005). As a major difference to lymph nodes, spleens are missing HEVs, and newly arriving T cells from the blood are released with the arterial blood supply. Almost 50 years ago, it was shown that intravenously injected ink particles primarily label the MZ but that at some sites, WP channels penetrate the MZ envelope and directly enter the RP. Similarly, injection of radiolabeled