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Contribution of IRF-3 Mediated IFN β Production to DNA Vaccine Dependent Cellular Immune Responses

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Abstract

The mechanism(s) by which DNA vaccines activate Ag-specific cellular immune responses is incompletely understood. Current findings indicate that IRF-3 plays an important role in this process. The IRF-3 dependent signaling pathway is triggered by the presence of intracytoplasmic DNA, and culminates in the production of type I IFNs. DNA vaccination of IRF-3 KO mice elicits a strong Ag-specific humoral response, yet CD4 and CD8 T cell responses (including the production of Th1, Th2 and Th17 cytokines) are severely impaired. Although expression of the immunogenic protein encoded by the DNA vaccine was similar in IRF-3 KO vs wild type mice, antigen presentation was severely impaired in the KO animals. This defect was remedied by the co-delivery of an IFN β encoding plasmid. These findings suggest that the IRF-3/IFN β pathways are key to the induction of cellular immunity following DNA vaccination.

Keywords

DNA Vaccine; IRF-3; Type1 IFNs

INTRODUCTION

There is considerable interest in developing DNA vaccines capable of inducing cellular and humoral immune responses against infectious pathogens and tumor antigens [1;2]. DNA vaccines are constructed such that a strong promoter drives the expression of an Ag-encoding gene, resulting in the transcription and translation of the encoded protein by transfected cells *in vivo* [3;4]. An important feature of the immune response elicited by DNA vaccines is the generation of IFN γ -secreting CTL.

While many factors influence the immunogenicity of DNA plasmids, recent results suggest that elements in the plasmid backbone play an important contributory role [5–7]. Nucleic acids have multiple and complex effects on the immune system. Bacterial and viral nucleic acids interact with intracellular Toll-like receptors (TLRs) 3, 7, 8 and 9, thereby triggering an innate immune response via the MyD88 dependent pathway [8]. In addition, intracytoplasmic dsRNA and dsDNA can be recognized via IRF-3 [9;10]. Activation of IRF-3 triggers the production

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of type I IFNs, such as IFN β which under physiological conditions facilitate the clearance of infectious viruses [11]. The activation of IRF-3 to produce IFN β can proceed via both TLR dependent (TLRs 3 and 4) and independent (RIG-I and Mda5) pathways [12].

Previous *in vitro* studies established that DNA vaccines delivered into the cytoplasm of target cells activated CD8⁺ T cells via an IRF-3 mediated pathway to produce IFN β [13;14]. The current work was undertaken to determine whether the immunogenicity of plasmid DNA vaccines, whose activity is dependent on intracytoplasmic uptake, utilized this pathway *in vivo*.

MATERIALS AND METHODS

Animals

C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD) and Japan SLC (Shizuoka, Japan) and studied at 6–10 wk of age. IRF-3-deficient mice [15] (C57BL/6 mice background) were kindly provided by T. Taniguchi (Tokyo, Japan) and IFN α/β Receptor-deficient mice (C57BL/6 mice background) were kindly provided from H. Rosenberg (NIAID/NIH, MD). OT-1 mice carrying OVA-TCR-transgenic T cells on a RAG-1 $-/-$ background were obtained from The Jackson laboratory (Bar Harbor, NE). All studies were approved by the Center for Biologics Evaluation and Research (CBER) Animal Care and Use Committee and Tohoku University Animal Experiment Ethics Committee. For immunization, plasmids were injected bilaterally into the femoral muscle of anesthetized mice.

Preparation of OT-1 cells for adoptive transfer

OT-1 cells were isolated from the spleens of OT-1/RAG1 $-/-$ mice by negative selection using a combination of anti-MHC class II and anti-DX5 microbeads (Miltenyi Biotec). OT-1 cells were labeled with CFSE (Invitrogen) according to the manufacturers' instructions. 2 days after DNA vaccination, 2×10^6 CFSE-labeled OT-1 cells were transferred i.v. into recipient mice. Recipients were sacrificed 3 days later, lymphocytes harvested from their inguinal lymph nodes, and cell division analyzed using a FACSCalibur to monitor cellular CFSE content.

Plasmids and reagents

An OVA-encoded plasmid, p2/386OVA was constructed by subcloning a 1.4-kb EcoRI (filled-in) - XbaI (filled-in) fragment, containing the entire encoding sequence for ovalbumin, from plasmid pAc-neo-OVA [16] into StuI (phosphatase-treated) site of CpG free plasmid pCpG-mcs G2 (Invivogen, San Diego, CA). A CpG-free plasmid encoding murine IFN β pORF-mIFN β was obtained from Invivogen. The luciferase encoded plasmid, pSV2-Luc, was constructed by S.K. Nordeen [17]. Plasmid DNA was purified using an EndoFree plasmid mega/giga kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and treated with Triton-X-114 to remove endotoxin, as previously described [13;14]. Less than 0.1 U/ug endotoxin was present in the plasmid DNA preparations as determined by Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, NJ). The peptide SIINFEKL (H-2Kb-restricted OVA peptide 257–264) was synthesized at the CBER Core Facility.

Ag-specific ELISA

IgG and IgM anti-OVA antibody titers were measured in mouse serum by ELISA, as previously described [18]. Briefly, 96 well Immulon II plates were coated with 2 ug/ml of OVA and then blocked with PBS 1% BSA. Plates were washed and overlaid with serially diluted serum for 2 h at RT. After thorough washing, bound Abs were detected by HRP-labeled goat anti-mouse IgG, IgG1, IgG2a and IgM (Southern Biotechnology, Birmingham, AL), followed by ABTS substrate (Kirkegaard & Perry, Gaithersburg, MD). Antibody titers were determined by

comparison to a standard curve generated using pooled sera from hyper-immunized mice, and were expressed as the reciprocal of the end point dilution following background subtraction. All samples were analyzed in duplicate.

Cytokine ELISpot assay

Single cell suspensions were prepared from whole spleen. In some cases, these were separated into CD4⁺ or CD8⁺ T cell populations by negative selection using anti-CD4 or anti-CD8 microbeads (Miltenyi Biotec, Auburn, CA). The resulting cell populations contained <0.2% CD8⁺ or CD4⁺ cells, as measured by flow cytometry. $1.5 - 6.0 \times 10^5$ splenocytes/well were stimulated for 12–14 h with OVA (Sigma Chemical Co., St. Louis, MO) or SIINFEKL peptide in 96-well Multiscreen HTS plates (Millipore, Billerica, MA) previously coated with anti-IFN γ anti-IL-4 or anti-IL-17 antibody. The plates were then washed and treated with biotinylated anti-IFN γ anti-IL-4 or anti-IL-17 antibody, followed by streptavidin-alkaline phosphatase. Spots were visualized by the TMB peroxidase substrate (Moss, Inc) and counted by an automated ELISPOT reader system (Carl Zeiss MicroImaging, Inc., NY) with KS ELISPOT software.

Imaging and quantification of bioluminescence data

To monitor luciferase gene expression *in vivo*, the area above the femoral muscle of anesthetized mice was shaved. The muscle was then injected with 20 μ g of pSV2-luc in 20 μ l. Two days later, anesthetized mice were injected i.p. with 3 μ g of firefly D-luciferin in 100 μ l. Ten minutes later, mice were studied using an IVIS Imaging System (Xenogen, Alameda, CA), consisting of a cooled CCD camera mounted on a light-tight specimen chamber (dark box), a camera controller, a camera cooling system, and a Windows-based computer system. Imaging parameters were maintained for comparative analysis. Gray scale-reflected images and bioluminescence-colored images were superimposed and analyzed using the Living Image software (Xenogen). A region of interest (ROI) was manually selected based on signal intensity. The area of the ROI was kept constant, and the intensity was recorded as maximum photon counts within each ROI.

Statistical analysis

Student's t test was used to analyze all results.

RESULTS

Influence of IRF-3 on the cellular immune response induced by DNA vaccination

To investigate whether IRF-3 contributes to the induction of cellular immunity following DNA vaccination, spleen cells were isolated from C57Bl/6 and congenic IRF-3 KO mice 2 wk after immunization with a CpG-free OVA-encoding plasmid. These cells were then cultured *in vitro* with OVA or SIINFEKL peptide and monitored for IFN γ production by ELISpot assay. The number of spleen cells spontaneously secreting IFN γ was similar in control and vaccinated mice (Fig 1). When splenocytes from DNA vaccinated C57Bl/6 mice were cultured with SIINFEKL peptide or OVA, the number of cells secreting IFN γ rose by 5- and 9-fold, respectively (p. <0.001, Fig 1A). In contrast, spleen cells from unvaccinated mice failed to respond when stimulated with OVA protein or peptide.

The response of spleen cells from DNA vaccinated IRF-3 KO mice was then examined. IFN γ production by IRF-3 derived splenocytes following SIINFEKL peptide or OVA re-stimulation was 4- and 6-fold lower than that of cells from identically treated B/6 mice (p. <0.001, Fig 1A). This reduction in IFN γ secreting cell frequency was not due to a defect in the ability of IRF-3 KO mice to respond to OVA or produce IFN γ . As seen in Fig 1B, the response

of spleen cells from IRF-3 KO mice immunized with OVA protein in IFA (rather than following DNA vaccination) when re-stimulated with SIINFEKL or OVA was indistinguishable from that of similarly vaccinated B/6 mice. Thus, IRF-3 KO mice can mount a normal cellular immune response to OVA protein, but not to OVA encoded by a DNA vaccine.

Additional studies were performed to clarify the nature of this loss of T cell activity. Spleen cells from DNA vaccinated mice were selectively depleted of CD4 or CD8 cells (contamination <0.2% as determined by FACS). Although CD8 T cells were the dominant source of IFN γ following DNA vaccination, both CD4 and CD8 T cells from IRF-3 KO mice were significantly impaired in their ability to produce IFN γ when re-stimulated *in vitro* with OVA (Fig 1C). This defect extended to the production of the Th2 cytokine IL-4 and the Th17 cytokine IL-17. Consistent with previous reports, Ag specific IL-4 and IL-17 producing cells were induced by DNA vaccination of normal mice [19;20]. When re-stimulated *in vitro* with OVA, spleen cells from DNA vaccinated IRF-3 KO mice showed a significant impairment in the production of both of these cytokines (Fig. 1D,E). These findings suggest that IRF-3 plays a broad role in the activation of antigen specific T cells following DNA vaccination.

The mechanism by which IRF-3 influences T cell activation following DNA vaccination was examined. To determine if Ag presentation was altered, resting OVA specific OT-1 CD8⁺ T cells were isolated, labeled with CFSE, and adoptively transferred into DNA vaccinated normal and IRF-3 KO mice. The proliferation of these OT-1 cells was significantly reduced in the IRF-3 KO recipients (Fig 2). These results confirm previous *in vitro* studies [14] and indicate that the IRF-3 pathway is involved in the MHC class I presentation following DNA vaccination.

IRF-3 does not influence humoral immunity following DNA vaccination

To examine whether the humoral immune response elicited by DNA vaccination is IRF-3 dependent, serum from B/6 and IRF-3 KO mice immunized with the OVA-encoding plasmid was examined. No significant difference in the magnitude or isotype of the resultant OVA-specific Ab response was observed between strains (Fig 3). This finding suggests that IRF-3 does not impact the induction of Ag specific humoral immunity following DNA vaccination.

IRF-3 does not alter plasmid dependent protein production

To clarify the basis for the reduced cellular immune response observed following DNA vaccination of IRF-3 KO mice, the production of plasmid encoded protein was explored. To maximize the sensitivity of protein detection, mice were vaccinated i.m. with a luciferase-encoding (rather than OVA-encoding) DNA plasmid, and luciferase expression monitored *in vivo*. As seen in Fig 4, the level of luciferase expression was slightly higher in vaccinated IRF-3 KO than congenic B/6 mice, although this difference did not achieve statistical significance. Studies at multiple time points, and by other labs, confirm this observation ([21] and data not shown). Thus, it does not appear that transfection, transcription or translation of the plasmid-encoded gene are defective in IRF-3 KO mice. This finding is consistent with the observation that IRF-3 KO mice mount normal humoral immune responses following DNA vaccination.

Contribution of IFN β to the cellular immune response induced by DNA vaccination

Phosphorylation of IRF-3 culminates in the production of type I IFNs, particularly IFN β [22]. To examine whether the induction of type I IFNs might influence the immune response elicited by DNA vaccines, IFN α/β R KO mice were immunized with a CpG-free OVA encoding DNA plasmid. Consistent with earlier results, the humoral immune response of B/6 and IFN α/β R KO mice was almost identical ([23] and data not shown). Nevertheless, the ability of spleen cells from IFN α/β R KO mice to secrete IFN γ when re-stimulated *in vitro* with OVA was significantly reduced when compared to WT controls (Fig 5A, p. <0.005). This finding suggests that the production of type I IFNs may contribute to the overall immunogenicity of DNA

vaccines, perhaps through an IRF-3-mediated process. To pursue this possibility, IRF-3 KO mice were co-immunized with the OVA-encoding DNA vaccine plus a plasmid encoding IFN β . As seen in Fig 5B, the inclusion of IFN β corrected the defect in DNA vaccine responsiveness observed in IRF-3 KO mice in a dose dependent manner.

DISCUSSION

The mechanism(s) by which DNA vaccines induce cellular immune responses is incompletely understood. Initial reports indicated that CpG motifs present in the bacterial plasmid backbone enhanced vaccine induced immunity, yet more recent studies utilizing plasmids lacking CpG motifs and mice lacking TLR9 do not support that conclusion [24;25]. Current findings provide evidence for an alternative hypothesis: that the cellular immune responses induced by DNA vaccines is mediated, at least in part, through the IRF-3 dependent recognition of intracytoplasmic plasmid, culminating in the production of IFN β and other cytokines.

IRF-3 is known to play a critical role in mediating the activation pathway triggered by the recognition of intracytoplasmic nucleic acids [13;14]. Under physiological conditions, this recognition likely contributes to the detection and eradication of infectious viruses [11]. We previously showed that cells transfected *in vitro* with a plasmid DNA vaccine were stimulated to produce IFN β which resulted in the activation of CD8⁺ T cells through an IRF-3 dependent mechanism [14]. As expected, cells lacking IRF-3 showed no such response. In the current study, IRF-3 deficient mice were immunized with either OVA protein or an OVA-encoding DNA vaccine (the later free of immunostimulatory CpG motifs). While responding normally to vaccination with the adjuvanted protein, the cellular response of IRF-3 KO mice following DNA vaccination was significantly impaired (Figs 1,2). These results are consistent with the recent findings of Ishii et al. showing that DNA vaccinated Tbk1 KO mice generate poor T cell responses [26].

Current findings further indicate that the IRF-3 pathway contributes to the activation of CD4 as well as CD8 T cells, and the production of Th2 and Th17 as well as Th1 cytokines following DNA vaccination (Fig. 1). All of these findings are consistent with the observation that antigen presentation is impaired in IRF-3 KO mice (Fig. 2), and are consistent with previous *in vitro* studies showing that plasmid DNA directly activates APCs (including muscle cells) via an IRF-3 dependent pathway [14].

Two observations indicate that the transfection, translation and transcription of DNA plasmids is normal in IRF-3 mice. First, *in vivo* studies utilizing a luciferase encoding plasmid demonstrate that protein production is normal in this strain. Second, DNA vaccinated IRF-3 mice mount normal humoral immune responses to OVA (suggesting that both B and helper T cell responses are intact [15]). Instead, current findings support the hypothesis that internalized plasmid is recognized by IRF-3, triggering a signaling pathway that culminates in the production of type 1 IFNs (particularly IFN β). Consistent with such a model, IFN α/β R KO mice responded poorly to DNA vaccination (Fig 5). This confirms earlier findings by Leitner et al. showing that DNA vaccinated IFN α/β R KO mice fail to generate Ag-specific CTL activity, strongly suggesting that type 1 IFNs play a critical role in the induction of CTL [23]. Moreover, the IRF-3 dependent defect in IFN γ production could be corrected by providing an alternative source of IFN β . As seen in Fig 5, co-delivery of an IFN β encoding plasmid along with the OVA DNA vaccine resulted in a normal IFN γ response. Of particular note, IRF-3 did not impact the induction of Ag specific humoral immunity following DNA vaccination in the current study or that of Leitner et al. This suggests that IFN β plays an important role in the generation of IFN γ secreting cellular immune response, but not in the generation of the helper T cells needed to support Ab production.

Type 1 IFNs make broad contributions during both innate and adaptive immune responses. In particular, these cytokines play a key role during the induction of cellular immunity. IFN β up-regulates the expression of TAP, LMP, and MHC class I molecules through both autocrine and paracrine pathways, thereby improving Ag presentation. IFN β also up-regulates CD40 and B7 expression, and these co-stimulatory molecules facilitate the induction of CD4-independent CTL responses [27;28]. Thus, mice lacking type I IFNs receptors (or elements of the associated signaling cascade) exhibit defects in their capacity to eliminate virus infect cells and cancer cells.

To design of optimally effective DNA vaccines requires an understanding of the factors that influence the induction of humoral and cellular immunity. Current results indicate that the recognition of intracytoplasmic plasmid by cells transfected *in vivo* following DNA vaccination is mediated by IRF3, and that this process plays a critical role in the subsequent generation of an effective CTL/cellular immune response. Thus, methods designed to optimize DNA vaccine uptake (such as electroporation) should result in qualitatively as well as quantitatively improved immune responses that are better suited DNA vaccine mediated elimination of virus infections and cancer.

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Figure 1. Ag-specific cytokine response of cells from C57Bl/6 and congenic IRF-3 KO mice immunized with an OVA-encoding DNA vaccine or OVA protein in IFA

Mice were immunized bilaterally in the femoral muscles with 50 μ g/muscle of a CpG-free OVA-encoding DNA plasmid (A, C, D and E) or i.p. with 10 μ g of OVA in IFA (B). Two weeks after immunization, spleen cells (A, B, C and D), or CD4⁺ or CD8⁺ T cell depleted spleen cells (E) from these mice were re-stimulated *in vitro* with 100 μ g/ml of OVA or 1 ng/ml of SIINFEKL for 12 hrs. The number of spleen cells stimulated by Ag to secrete IFN γ IL-4 and IL-17 was monitored by ELISpot assay. Data represent the combined mean $\pm$ SD of 7 – 12 independently analyzed mice per group from 2 – 3 independent experiments.

* p < .05, ** p < .001.

Figure 2. Ag-specific CD8 T cell proliferation from C57Bl/6 and congenic IRF-3 KO mice immunized an OVA-encoding DNA vaccine

Mice were immunized as described in Fig 1. Two days later, 2.0×10^6 resting CFSE-labeled OT-1 cells were transferred i.v. into these animals. 3 days later, popliteal LN cells were stained with anti-Vα2Vβ5-APC and analyzed by flow cytometry. The upper panel shows Vα2Vβ5⁺ CFSE⁺ gated T cells. Cells that divided >2 times are shown in the boxed region in the upper panels, and are quantified (mean ± SD of 6 – 7 mice/group from 3 independent experiments) in the lower panel.

** p < .001.

Figure 3. Serum anti-OVA Ab titers in C57Bl/6 and congenic IRF-3KO mice immunized with an OVA-encoding DNA vaccine

Mice were immunized as described in Fig 1. Four weeks later, serum obtained from these animals was analyzed by ELISA for OVA specific Ig titers. Data represent the mean $\pm$ SEM of 7–8 independently analyzed mice per group from 2 independent experiments and were confirmed in two additional experiments. Note that there were no significant differences between strains.

Figure 4. Effect of IRF-3 on plasmid encoded protein production

Mice were immunized bilaterally in the femoral muscles with 20 ug of a luciferase encoding plasmid. Two days later, mice were injected i.p. with 3 ug of firefly luciferin and transgene expression was monitored *in vivo* using an IVIS imaging system. Total proton flux in each leg (protons/sec) was measured in regions of interest (red circles). Data represent the mean $\pm$ SD of four independently analyzed legs per group. Results were confirmed in 2 additional experiments, and in the analysis of additional time points.

Figure 5. Effect of IRF-3 and type I IFNs on DNA vaccine immunogenicity

A) C57Bl/6 and congenic IFN α/β R KO mice were immunized with the OVA encoding plasmid as described in Fig 1. B) IRF-3 KO mice were co-immunized i.m. with 100 μ g of the OVA encoding plasmid plus the indicated dose of an IFN β encoding plasmid. Two weeks after vaccination spleen cells from A) and B) were re-stimulated *in vitro* with 100 μ g/ml of OVA or 1 ng/ml of SIINFEKL for 12 hrs, and IFN γ secreting cells quantified by ELISpot assay. Data represent the mean $\pm$ SD of 4–9 mice/group from 2 independent experiments.

* $p < .05$, ** $p < .001$