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SHORT COMMUNICATION

STAT4 is required for the generation of T_h1 and T_h2, but not T_h17 immune responses during monophosphoryl lipid A adjuvant activity

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Abstract

STAT4 is critical for the production of IFN-γ during the generation of T_h1 immune responses. We investigated the role of STAT4 in mediating T_h1-inducing activity of a vaccine adjuvant monophosphoryl lipid A (MPL-A) using the standard antigen ovalbumin (OVA) in STAT4KO mice. Our results show that splenocytes from STAT4KO mice displayed lower OVA-specific T-cell proliferation and IL-2 production compared with wild-type (WT) mice. Further, IFN-γ production was diminished in STAT4KO-derived splenocytes but the levels of IL-12 and TNF-α were similar compared with WT mice. Interestingly, STAT4 deficiency also led to a decrease in IL-10 and T_h2 cytokines such as IL-4 and IL-13 upon MPL-A immunization, although IL-17 production was similar between WT- and STAT4KO-derived splenocytes. Our observations for defective T_h1 and T_h2 responses in STAT4KO mice were further supported by the low levels of T_h1-associated IgG2a and T_h2-associated IgG1 in the sera of these mice. Taken together, our results show that STAT4 plays a critical role in mediating both T_h1 and T_h2 responses upon immunization with MPL-A. Our study provides a better understanding of how MPL-A mediates T-cell activation which will be critical for future vaccine development.

Keywords: IFN-γ, IL-2, IL-4, T cells, vaccine

Introduction

Monophosphoryl lipid A (MPL-A) is a chemically modified derivative of lipopolysaccharide (LPS) that has minimal toxicity while maintaining the immunostimulatory properties of LPS (1, 2). MPL-A is a component of the adjuvant AS04 which has successfully been employed in vaccines against hepatitis B virus and human papilloma virus-mediated cervical cancer (3–5). Further, it has demonstrable efficacy in many pre-clinical and clinical studies targeting cancer and infectious diseases such as HIV (6–8). Clinical trials have shown that MPL-A is an effective and well-tolerated adjuvant that can be used in a broad range of candidate vaccines. It triggers both cellular and humoral effector arms of the immune response and has the capacity to induce T_h1 and T_h2 immune responses (9, 10).

Previous studies that define the cellular targets of MPL-A have demonstrated that it activates antigen-presenting cells (APCs) and T cells (9, 10). APCs such as dendritic cells (DCs) respond to MPL-A stimulation through up-regulation of co-stimulatory molecules, increased production of the T_h1-inducing cytokine, IL-12, and enhanced cellular migration. Therefore, MPL-treated DCs are more efficient at stimulating T-cell proliferation and producing the T_h1 cell-derived cytokine, IFN-γ. MPL-A also robustly activates macrophages to enhance T_h1 and T_h2 immune responses. In addition to promoting T-cell responses by acting on innate immune cells, MPL-A directly targets T cells to enhance surface CD40L expression. It is known that MPL-A engages the TLR-2 and TLR-4 pathways in DCs and macrophages leading to the sequential activation of signaling molecules such as p38 MAP kinase, ERK1/2 and NF-κB (9–12). However, the mechanism by which MPL-A regulates the function of T cells has not been fully defined. Further studies into the mechanisms by which

MPL-A modulates T-cell function will improve our understanding of its adjuvant activity, thereby providing us with critical knowledge that will facilitate improved vaccine development.

STAT proteins are transcription factors which are activated in response to cytokines. The transcription factor, STAT4, is expressed in T cells and is phosphorylated primarily in response to IL-12. Binding of IL-12 to the receptors IL-12Rβ1 and IL-12β2 induces the heterodimerization of the JAK kinases, JAK2 and TYK2. These kinases further phosphorylate STAT4 which forms homodimers and translocates to the nucleus to activate IFN-γ production (13–15). The requirement for STAT4 in the development of T_h1 responses was demonstrated by a study showing impaired IFN-γ production and T_h1 cell generation in STAT4-deficient mice (16). Since IL-12 and IFN-γ synthesis are hallmarks of MPL-A adjuvant activity, we hypothesized that STAT4 plays a role in the generation of T_h1 responses upon MPL-A administration. We therefore investigated the role of STAT4 in the generation of these immune responses using a MPL/ovalbumin (OVA) immunization model in wild-type (WT) and STAT4KO mice.

Methods

Animals

Female WT BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN, USA). STAT4KO BALB/c mice were gift from Dr Mark H. Kaplan (Indiana University, Bloomington, IN, USA). Mice were maintained and bred at the Ohio State University animal facility according to animal protocols and University Laboratory Animal Resources (ULAR) regulations. The experiments were performed using 8- to 10-week-old sex-matched mice according to the institutional guidelines for animal research.

Immunization of mice

MPL-A, derived from *Salmonella minnesota* R595, was purchased from InvivoGen (San Diego, CA, USA). Mice were immunized with an emulsified solution of 50 μg of MPL-A with 100 μg of EndoGrade OVA from Biovendor LLC (Ashville, NC, USA) on the basis of a protocol described previously (17). Briefly, mice were injected subcutaneously in the back with MPL-A (50 μg) and OVA (100 μg) at day 0. The same dosage formula of MPL-A and OVA was used for subsequent boosting at week 2 and week 4. Mice were sacrificed at week 6, 2 weeks after the second boosting dose, to harvest spleens in order to evaluate T-cell proliferation and cytokine responses.

Evaluation of OVA-specific cellular proliferation and cytokine responses in splenocytes

Mouse spleens were excised and mashed to get single-cell suspensions which were treated with ACK lysis buffer to lyse RBCs. Cells were then washed, plated in 96-well plates at the concentration of 5×10^6 cells ml^{-1} in RPMI medium (supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, 1% HEPES and 0.1% β-mercaptoethanol), then stimulated with 1 mg ml^{-1} OVA for 72 h at 37°C. At 48 h, cellular proliferation was analyzed by the reduction of alamar blue using a colorimetric assay. At this time point, 10% alamar blue (Life Technologies, Grand Island, NY, USA) was added to all the wells and plates were incubated for 6–8 h before measuring OD values using a Spectramax Microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) at absorbance wavelengths of 570 and 600 nm. Cellular proliferation was analyzed using Softmax Pro software (Molecular Devices LLC) and an online alamar blue reduction calculator (AbD Serotec, Raleigh, NC, USA). At 72 h, culture supernatants were harvested to analyze production of cytokines by ELISA. Concentrations of IFN-γ, IL-2, IL-4, IL-10, IL-12, IL-13, IL-17 and TNF-α cytokines were determined using capture and detection antibodies purchased from Biolegend (San Diego, CA, USA) and cytokine standards purchased from BD Biosciences (San Jose, CA, USA). ELISA results were analyzed using the Softmax Pro software (Molecular Devices LLC).

Flow cytometry

Flow cytometry was performed from single-cell suspensions prepared from the spleens of WT and STAT4KO mouse experimental groups at the 6-week endpoint. Spleens were briefly mashed and RBCs were lysed with ACK lysis buffer. Normal mouse serum was used to block the Fc receptors followed by staining with the appropriate antibody cocktail. CD19, CD11b, CD11c, MHCII, CD40, CD80 and CD86 antibodies were purchased from Biolegend. Cells were acquired by BD LSRII flow cytometer (BD Biosciences). Analysis was later performed with Flow Jo software (Tree Star Inc., Ashland, OR, USA).

Determination of OVA-specific IgG1, IgG2a antibodies

Mice were bled at weeks 2, 4 and 6 from all groups, 2 days before the subsequent boosting, to collect blood sera which were incubated at 4°C overnight and centrifuged to obtain serum. OVA-specific IgG1 and IgG2a antibody titers in serum were determined by ELISA using HRP-conjugated antibodies (Biolegend) and TMB substrate (KPL Inc., Gaithersburg, MD, USA). ELISA results were analyzed using the Softmax Pro software (Molecular Devices LLC).

Statistical analysis

All statistical analyses were done using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Student's unpaired *t*-test was used to determine statistical significance of samples values obtained. P values <0.05 were considered statistically significant.

Results and discussion

The splenocytes of MPL/OVA-immunized STAT4-deficient mice display reduced cellular proliferation after ex vivo re-stimulation

To determine whether STAT4 plays a role in MPL-mediated activation of adaptive immune responses, we immunized WT and STAT4KO mice with MPL-A and OVA and evaluated the OVA-specific cellular proliferation at week 6 post-immunization. We observed that MPL-A administration led to strong cellular proliferation in WT-derived splenocytes while

STAT4KO-derived splenocytes showed significantly lower cellular proliferation (Fig. 1A). Since IL-2 is critical for promoting T-cell proliferation, we examined whether diminished T-cell proliferation in STAT4KO correlated with lower IL-2 production in these mice (18). As expected, IL-2 levels were significantly lower in *ex vivo* re-stimulated STAT4KO-derived splenocytes compared with WT counterparts (Fig. 1B). Further, MPL immunization had little or no effects on cellular proliferation and concomitant IL-2 production in STAT4-deficient re-stimulated splenocytes (Fig. 1A and B). These data demonstrate that STAT4 is critical for MPL-mediated proliferation of adaptive immune T cells following MPL–OVA immunization.

STAT4 deficiency results in diminished T_h1 and T_h2 responses but not T_h17 cytokine response

Since STAT4 is critical for the development of a T_h1 response and MPL-A administration enhances T_h1 immune responses (9–11, 16), we determined whether STAT4KO mice are able to mount a T_h1 response in response to MPL-A treatment. We observed that there was approximately a 3-fold decrease in IFN-γ production in STAT4KO-derived splenocytes compared with WT-derived splenocytes upon MPL-A administration (Fig. 2A). Interestingly, STAT4 deficiency did not disrupt innate cytokine production, specifically IL-12 and TNF-α (Fig. 2B and C), which are critical for promoting T_h1 cell differentiation.

Next, we determined whether STAT4 deficiency affects the development of T_h2 and T_h17 responses upon MPL-A/OVA immunization. MPL-A-treated STAT4KO-derived splenocytes displayed significantly impaired IL-4, IL-6 and IL-13 production (Fig. 2D, G and F) compared with MPL-A-treated WT mice. Further, STAT4KO-derived splenocytes displayed lower IL-10 production compared with WT-derived splenocytes upon MPL administration (Fig. 2E), but there was no significant difference in IL-17 production between the two groups (Fig. 2H).

Finally, we determined whether STAT4 deficiency affects antibody isotype production in response to MPL-A/OVA immunization. We observed that antibody titers of T_h2-associated IgG1 and T_h1-associated IgG2a increased over time in MPL-A-treated WT mice (Fig. 3A and B). However, MPL-A-treated STAT4KO mice showed significantly reduced production of OVA-specific IgG1 and IgG2a antibody. Taken together, these results show that STAT4 deficiency inhibits T_h1 and T_h2, but not T_h17 immune responses in response to OVA/MPL-A immunization.

Absence of STAT4 inhibits MPL-induced expression of co-stimulatory molecules on DCs

Since MPL-A is known to activate APCs and enhance their expression of co-stimulatory molecules, we evaluated the role of STAT4 in DC and B-cell activation by MPL. OVA re-stimulated cultures from MPL-A-immunized WT and STATKO mice were analyzed by flow cytometry for co-stimulatory markers CD40, CD80 and CD86 on B cell and DC populations. We found no differences in co-stimulatory molecule expression on B cells between MPL-A/OVA-immunized WT- and STAT4KO-derived splenocytes (Fig. 4A and C). Interestingly, in the DC population, CD80 and CD86 were expressed at a much lower rate in *ex vivo* re-stimulated STAT4KO-derived splenocyte cultures compared with WT (Fig. 4B and D). No differences were found in the expression of CD40 between re-stimulated WT and STAT4KO DCs. In WT DCs, expression of CD80 and CD86 was significantly higher in MPL-A/OVA-immunized WT mice compared with WT mice immunized with OVA alone. This difference was not observed in DC cultures of MPL-A/OVA-immunized STAT4KO mice and OVA-alone-immunized STAT4KO mice (Fig. 4B and D). Our results demonstrate that MPL-A-mediated enhancement of co-stimulatory molecules CD80 and CD86 in DCs is mediated partly by STAT4. The reduced expression of co-stimulatory molecules in MPL-immunized STAT4-deficient mice provides a potential mechanism underlying the deficient T-cell activation, proliferation and cytokine production observed in the absence of STAT4 (Figs. 1 and 2). Taken together, our data demonstrate the role STAT4 plays in DC activation and subsequent T-cell immune responses following immunization with MPL-A.

Our present study indicates that MPL-A mediates its adjuvant activity and stimulation of adaptive immune responses through

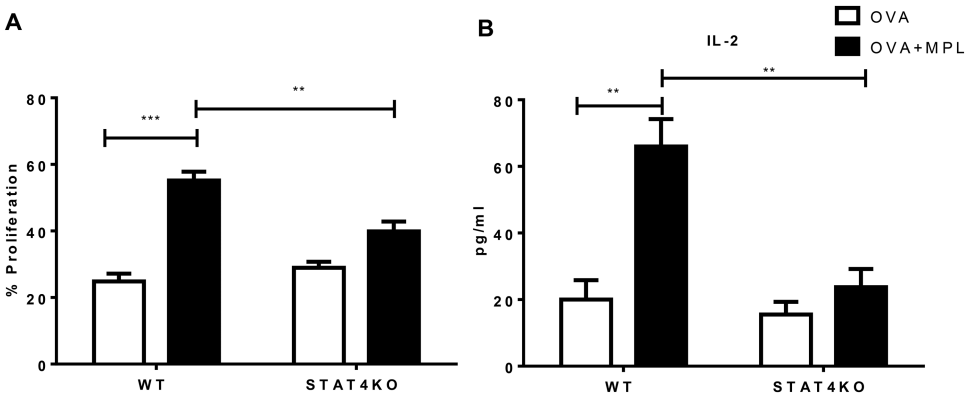

Fig. 1. Evaluation of OVA-specific cellular proliferation and IL-2 production upon MPL-A/OVA immunization. Splenocytes from MPL-A/OVA-immunized WT and STAT4KO mice were harvested at 6 weeks post-immunization and stimulated with 1 mg ml^{-1} OVA to analyze OVA-specific (A) cellular proliferation and (B) IL-2 production. At 48 h of OVA stimulation, cellular proliferation was evaluated based on the reduction of alamar blue. At 72 h of OVA stimulation, IL-2 production was assayed in the culture supernatant by ELISA. Data represented are the combined mean ± SEM values from three independent experiments (n = 5 per group). P values are represented by: $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

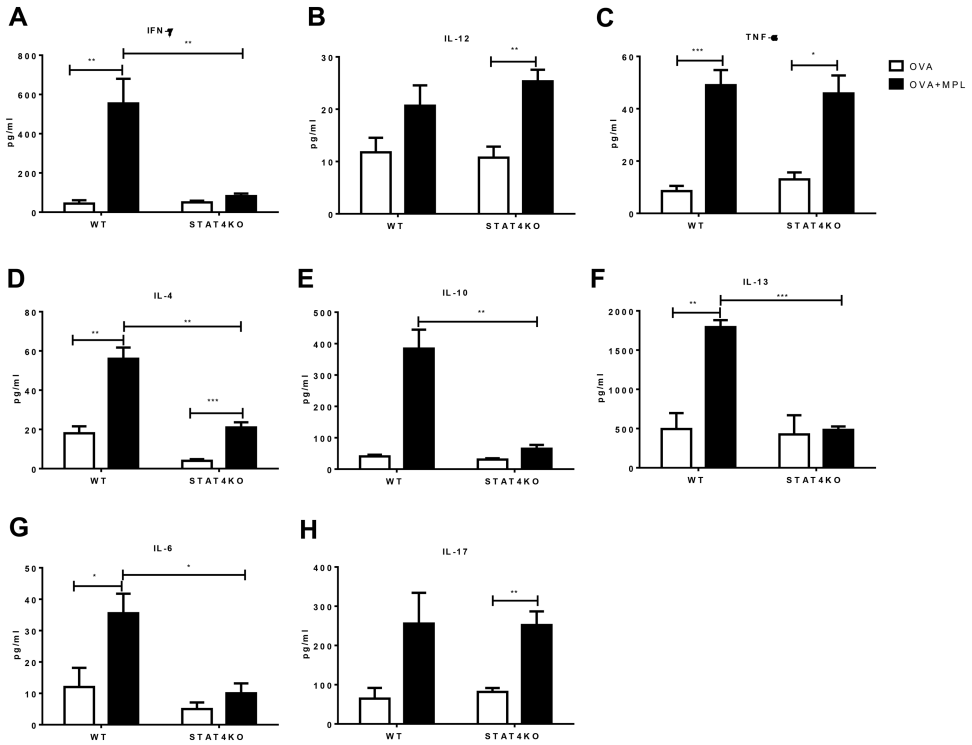

Fig. 2. STAT4 deficient derived splenocytes display impaired IFN-γ, IL-4, IL-6, IL-10 and IL-13 but similar IL-17 production in response to MPL-A administration. Splenocytes from MPL-A/OVA-immunized WT and STAT4KO mice were harvested at 6 weeks post-immunization and stimulated with 1 mg ml^{-1} OVA to analyze OVA-specific T_h1, T_h2 and T_h17 cytokine production. At 72 h of OVA stimulation (A) IFN-γ, (B) IL-12, (C) TNF-α, (D) IL-4, (E) IL-10, (F) IL-13, (G) IL-6 and (H) IL-17 cytokines were assayed in the culture supernatant by ELISA. Combined data from three independent experiments each with duplicate sample values are shown and are presented as the mean ± SEM ($n = 5$ per group). *P* values are represented by: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

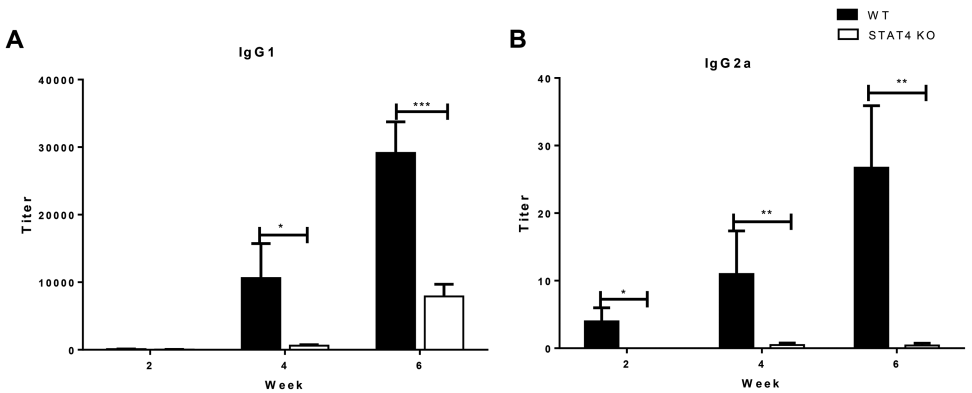

Fig. 3. STAT4 deficiency leads to lower IgG antibody responses upon MPL-A administration compared with WT mice. WT and STAT4KO mice immunized with OVA and MPL-A were bled at weeks 2, 4 and 6 to determine OVA-specific (A) IgG1 and (B) IgG2a antibody production by ELISA. Data represented are the combined mean ± SEM values from three independent experiments ($n = 5$ per group). *P* values are represented by: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

STAT4. We demonstrate that T cells from STAT4KO mice show substantial decrease in IFN-γ production upon MPL-A administration and this is consistent with previous reports of deficient T_h1 responses in STAT4KO mice (16). Our results further suggest that a deficiency in co-stimulatory molecule expression in DCs contributes to the defective T-cell activation and diminished IFN-γ production by T cells in STAT4KO mice after immunization with MPL-A. This general defect in T-cell activation by DCs can also explain the reduced production of T_h2 cytokines in the absence of STAT4. Consequently, although levels of IL-12 and TNF-α were similar in WT and STAT4KO mice immunized with MPL-A, T-cell immune responses were attenuated in STAT4-deficient mice. This supports the notion that production of these cytokines following MPL-A immunization is not dependent on STAT4, an idea further supported by a previous report demonstrating that LPS-treated STAT4KO mice

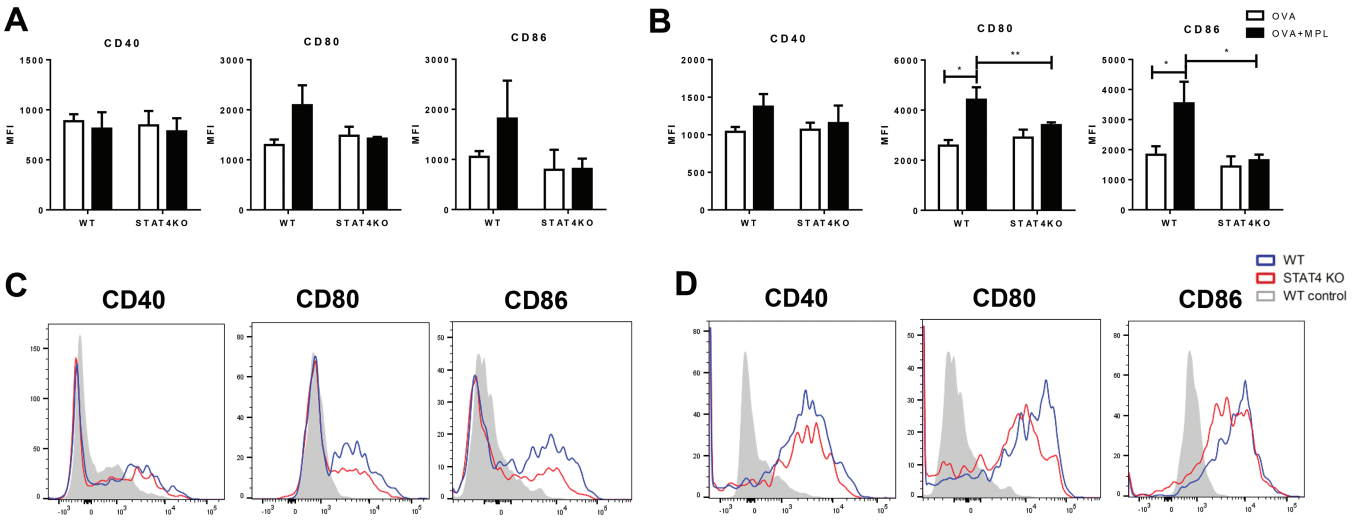

Fig. 4. STAT4KO-derived splenocytes show reduced expression of co-stimulatory molecules compared with WT-derived splenocytes upon immunization with MPL-A adjuvant. Flow cytometry data of splenocytes from WT and STAT4KO mice immunized with MPL-A–OVA or OVA alone harvested at 6 weeks post-immunization and re-stimulated with 1 mg ml^{-1} OVA. Expression of CD40, CD80 and CD86 co-stimulatory molecules on B cells (A and C) and DCs (B and D) are shown. Bar graphs (A and B) show mean fluorescence intensities (MFI) of cell surface markers from re-stimulated splenocytes labeled with B cell and DC markers. Data are from one experiment and presented as the mean ± SEM (n = 5 per group). Histogram plots (C and D) show expression of co-stimulatory molecules from a representative mouse in each group. P values are represented by: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

have no defects in IL-12 and TNF-α synthesis (19). However, we also observed that IFN-γ synthesis is not completely abrogated in the absence of STAT4 (Fig. 2A), highlighting the possible role of STAT4-independent pathways in promoting T_h1 immune responses upon MPL-A treatment. Indeed, such STAT4-independent pathways have been implicated in the development of T_h1 responses in STAT4KO mice infected with Gram-negative bacteria (20). These responses could be mediated by IL-18, which has been shown to be an important promoter of T_h1 differentiation (21) and critical for LPS-induced IFN-γ synthesis (22), possibly by the direct activation of AP-1 in the absence of STAT4 (23).

We observed reduced IL-4, IL-10 and IL-13 production upon MPL-A/OVA immunization in STAT4KO-derived splenocytes (Fig. 2D–F). It is known that T_h2 responses are generated upon activation of macrophages by MPL-A administration (9). However, the role of STAT4 in the generation of T_h2 immune responses is not well defined and appears to be dependent in part on the type of pathogen-associated molecular patterns (PAMPs). *Listeria*-infected STAT4KO mice display defective T_h1 responses and enhanced T_h2 responses (16). Similar results were also observed in infection of the helminth parasite, *Taenia crassiceps* (24). In contrast, a study by Myers *et al.* (25) demonstrated that STAT4KO mice in BALB/c background display deficient IL-4 and IL-13 production in response to infection with *Pneumocystis murina*. Interestingly, the diminished T_h2 responses correlated with reduced IL-2 responses in the STAT4KO-derived splenocytes which we also observed in our study (Fig. 1B). The importance of IL-2 in the differentiation of T_h2 cells has been described previously (26). Hence, it is possible that the diminished IL-2 production that we observed in MPL-A-treated STAT4KO-derived splenocytes could be responsible for the lack of T_h2 cell differentiation and subsequently, reduction in T_h2 cytokines responses in our study. Nevertheless, our study demonstrates that in MPL-A adjuvant administration, STAT4 plays a role in T_h2 immune response development.

Our results therefore highlight the potential benefit of exploiting the immunomodulatory properties of MPL-A in vaccine design for intracellular pathogens. This area of research is problematic, partly due to the complex biology of parasites and the requirement for activation of different arms of the immune response to confer protection, especially the cell-mediated arm of the immune response (27). The use of MPL adjuvant can be potentially beneficial in vaccine design due to its ability to induce both T_h1 and T_h2 cell-mediated immune responses. Given that STAT4 plays a role in immunity to a number of pathogens (16, 24, 28–30), MPL adjuvant activity could be potentially beneficial. In this regard, MPL-A was shown to be effective at overcoming immune non-responsiveness in mice vaccinated against the malaria parasite (31). Another recent study demonstrated that addition of MPL to a liposomal anti-leishmania vaccine preparation enhanced antigen presentation by DCs and subsequent antigen-specific $CD8^+$ T-cell-mediated immune responses (32). These studies highlight the value of MPL adjuvant in vaccination strategies and it is likely to be a future direction for vaccine design against intracellular pathogens.

In summary, our study indicates that STAT4 contributes to the development of both T_h1 and T_h2 responses upon MPL-A/OVA immunization. The observations of our study enhance our understanding of the mechanism by which the adjuvant MPL-A modulates T-cell activation. This study and other studies that define the mechanisms of MPL-A-mediated adjuvant activity are critical in enhancing the application of this adjuvant beyond its current use in the development of vaccines for cancer and infectious disease prevention (3–8).

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