ReGLUation of cGAS

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The detection of cytosolic DNA by the sensor cGAS triggers potent antiviral responses. New data now propose that cGAS is regulated on a post-translational level by glutamylation.

The sensing of foreign nucleic acids is a major strategy by which the innate immune system senses viral infection. Distinct receptor systems for viral RNA and viral DNA trigger antiviral responses, including the production of type I interferons. In the cytosol, members of the family of RIG-I-like helicases detect viral RNA, whereas the presence of cytosolic double-stranded DNA is sensed by cGAS. To induce the expression of type I interferons, both sensing systems rely on the coordinated activation of transcription factors, including NF-κB and IRF3. However, the cGAS pathway is unusual in that its most upstream components are not linked through classic protein-protein interactions. Instead, cGAS is an enzyme that, after binding DNA, catalyzes the production of a second-messenger molecule, 2′3′-cGAMP, which engages the endoplasmic reticulum adaptor STING[1]. The cGAS-STING cascade is not restricted to signaling in a cell-intrinsic fashion but instead, by means of incorporation into viral particles or through transfer via gap junctions, also functions in a paracrine and systemic manner[2–4]. Thus, tiny amounts of DNA can be sufficient to induce strong systemic immune responses. Given this amplificatory nature of the cGAS–STING–type I interferon pathway, it seems reasonable that regulatory mechanisms would exist to limit excessive activation of this signaling cascade. In this issue of *Nature Immunology*, a report by Fan and colleagues proposes just such a mechanism by which cGAS is regulated[5].

An interesting outcome of research on the role of DNA sensing in controlling pathogen infection has been the appreciation that in addition to viral (or pathogen-derived) DNA, host DNA can also activate cGAS and upregulate cytokine production. Indeed, once naturally occurring turnover of DNA is dysregulated and self DNA accumulates in the cytosol, it can contribute to the pathogenesis of autoimmune diseases[6]. Although such studies have emphasized the important role of DNA-degrading enzymes in the prevention of chronic inflammatory responses, little is known about the regulatory mechanisms that operate directly on cGAS. Controlling the expression of cGAS appears to be one mechanism at play, and indeed cGAS expression undergoes substantial induction after viral infection or stimulation with type I interferons. Cytosolic DNA has also been found to trigger autophagy, and the recruitment of cGAS into autophagosomes and its consequent degradation might constitute another way of limiting its activity[7]. Another means of controlling protein function in a rapid and reversible manner is post-translational modification (PTM). Phosphorylation is a widely applied PTM that controls diverse signal-transduction pathways, and a published study has highlighted the phosphorylation of cGAS by the kinase AKT[8]. Fan and colleagues now propose an alternative mechanism for the regulation of DNA-triggered antiviral responses by identifying glutamylation as a mechanism of controlling cGAS activity[5].

Glutamylation is a reversible PTM in which glutamate side chains of variable length are formed at specific sites on a target protein. The presence and length of the glutamate side chains are regulated in a dynamic fashion by the tubulin-tyrosine ligase–like (TTLL) family of enzymes, which catalyze glutamylation, and by members of the cytosolic carboxypeptidase (CCP) family, which remove the modification. Although it was initially discovered on tubulins, it is now clear that glutamylation is a regulator of diverse cellular processes; however, its involvement in innate immunological signaling pathways has not yet been explored.

On the basis of published work demonstrating that glutamylation regulates megakaryopoiesis[9], Fan and colleagues investigate its possible involvement in signaling via the innate immune system[5]. In an attempt to systematically explore this, the authors start with mice that have individual deletion of each of the six CCP-encoding genes and infect these mice with the DNA virus herpes simplex virus type 1 or vaccinia virus. They observe that absence of CCP5 or CCP6 results in enhanced susceptibility to infection with either of the two viruses, which is paralleled by decreased production of type I interferons. These findings are also evident on a cellular level, with compromised production of type I interferons in bone marrow–derived macrophages or dendritic cells deficient in either CCP5 or CCP6 that are either challenged with DNA virus or stimulated with synthetic double-stranded DNA. *In vivo* and *in vitro* responses to RNA viruses or to the synthetic RNA duplex poly(I:C) are not affected by the absence of either CCP5 or CCP6, which indicates that both enzymes are specifically involved in regulating DNA-sensing pathways but not in regulating RNA-sensing pathways.

Fan and colleagues next focus their attention on identifying the deglutamylation substrate(s) of CCP5 and CCP6. A combination of immunoprecipitation with antibody to glutamate side chains and mass spectrometry results in the identification of cGAS as a glutamylated target of CCP5. In a series of experiments, the authors next characterize two glutamylation sites within cGAS (Glu272 and Glu302 in mice, and Glu286 and Glu372 in humans) and assign specific roles for deglutamylation of these residues by CCP5 and CCP6, with the latter being responsible for removal of polyglutamate chains from Glu272 (human Glu286) and the former being in charge of

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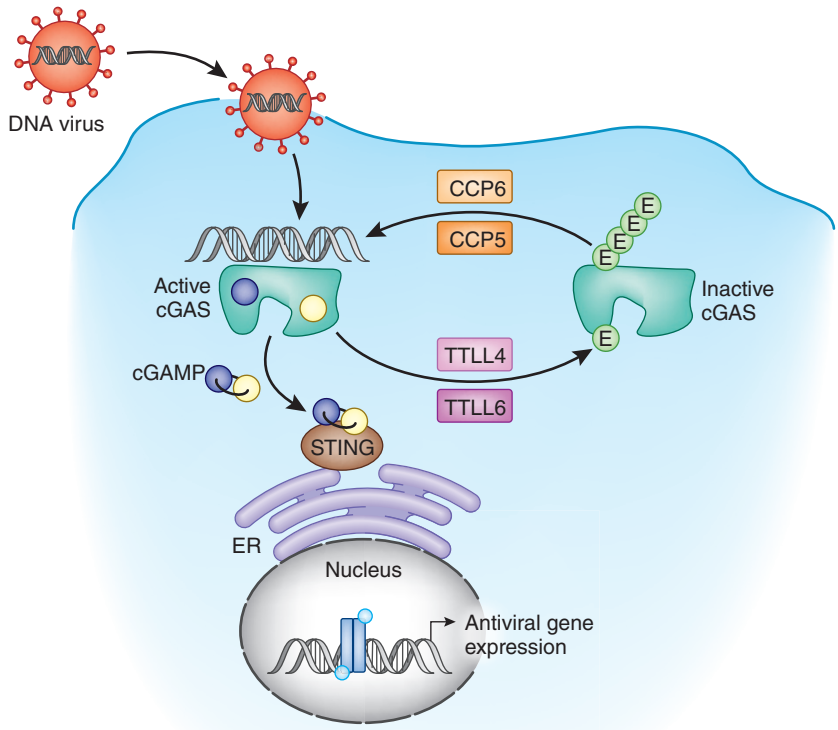

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Figure 1 Glutamylation regulates the activity of cGAS. The carboxypeptidases CCP5 and CCP6 promote cytosolic DNA sensing by removing glutamate side chains from cGAS, thereby enabling DNA binding and cGAMP synthesis. This process is antagonized by the enzymes TTLL4 and TTLL6, which mediate mono- and polyglutmatylation of cGAS. Upon infection with herpes simplex virus, deglutamylated cGAS binds to cytosolic DNA and produces cGAMP, which leads to antiviral gene expression via STING and IRF3. ER, endoplasmic reticulum.

hydrolyzing the monoglutamate chain at Glu302 (human Glu372).

But which enzymes are responsible for the glutamylation of cGAS? To address this, Fan and colleagues analyze nine members of the TTLL family, well known for their ability to modify and mark proteins by glutamylation, assessing their interaction with cGAS. This approach yields the monoglutamylase TTLL4 and the polyglutamylase TTLL6, but not other TTLL enzymes, as cGAS-interaction partners. The authors show that TTLL4 and TTLL6 have non-redundant roles in the glutamylation of cGAS, with TTLL4 mediating the monoglutamylation of cGAS at Glu302, and TTLL6 adding polyglutamylation chains on Glu272. Thus, it seems that specific TTLL enzymes and CCPs work in opposition to each other in modifying the glutamylation status of cGAS.

The authors next seek to determine how glutamylation affects the activity of cGAS by focusing on its two core functions: DNA binding and catalysis of cGAMP. Through complementary experiments, they conclude that the two TTLL-CCP modules—TTLL4–Glu302-CCP5 and TTLL6–Glu272-CCP6—regulate both functions of cGAS independently of each other (**Fig. 1**). Thus, polyglutamylation at Glu272 blocks the ability of cGAS to bind to double-stranded DNA, whereas monoglutamylation at Glu302 interferes with its catalytic function. Accordingly, preventing polyglutamylation at Glu272 (via mutant cGAS with the E272A substitution or knockout of TTLL6) increases the binding ability of cGAS, whereas interfering with the monoglutamylation of Glu302 (via mutant cGAS with the E302A substitution or knockout of TTLL4) augments the production of cGAMP. Impairment of glutamylation of cGAS by knockout of TTLL4 or TTLL6 'translates' into greater production of type I interferons and more efficient blocks an the replication of herpes simplex virus type 1 relative to that of wild-type mice, and this effect is even greater in mice with double knockout of both TTLL4 and TTLL6. Conversely, mice with double knockout of both CCP5 and CCP6 display lower production of type I interferons and diminished resistance to infection with a DNA virus relative to that of wild-type mice.

On the basis of those findings, Fan and colleagues propose that the glutamylation status of cGAS is a critical determinant in controlling its functional capability and in balancing the host response to DNA[5]. The catalog of post-translational alterations that affect immunological signaling pathways is a complex one. However, the idea of 'fine-tuning' the cGAS-STING pathway in a rapid and reversible manner is of special attractiveness, given that by itself cGAS cannot reliably distinguish between self (or harmless) triggers or situations and those that are non-self (or dangerous). Enzymes that control the PTM could thus function as molecular 'switches' that integrate important environmental cues and alter the functional ability of cGAS, adjusted to the needs and requirements of the host. Such a system would allow rapid recruitment of 'active' cGAS in the setting of pathogen infection. On the other hand, it could transiently turn off the DNA-sensing pathway during mitosis or apoptosis, scenarios in which cytosolic DNA should be made 'invisible' to cGAS.

These findings raise the next question: what controls the enzymes that control cGAS? Fan and colleagues offer a partially resolved solution[5]. They observe that in resting cells, specific TTLL enzymes and CCPs are in competition to modify cGAS activity, with the result being the coexistence of both glutamylated (inactive) cGAS and deglutamylated (active) cGAS. Stimulation of cells leads to downregulation of TTLL expression and thereby passively generates enrichment for 'active versions' of cGAS. Thus, it seems that the ratio between TTLL enzymes and CCPs dynamically controls the pool of DNA-binding, competent and enzymatically active cGAS. How the expression and functionality of these enzymes is regulated remains an open issue. Along these lines, given the substantial effect of glutamylation on the outcome of infection with a DNA virus, it is very likely that viruses have developed countermeasures against this regulatory step. Thus, one could speculate about the existence of virus-encoded TTLL enzymes or factors that intercept with CCPs. The discovery of this role of cGAS marks an important breakthrough in the understanding of DNA sensing. Although the cGAS product cGAMP holds great 'translational' potential as an adjuvant, it is also a promising target for pharmacological intervention in autoinflammatory syndromes. Although the relevance of glutamylation of cGAS still needs to be validated in other experimental settings, the study by Fan and colleagues[5] opens the door to more research on non-canonical PTMs. Thus, there might be a plethora of non-canonical PTMs that could be equally important in the regulating

the sensing of nucleic acids. In the future, it will be important to determine whether additional members of the DNA-sensing cascade are substrates of these PTMs and to also find ways of modulating these activities for creating novel therapeutic opportunities.

COMPETING FINANCIAL INTERESTS
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Barrier regulation: tolerance stops at cell death

Johanna Pott, Kevin J Maloy & Ana Izcue

The accumulation of intestinal Foxp3$^+$ regulatory T cells (T_{reg} cells) in response to the microbiota is tightly regulated. Epithelial apoptosis inhibits the production of tolerogenic interferon-β by myeloid cells and thereby reduces the frequency of T_{reg} cells and lowers the threshold for inflammatory responses.

Barrier surfaces, such as the gut, skin and lungs, are home to a rich commensal microbiota but also represent potential entry points for pathogens. Therefore, the local immune system is constantly challenged to 'decide' between tolerance and inflammation. Foxp3$^+$ regulatory T cells (T_{reg} cells) are key modulators of such 'decisions'. Commensal microbiota promote T_{reg} cell differentiation, but how the accumulation of T_{reg} cells is controlled at barrier sites remains incompletely understood. In this issue of *Nature Immunology*, Nakahashi-Oda and colleagues propose a novel circuit in which the apoptosis of epithelial cells reduces the microbiota-induced accumulation of T_{reg} cells[1].

The authors postulate that the high level of constitutive apoptosis at barrier surfaces might modulate local immunological homeostasis. To investigate this, they study mice that lack CD300a, a conserved inhibitory receptor expressed by immune cells that recognizes phosphatidylserine (PS) exposed on the membrane of apoptotic cells[2]. They find that CD300a-deficient mice have an increased frequency of T_{reg} cells in the intestinal lamina propria. Strikingly, this effect is not restricted to the intestine, as CD300a-deficient mice also have an increased frequency of T_{reg} cells in lungs and skin, whereas the frequency of T_{reg} cells is unchanged in systemic lymphoid tissues not exposed to microbes, such as the spleen and lymph nodes. The authors observe similar increases in barrier-site T_{reg} cells in wild-type mice following local application of a protein that blocks the interaction of CD300a with PS. In addition, they also find that the frequency of T_{reg} cells is not increased in germ-free CD300a-deficient mice, which indicates that CD300a modulates a microbiota-dependent circuit. Hence, the authors propose a mechanism in which CD300a signals limit commensal microbiota–induced accumulation of T_{reg} cells at various barrier surfaces.

Interestingly, the main cell population in gut, skin and lung that shows exposed PS is epithelial cells. Despite the high constitutive cell-death rate in epithelia, this result is surprising, as epithelial cells are rapidly shed into the external environment before apoptosis is completed. Therefore, it would not be expected that apoptotic epithelial cells would strongly influence the biology of the underlying tissue. However, the authors identify CD300a$^+$CD11c$^+$ cells in close apposition to PS$^+$ epithelial cells, suggestive of direct interactions between local myeloid cells and apoptotic epithelial cells. Transfer experiments and conditional ablation of *Cd300a* in CD11c$^+$ cells confirm that myeloid cells mediate the inhibitory effect of CD300a on the accumulation of T_{reg} cells. To gain mechanistic insight, the authors compare the gene expression of CD300a-deficient and wild-type intestinal myeloid cells and find that CD300a-deficient myeloid cells have higher expression of the gene encoding interferon-β (IFN-β), a cytokine with dual effects. Although it can promote inflammatory responses, it has also been shown to have anti-inflammatory activities. Several reports have indicated that interferon signals can enhance the accumulation of T_{reg} cells[3,4] but have reached different conclusions on whether IFN-β acts directly or indirectly on T_{reg} cells. Nakahashi-Oda and colleagues find that fecal contents stimulate the production of IFN-β by myeloid cells *in vitro* through activation of the Toll-like receptor 4 (TLR4)–transcription factor TRIF signaling pathway that is triggered in response to lipopolysaccharide. Notably, this IFN-β production is inhibited by CD300a signaling, which confirms that IFN-β is susceptible to negative regulation by apoptotic cells. Moreover, blockade of IFN-β decreases the induction of T_{reg} cells by CD300a-deficient myeloid cells *in vitro* and reduces their accumulation in CD300a-deficient mice *in vivo*. Thus, CD300a signals inhibit microbe-induced IFN-β production in myeloid cells and thereby limit the frequency of T_{reg} cells. Although the analyses here focus on T_{reg} cells, it will be important to determine whether other populations of immune cells at barriers are also influenced by CD300a signaling or by the altered cytokine milieu.

A key issue is the functional relevance of the CD300a-mediated decrease in epithelial T_{reg} cells. Since these cells are crucial for preventing intestinal inflammation, even moderate alterations in their frequency might affect intestinal homeostasis. Indeed, in an experimental model of colitis induced by the administration of dextran sodium sulfate, the authors observe attenuated intestinal inflammation in CD300a-deficient mice. Notably, CD300a-deficient mice also exhibit diminished pathology in the skin and lungs when assessed via models of atopic dermatitis and allergic-airway inflammation, respectively. Hence, the detection of apoptotic cells seems to be a conserved mechanism for limiting tolerance at different barrier surfaces, and loss of CD300a has marked functional consequences. These results contrast, however, with a published report showing that a lack of CD300a increases intestinal inflammation in mice fed a high-fat diet[5]. However, as CD300a is expressed by a variety of cells, including mast cells and macrophages, it is possible that in the high-fat model[5], the inhibition of pro-inflammatory cytokine production by CD300a dominates over the IFN-β-mediated accumulation of T_{reg} cells.

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