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Identification of Aim2 as a Sensor for DNA Vaccines

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Recent human study data have re-established the value of DNA vaccines, especially in priming high-level Ag-specific Ab responses, but also raised questions about the mechanisms responsible for such effects. Whereas previous reports have shown involvement of downstream signaling molecules in the innate immune system, the current study investigated the role of absent in melanoma 2 (Aim2) as a sensor for DNA vaccines. The Aim2 inflammasome directs maturation of the proinflammatory cytokines IL-1β and IL-18 and an inflammatory form of cell death called pyroptosis. Both the humoral and cellular Ag-specific adaptive responses were significantly reduced in Aim2-deficient mice in an IL-1β/IL-18–independent manner after DNA vaccination. Surprisingly, Aim2-deficient mice also exhibited significantly lower levels of IFN-α/β at the site of injection. These results indicate a previously unreported link between DNA vaccine–induced pyroptotic cell death and vaccine immunogenicity that is instrumental in shaping the Ag-specific immune response to DNA vaccines. The Journal of Immunology, 2015, 194: 630–636.

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Abbreviations used in this article: Aim2, absent in melanoma 2; Aim2−/−, Aim2-deficient; BMMD, bone marrow–derived macrophage; DAMP, damage-associated molecular pattern; HA, hemagglutinin; LDH, lactate dehydrogenase; poly(dA-dT), poly(deoxyadenylic-deoxythymidylic) acid; PRR, pattern recognition receptor; TBK1, TANK-binding kinase 1; UMMS, University of Massachusetts Medical School.

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data therefore establish a novel role for Aim2 as a key player in the regulation of DNA vaccination.

Materials and Methods

Animals

C57BL/6 mice were obtained from Taconic Laboratories. Aim2<sup>−/−</sup> mice were generated in house by K. Fitzgerald’s group at the University of Massachusetts Medical School (UMMS) as previously described (26). Aim2<sup>−/−</sup> mice were on a mixed B6/129 background, and therefore B6 × 129 mice were used as controls. B6.129 (hereafter referred to as Aim2<sup>+/+</sup>) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at UMMS. IL-1R, IL-18R, and Aim2<sup>−/−</sup> mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at UMMS. IL-1R, IL-18R, and Asc−/− mice were produced in house. All mice were maintained in the Department of Animal Medicine at UMMS according to Institutional Animal Care and Use Committee–approved protocols. Mice received 100 μg codon-optimized H1HA DNA vaccine expressing the full-length wild-type HA protein from A/Texas/04/09 (pH1HA), divided between quadriceps, at 2 and 4 wk. A third boosting immunization was delivered 1 wk prior to sacrifice.

Gene induction analysis

C57BL/6 mice were shaved and immunized with 100 μg H1-TX04-09.iPA DNA vaccine plasmid i.m. injected into the hind quad muscle. Punch biopsies were harvested from the site of immunization at 6, 12, and 24 h postimmunization and snap frozen. RNA was isolated from tissue biopsies using TRIzol Reagent (Life Technologies). We analyzed gene expression using the Nanostring nCounter Analysis system (Nanostring Technologies). Each reaction contained 100 ng RNA in a 5-μl aliquot, plus reporter and capture probes. We also included six pairs of positive control and eight pairs of negative control probes. Gene induction analysis and normalization was conducted using nSolver Analysis Software v1.1. Raw counts were normalized to naive mice using three reference genes: Gapdh, Gusb, and Hprt1.

Cell culture and cytokine ELISA

Mouse bone marrow–derived dendritic cells were generated from Aim2<sup>+/+</sup> or Aim2<sup>−/−</sup> mice by culturing fresh bone marrow in R10 medium containing GM-CSF for 8 d at 37°C. Aim2<sup>−/−</sup> and Aim2<sup>+/+</sup> immortalized bone marrow–derived macrophages (BMDM) were produced in-house. Cells were first primed with 200 ng/ml LPS (Sigma-Aldrich, St. Louis, MO) for 4 to 5 h. prior to treatment with appropriate stimuli. All media was removed from the cells, and the appropriate stimulus was added. Poly (deoxyadenylc-deoxycytidylic) acid [poly (dA-dT)] (Sigma-Aldrich) DNA and H1-TX04-09.iPA DNA vaccine plasmid were transfected using Lipofectamine 2000 at a concentration of 1.5 μg/ml. ATP was added at a concentration of 1.25 μM. Cultures were incubated 16–18 h at 37°C, and supernatants were harvested. Cell-culture supernatants were assayed for IL-1β (BD Biosciences, Franklin Lakes, NJ) by ELISA. Lactate dehydrogenase (LDH) release was used to measure pyroptotic cell death. LDH assays were performed using the Promega CytoTox96 Nonradioactive Cytotoxicity Kit according to the manufacturer’s directions (Promega, Madison, WI).

ELISA

Microtiter plates were coated with transiently expressed H1HA Ag at ~1 μg/ml in PBS for 1 h at room temperature and assayed as previously described (29). NaSCN displacement was performed at a serum dilution of 1:100. After washing of serum samples, NaSCN was added at various (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 M) concentrations in PBS for 15 min followed by five washes in ELISA wash buffer. The assay was then completed as above.

ELISPOT assay

Splenocyte T and B cell ELISPOT reagents were obtained from Mabtech (Mariemont, OH). HIHA-specific T cells were quantified per the manufacturer’s instructions. Positive controls were stimulated with 20 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich). The HIHA relevant peptide used was a CD8<sup>+</sup> cell-restricted HA peptide (YSTVASSL). HIHA-specific Ab-secreting cells were detected by coating of MAIPSWU (Millipore, Billerica, MA) plates with the transiently expressed H1HA Ag used for ELISA (~1.0 μg/well). Positive spots were visualized on a CTL imager, and counting was performed using Immunoscore software (Cellular Technology, Shaker Heights, OH).

In vivo caspase-1 activation

Aim2<sup>+/+</sup> and Aim2<sup>−/−</sup> mice were shaved and immunized with 100 μg H1-TX04-09.iPA DNA vaccine plasmid i.m. injected into the hind quad muscle. Punch biopsies were harvested from the site of immunization at 6, 12, and 24 h postimmunization and snap frozen. Cryopreserved tissue sections were generated and adhered to glass slides. Samples were then stained with a caspase-1 FAM/FLICA kit according to the manufacturer’s instructions (ImmunoChemistry Technologies, Bloomington, MN). Stained slides were visualized on a confocal microscope. Sixteen independent fields were analyzed for fluorescence.

Quantitative real-time PCR

Aim2<sup>+/+</sup> and Aim2<sup>−/−</sup> mice were shaved and immunized with 100 μg H1-TX04-09.iPA DNA vaccine plasmid i.m. injected into the hind quad muscle. Punch biopsies were harvested from the site of immunization at 6, 12, and 24 h postimmunization and snap frozen. RNA was isolated from tissues biopsies using TRIzol Reagent (#15596-026; Life Technologies), and cDNA was generated using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). CDNAs were then used for RT-PCR reactions on a Bio-Rad CFX-96 cycler (Bio-Rad). Primers sequences are available upon request.

Statistical analysis

All data are presented as the mean of individual mice ± SEM. Statistical analysis was performed using a Student t test, a one-way ANOVA followed by a Tukey posttest, or a two-way ANOVA followed by a Bonferroni posttest.

Results

DNA vaccine plasmid induces expression of Aim2, caspase-1, and the inflammasome

Although previous studies have mainly used noncoding DNA plasmid or DNA vaccines coding for marker proteins to study DNA-elicited innate immune responses, the current study tested a DNA vaccine (pH1HA) expressing the HA Ag of the type A influenza virus subtype H1N1 virus, which was responsible for a pandemic influenza in 2009. HA is the major protective Ag in clinically licensed inactivated and live-attenuated influenza vaccines. DNA vaccines expressing HA have been shown to be immunogenic in eliciting HA-specific Abs in both animal and human studies (2, 29–33). The expression of HA Ag by pH1HA used in the current study was confirmed by Western blot, and its immunogenicity to elicit HA-specific Ab response was verified in a pilot mouse study (data not shown).

We first wanted to profile key immune response genes following DNA vaccine pH1HA using the Nanostring nCounter gene expression system (Nanostring Technologies), which includes a custom array encoding 50 innate immunity targets. Gene induction was quantified from wild-type C57BL/6 mice immunized with the pH1HA DNA vaccine. mRNA was isolated, the expression of innate immune genes profiles using the Nanostring nCounter, and changes in gene induction quantified. Notably, Aim2 was induced ~6-fold within 12 h of immunization when compared with naive samples. Aim2 is a type I IFN–inducible gene, suggesting a potent ability of cells at the site of vaccination to recognize cytosolic plasmid vaccines (Fig. 1A). In accordance with the induction of Aim2, caspase-1 was also highly upregulated. (Fig. 1B). Most striking were the high levels of the inflammatory cytokines IL-1α and IL-1β (Fig. 1C, 1D). These observations indicate that inflammasome components were present at the site of vaccination. To test if the inflammasome pathway was active at the site of vaccination, we used a caspase-1–specific FAM/FLICA fluorescent stain to covalently label catalytically active caspase-1. Mature caspase-1 became apparent within 6 h of immunization and reached a peak at 12 h (Supplemental Fig. 1). Collectively, these results led us to examine the role of the Aim2 inflammasome pathway in Ag-specific immune responses elicited by pH1HA DNA vaccination.
Involvement of Aim2 in cellular responses to DNA vaccine plasmid

We first evaluated the ability of macrophages and dendritic cells to recognize pH1HA DNA vaccine by performing in vitro experiments. IL-1β production in response to pH1HA DNA vaccine was evaluated in BMDM collected from either Aim2^+/+ or Aim2^-/- mice (Fig. 2A). As expected, both the synthetic B-form dsDNA poly(dA-dT) and pH1HA DNA vaccine induced a robust IL-1β response in Aim2^+/+ BMDM as measured by ELISA. However, IL-1β production was abolished in Aim2^-/- BMDM. Similar results were seen in bone marrow–derived dendritic cells (data not shown). Next, the pan-caspase inhibitor Z-VAD-FMK was included in BMDM cultures prior to adding pH1HA DNA vaccine (Fig. 2B). This resulted in inhibited IL-1β production in Aim2^+/+ macrophages, yielding IL-1β levels comparable to Aim2^-/- wells, supporting the role of Aim2 in DNA vaccine–mediated IL-1β maturation. Finally, pH1HA DNA vaccine induced an inflammatory form of cell death (pyroptosis) in Aim2^+/+ macrophages, as measured by LDH release (Fig. 2C). This response was attenuated in Aim2^-/- cells. Collectively, these data indicate that Aim2 acts as a sensor of DNA vaccine plasmid and regulates caspase-1–dependent IL-1β production and pyroptotic cell death in response to pH1HA DNA vaccines in vitro.

Effects of Aim2 deletion on pH1HA induced HA-specific immune responses

As it is evident that the Aim2 inflammasome recognizes and responds to pH1HA DNA vaccine by performing in vitro experiments, IL-1β production in response to pH1HA DNA vaccine was evaluated in BMDM collected from either Aim2^+/+ or Aim2^-/- mice (Fig. 2A). As expected, both the synthetic B-form dsDNA poly(dA-dT) and pH1HA DNA vaccine induced a robust IL-1β response in Aim2^+/+ BMDM as measured by ELISA. However, IL-1β production was abolished in Aim2^-/- BMDM. Similar results were seen in bone marrow–derived dendritic cells (data not shown). Next, the pan-caspase inhibitor Z-VAD-FMK was included in BMDM cultures prior to adding pH1HA DNA vaccine (Fig. 2B). This resulted in inhibited IL-1β production in Aim2^+/+ macrophages, yielding IL-1β levels comparable to Aim2^-/- wells, supporting the role of Aim2 in DNA vaccine–mediated IL-1β maturation. Finally, pH1HA DNA vaccine induced an inflammatory form of cell death (pyroptosis) in Aim2^+/+ macrophages, as measured by LDH release (Fig. 2C). This response was attenuated in Aim2^-/- cells. Collectively, these data indicate that Aim2 acts as a sensor of DNA vaccine plasmid and regulates caspase-1–dependent IL-1β production and pyroptotic cell death in response to pH1HA DNA vaccines in vitro.

Effects of Aim2 deletion on pH1HA induced HA-specific immune responses

As it is evident that the Aim2 inflammasome recognizes and responds to pH1HA DNA vaccine in cultured cells, the role of Aim2 in pH1HA DNA vaccination was next examined in Aim2^-/- and wild-type Aim2^+/+ mice. The pH1HA DNA vaccine induced high-level HA-specific Ab responses in Aim2^+/+ mice, but significantly lower Ab titers in Aim2^-/- mice (Fig. 3A). This reduction is isotype-independent as Aim2^-/- mice exhibited significantly lower levels of HA-specific IgG1, IgG2b, and IgG2c responses (data not shown). Likewise, Aim2^-/- mice exhibited significantly reduced HA-specific circulating B cells as well as IFN-γ–secreting CD8^+ T cells in the spleen (Fig. 3C, 3D). The role of Aim2 in regulating the maturation process of pH1HA-induced Ab responses was further confirmed by measuring the avidity of serum HA-specific Abs in these mice (Fig. 3B). Aim2^+/+ mice required high concentrations of the chaotropic agent NaSCN to disrupt Ag/Ab complexes, whereas much lower concentrations of NaSCN were required for dissociation in Aim2^-/- mice. To confirm the requirement for inflammasome signaling in DNA vaccine immunogenicity, we also quantified the adaptive response in Asc^-/- mice. Asc deletion similarly inhibited the generation of optimal HA-specific immune responses (Supplemental Fig. 2A–C).
Aim2<sup>−/−</sup> mice fail to cleave caspase-1 into its active form

Because DNA vaccination resulted in high levels of caspase-1 activation in Aim2<sup>+<//+</sup> mice (Supplemental Fig. 1), we analyzed Aim2<sup>−/−</sup> mice for their ability to generate catalytically active caspase-1 using the FAM/FLICA assay (Fig. 4). Aim2<sup>−/−</sup> mice demonstrated a clear reduction in caspase-1 activation at the 12-h peak time point when compared with Aim2<sup>+<//+</sup> controls.

Effects of IL-1 and IL-18 deletion on vaccine-induced HA-specific immune responses

As inflammasome signaling ultimately results in the downstream cleavage of pro–IL-1β and pro–IL-18 into their respective active forms, the role of IL-1β and IL-18 signaling in DNA vaccine was next investigated (Supplemental Fig. 3). Surprisingly, both of these cytokines were dispensable for the DNA vaccine response as mice lacking the IL-1R or the IL-18R mounted normal vaccine responses. Total serum HA-specific IgG titers were similar to wild-type C57BL/6 mice in both Il<sup>−1r</sup><sub>−/−</sub> and Il<sup>−18r</sup><sub>−/−</sub> mice. Likewise, no significant difference was seen in total HA-specific B or CD8<sup>+</sup> T cell numbers as measured by ELISPOT. These data are in line with previously published reports demonstrating little impact on DNA vaccination following MyD88 deletion (19).

The immune response is lineage dependent

Previously published data have demonstrated the requirement for both hematopoietic and non-hematopoietic cell lineages in DNA vaccination (19). To further elucidate the role of Aim2, bone marrow chimeric mice were generated by transferring bone marrow from Aim2<sup>−/+</sup> mice into Aim2<sup>−/−</sup> mice, or vice versa (Fig. 5). Aim2<sup>−/+</sup> and Aim2<sup>−/−</sup> mice reconstituted with Aim2<sup>−/−</sup> bone marrow exhibited strong defects in both the T cell response and HA-specific IgG production. Interestingly, transfer of Aim2<sup>−/+</sup> bone marrow into Aim2<sup>−/−</sup> rescued the T cell response against DNA vaccination following MyD88 deletion (19).

FIGURE 3. Optimal DNA vaccine immunogenicity requires Aim2.

Wild-type Aim2<sup>+/+</sup> and Aim2<sup>−/−</sup> mice were immunized i.m. with a pH1HA encoding DNA vaccine at weeks 2 and 4. (A) HA-specific IgG titers were analyzed 14 d post-second immunization. Anti-HA binding avidity was quantified via ELISA and reported as molar concentration of sodium thiocyanate required to displace anti-HA serum Abs to 2× prebleed levels (B). For ELISPOT, spleens were harvested at termination 7 d following a third boosting immunization. HA-specific Ab-secreting B cells (C) or IFN-γ-secreting T cells (D) in mice immunized with either pH1HA or empty vector. Data are the averages ± SEM of five mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus control group.

FIGURE 4. Aim2<sup>−/−</sup> mice exhibit diminished caspase-1 activation at the site of immunization.

(A) PBS-injected controls were used for comparison. The site of injection was harvested and cryopreserved for tissue sectioning. Wild-type Aim2<sup>−/+</sup> (B) and Aim2<sup>−/−</sup> mice (C) were immunized i.m. with pH1HA vaccine, and caspase-1 activation was quantified by FAM/FLICA staining 12 h postimmunization. FAM/FLICA staining was visualized by confocal microscopy and is representative of three mice per group. Sections of 10 μm were stained with green fluorescent FLICA caspase-1 inhibitor. Nuclei were counterstained with DAPI. Low-power resolution presented (original magnification ×16).
The failure of Aim2−/− mice to generate optimal adaptive immune responses implies a defect in immune priming at the site of injection. Although the major function of the Aim2 inflammasome is to regulate caspase-1 activation, resulting in IL-1β, IL-18, and cell death pathways, our data indicate that IL-1β and IL-18 are not responsible for the Aim2-dependent effects we observed. We therefore endeavored to quantify IFN-α/β, as it has been reported to play a key role in the immune response to DNA vaccination (24, 34–36). In addition, it has been established that IFN-α/β signaling is required for DNA vaccination (19, 20). Aim2 does not control DNA-induced IFN-α/β production directly. Rather, the STING pathway mediates these effects. Because the IFN-α/β response is so critical for DNA vaccination, we performed a detailed kinetic analysis measuring IFN-α/β expression in Aim2+/+ and Aim2−/− mice. To ensure we only detect DNA vaccine-induced IFN-α/β, we limited our measurements to the site of immunization. Aim2+/+ and Aim2−/− mice were immunized with the pH1HA DNA vaccine, and punch biopsies were collected from the site of injection. Quantitative RT-PCR analysis of mRNA clearly shows that Aim2−/− mice have reduced IFN-α and IFN-β expression compared with Aim2+/+ controls, with expression peaking at 12 h postimmunization in wild-type mice (Fig. 6). Intriguingly, IFN-α/β expression in Aim2−/− mice peaked at 6 h postimmunization and remained static throughout the time course. Consistent with the decrease in IFN-α/β, there was a corresponding decrease in the IFN-stimulated gene IP-10. We also noticed a significant decrease in TNF. Asc−/− mice had similar levels of IFN-α/β, further confirming the requirement for inflammasome signaling (Supplemental Fig. 2D). These data suggest a previously unreported role for Aim2 in regulating local IFN-α/β levels following DNA vaccination. As Aim2 controls cell death at the site of infection, it is likely that Aim2-dependent cell death liberates endogenous DAMP danger signals, which might in turn elicit IFN-α/β via the Aim2-independent STING/TBK1 pathways. This broad defect in IFN-α/β signaling likely explains the defects we observed in Aim2−/− mice treated with DNA vaccines.

**Discussion**

The innate immune pathways governing DNA vaccination remain to be fully characterized. Recent reports have established the STING/TBK1/IFN-α/β axis as required for DNA vaccine immunogenicity (19, 20); however, the PRR(s) required for IFN-α/β production remain to be identified in this context. Likewise, the involvement of other innate immune signaling pathways is unclear. In particular, the requirement for the inflammasome signaling machinery in DNA vaccine–elicited Ag-specific immune responses has not been examined. In this study, we identified the Aim2 inflammasome as a DNA vaccine sensor with the ability to regulate the Ag-specific adaptive immune response. Whereas previous reports have focused on downstream signaling mole-
Aim2 through the STING/TBK1 signaling axis, further propagating the decreased pyroptotic cell death in Aim2 requires in both cell lineages for optimal humoral responses, as chimeric mice lacked high levels of anti-HA Abs. This may be required in both cell lineages for optimal humoral responses, as mice reconstituted with Aim2+/− bone marrow presented similar levels of IFN-γ to Aim2+/+. This provides further evidence for a defect in immune cell priming.

Disclosures
The authors have no financial conflicts of interest.

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