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How squalene GLAdly helps generate antigen-specific T cells via antigen-carrying neutrophils and IL-18

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Abstract

The mechanisms by which squalene, which in oil-and-water emulsions has been shown to be an excellent formulation for TLR agonists, enhances the magnitude and quality of adaptive immune responses are not thoroughly defined. In this issue of the *European Journal of Immunology* [Eur. J. Immunol. 2015. 45: XXXX-XXXX], Desbien et al. show that a squalene/TLR4-based adjuvant augments antigen-specific Th1 responses in vaccinated mice through a caspase/IL-18-dependent mechanism. This commentary will discuss the authors' findings in the context of elucidating the mechanism of action of squalene as an adjuvant, and the new questions that the work generates.

Keywords

Adjuvant; Caspase; IL-18; Neutrophil; Squalene; Th1; TLR4

Pop culture movie star objections notwithstanding, vaccination is arguably one of the most successful medical interventions in human history. The early years of vaccine development centered on the use of attenuated infectious agents as the mediators of immune protection [1]. However, numerous problems exist with these vaccines, ranging from vaccine production and storage issues to adverse reactions and reversion to virulence. As a result, more recent efforts at vaccine design are directed toward subunit vaccination [2], using non-infectious, molecularly defined portions of an infectious agent in a vaccine formulation. For close to 100 years, it has been recognized that successfully initiating immunity against the target proteins within these subunit vaccines required the incorporation of an adjuvant, an additive which when delivered in conjunction with the desired target proteins improves the rate and magnitude of protective immunity [3]. These adjuvants (e.g. monophosphoryl lipid A, PolyIC) induce innate immune cells such as dendritic cells and macrophages to produce the pro-inflammatory stimuli necessary to kick-start the slower acting adaptive immune cells

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(T cells and B cells) into action, leading to the eventual formation of immune memory that can protect the host against infectious challenge for years to come.

Critical experiments throughout the 1990s [4, 5] culminated in the identification of the prototypic Toll-like receptor (TLR) involved in the recognition of LPS. In the years since, not only were more TLRs identified, but multiple innate receptor pathways (NLRs, RLRs, ALRs, STING, etc) were identified as well, along with their ligands and downstream inflammatory mediators. Because of their capacity to induce robust inflammation through molecularly defined receptors and signaling pathways, ligands for many of these innate receptors have been tested as vaccine adjuvants in the hopes of developing improved vaccine platforms capable of generating robust immunity for hard-to-vaccinate-against chronic infections such as Hepatitis C, HIV, tuberculosis or malaria.

Against this backdrop, there are two aspects of modern vaccinology and adjuvant development that have evaded clarity. First, some of our most clinically successful adjuvants remain largely undefined with respect to their molecular mechanisms of action. A primary example of this is the squalene oil-in-water emulsion that is a major adjuvant component of vaccines applied to literally millions of patients over the last 14 years, and presently serving as the foundational adjuvant for a clinically approved flu vaccine for the elderly (Fluad) [[6]]. In spite of this wide use, the molecular and cellular underpinnings to the success of squalene have continued to evade full characterization. Second, while many adjuvants have the capacity to improve humoral immunity (antibody production), few are successful at eliciting meaningful CD4⁺ and CD8⁺ T-cell responses [2, 7]. While this is not an issue for infectious challenges where neutralizing antibody is the necessary immune correlate of protection, protection against the more recalcitrant infectious diseases mentioned above will most certainly require the production of robust and enduring T-cell responses.

In this issue of the *European Journal of Immunology*, Desbien et al. [8] identify mechanisms by which a squalene-based adjuvant produces CD4⁺ Th1 responses in mice. In conjunction with a synthetic TLR4 agonist, glucopyranosyl lipid adjuvant (GLA), the squalene-emulsified GLA (GLA-SE) was shown to induce a robust Th1-cell response to ID87, a tuberculosis subunit vaccine candidate. In contrast, vaccinating mice with an aqueous formulation of the GLA, or use of the SE without GLA, resulted in substantially reduced numbers of responding IFN- γ -producing T cells. Previous work by others indicated that the prototypic squalene-based adjuvant, MF59, produces immune responses dependent on MyD88 (implicating TLR and/or IL-1/18/33 signaling) and independent of the NLR inflammasome constituent NLRP3 [9, 10]. Desbien et al. [8] also found no role for NLRP3 in their vaccination, but through a somewhat circuitous route, they eventually concluded critical roles for the inflammasome, IL-18, and neutrophils in the complicated mechanism by which their adjuvant functions. The first signpost directing the authors toward these players was an IFN- γ -mediated inflammatory signature within the draining node early after vaccination. This production of IFN- γ was shown to be critical to the eventual formation of the Th1 response, as IFN- γ blockade with anti-IFN- γ antibody during vaccination substantially reduced the frequency of antigen-responsive T cells 7 days after vaccination. Furthermore, the time frame of IFN- γ production implicated inflammasome-mediated production of IL-18, known to induce antigen-independent memory T-cell IFN- γ

production. Use of IL-18R^{-/-} and caspase-1/11^{-/-} mice in this vaccination model confirmed important roles for both of these molecules in the early production of IFN- γ by CD8⁺ T cells, as well as in the eventual expansion of antigen-specific Th1 cells. Closer examination of the local node after vaccination revealed the surprising finding that neutrophils were the major population of IFN- γ -producing cells in the first 24 hours after vaccination. As with the memory CD8⁺ T cells, neutrophil production of IFN- γ was IL-18 and caspase-1/11-dependent. Perhaps even more surprising, neutrophils were found to engulf and process vaccine-related antigens and migrate into both the afferent lymphatics and deeper T-cell regions of the draining lymph node. Given their low expression of class II MHC, antigen associated with neutrophils probably has to be exchanged with professional antigen-presenting cells (APCs) in order to be presented to antigen-specific T cells. The end result is a complicated picture involving i) inflammasome/caspase activity and IL-18 production by an as-yet-undetermined cell type, ii) neutrophil recruitment, antigen uptake, induction of IFN- γ production and migration into local lymphoid tissues, and iii) stimulation of antigen-specific CD4⁺ T cells (possibly after antigen exchange between neutrophils and professional APCs) and subsequent Th1-cell differentiation as a result of either memory CD8- or neutrophil-derived IFN- γ (Figure 1).

While these data identify a molecular mechanism by which a squalene-based emulsion can elicit cellular immunity, many questions remain, chief among them being i) what form does the inflammasome take? ii) what are the cell types that produce IL-18 through inflammasome activity? and iii) which cells must receive signals through expression of an IL-18R? The identification of the IL-18-producing cell type was out of scope for the present article, but given the original description of IL-18 as a factor produced by activated macrophages [11], inflammatory monocytes/macrophages are possible/likely sources. A reasonable model (Figure 1) places neutrophils as the cell type necessary to be stimulated by IL-18, upstream of eventual T-cell activation and expansion. To be fair however, the data in Desbien et al. [8] fit in a number of different models and the cell intrinsic versus extrinsic requirements for IL-18, and even IFN- γ , in response to this vaccine adjuvant await full clarity.

The contribution of the TLR4 agonist GLA to inflammasome/caspase-1/11/IL-18 activity also remains to be clarified. Whereas previous reports indicated that the efficacy of the squalene adjuvant MF59 does not rely on caspase-1 activity, that work was performed in the absence of any added TLR stimuli [10]. Furthermore, whereas previous analysis of MF59 examined the necessary stimuli for the formation of antibody responses [10], Desbien et al. analyzed the requirements for the adjuvant to elicit T-cell responses. As mentioned above, inducing T-cell responses following subunit vaccination requires signals clearly divergent from those required by B cells, and the present report might suggest that caspase-1 activation represents one of those divergent signals. That said, Desbien et al.'s use of GLA-SE, and even the individual adjuvant components alone, produced detectable T-cell responses independent of caspase-1/11 or IL-18. Thus, the GLA-SE elicits innate signals other than IL-18 that are also important in T-cell generation. It seems likely that GLA-SE is representative of a growing class of combined adjuvants [12–14] in which the combination of adjuvant components are capable of producing signals that are distinct from those

produced by either adjuvant alone and that are critical for the development of cellular immunity.

The observation of neutrophil activity in response to GLA-SE is both timely and provocative. Timely, because neutrophil involvement in the response to vaccination, specifically with the activities of MF59 and Alum, has been recently documented [15]. Provocative, because it adds to the mounting evidence [6] that neutrophils are not just for bactericidal activity any more, particularly when it comes to vaccine adjuvants. In previously published work, neutrophils were found to be involved both at the injection site as well as in the draining lymphoid tissues after vaccination [6, 15], precisely as documented here by Desbien et al. The present observation that neutrophils are a robust producer of IL-18-elicited IFN- γ is particularly surprising and novel. Though it is unclear whether neutrophil-produced IFN- γ is necessary for the adjuvant activity of GLA-SE, the observation should at the very least inspire vaccinologists to stop putting PMNs into their FACS dump gates. It seems likely that adjuvants other than GLA-SE may induce some degree of neutrophil activity that has gone on unnoticed, necessitating some reexamination by the implementers of other adjuvant platforms.

The documentation of inflammasome activity by Desbien et al. also adds squalene-containing adjuvants to the growing list that have the activation of an NLR of one kind or another as central to their mechanism of action. Alum is the most recently added and notable member of such NLR-activating adjuvants, although the precise role of the inflammasome in motivating T-cell responses remains somewhat controversial [16, 17]. A recent report demonstrated the capacity for Alum to induce neutrophils to extrude their DNA and chromatin in what are known as extracellular traps (ETs) [18]. These data might possibly offer a model by which neutrophils, inflammasomes, caspase-1 and IL-18 can be integrated. It seems possible that these recruited neutrophils might also produce ETs. The extruded DNA/chromatin could be taken up by local monocytes/macrophages and activate a cytosolic DNA sensor, such as Aim2, which induces caspase-1 activity. Indeed, Aim2 has been shown to induce caspase-1 activation by forming an inflammasome-like structure with ASC [19, 20], an adapter already shown to be necessary for MF59 adjuvant activity. The occurrence of this within the injection site as well as the subcapsular spaces and T-cell region within the LN might also facilitate the exchange of antigen between the neutrophils and APCs. This model is of course highly speculative and is one of a number of possible means by which GLA-SE might influence cellular immunity. Importantly, the work done by Desbien et al. not only provides us with a broader spectrum of testable models, it identifies novel molecular and cellular targets for the ongoing process of vaccine adjuvant design and development.

Acknowledgments

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Figure 1.

Squalene emulsified GLA (GLA-SE) augments Th1 responses through caspase activation, neutrophil recruitment and IFN- γ production. (A) GLA-SE in the injection site, or draining lymphatics, induces IL-18 via caspase-1/11 from an as-yet-undermined cell type. A speculated source of IL-18 activity may be monocytes/macrophages activated by squalene-induced neutrophil extracellular traps (ETs). Neutrophils activated by IL-18 not only produce IFN- γ but also acquire and process antigen (AG). (B) Antigen-carrying, IFN- γ -producing neutrophils can be subsequently found in subcapsular lymphatics and T-cell regions of draining lymphoid tissue. The antigen is then presented to T cells either directly, which is less likely given that neutrophils are MHC class II^{lo}, or after antigen exchange with professional APCs. All red outlines, text, or figures represent speculated mechanisms.