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The Requirement of CD80, CD86, and ICAM-1 on the Ability of Adjuvant Formulations to Potentiate Antibody Responses to a *Plasmodium falciparum* Blood-Stage Vaccine

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Abstract

Many adjuvants are known to enhance expression of co-stimulatory and adhesion molecules secondarily to the activation of immune cells. Whether interactions via these molecules are obligatory in adjuvants' ability to potentiate vaccine immunogenicity is less clear. We investigated the ability of eight adjuvant formulations to potentiate the immunogenicity of a malaria vaccine in mice deficient in the prominent co-stimulatory molecules, CD80 and CD86; and the adhesion ligand, ICAM-1. While no adjuvants could bypass co-stimulatory requirements, more formulations exhibited dependency for CD86 than for CD80. In CD80 or CD86 KO mice, formulations with the saponin derivative, QS21 could efficiently default to the other B7 molecule. This effect was dominant over other adjuvant constituents. The requirement for ICAM-1 could be readily by-passed using adjuvant formulations containing immunomodulators; whereas this was not the case with emulsion-type adjuvants in which reduction in adjuvanticity was associated with decreases in antigen-specific IFN- γ responses. These studies may help guide the formulation of vaccine adjuvants to maintain effectiveness in hosts with altered immunological environment that often result from infections.

INTRODUCTION

Adjuvants are often indispensable components of many vaccine formulations. Recent research work have identified critical mechanisms of action of immunological adjuvants [1,2]. While a number of adjuvants such as the monophosphoryl lipid A (MPL) act by initial binding to specific ligands on immune cells, eg. the TLRs on APCs, resulting in upregulations of co-stimulatory molecules like CD80 and CD86 [1–4], the ensuing array or cascade of immunological activities will have much broader immuno-biological effects or consequences. It is however less clear what role and/or how important that these secondary events play to specifically augment the potentiation activities of immunological adjuvants. This is of considerable relevance since such events often influence the outcome of the nature of the immunological responses, which in turn help shape the protective effector mechanisms of vaccine-induced immunity. For example, the induction of CD8+ CTL effector responses can be influenced by a number of immunological parameters, including a battery of cytokines, co-stimulatory signals, and accessory cells [5–8]. A recent study show that CD8+ T cell responses can be synergistically augmented by two distinctive adjuvants, namely monophosphoryl lipid

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A (MPL) and α -GalCer glycolipid, which have very different initial cell and ligand binding specificities [4,9–18], but may have overlapping and non-overlapping secondary activities on DCs that in turn affect the generation of CD8⁺ responses [19]. Thus, further knowledge on which downstream immunological activities are critical for adjuvant activity will facilitate the design of adjuvant mixtures that may have synergistic effects.

We have studied a number of such “secondary” immunological signals; namely IL-4 and IFN- γ [20], as well as IL-6 [21] in terms of their influences on the potentiation activities of a number of well known adjuvant components and formulations to induce antibody responses to a *P. falciparum* blood-stage vaccine, MSP1 [22]. Results show that the effects are highly adjuvant-specific, and in some cases the role(s) of adjuvants on the growth and differentiation of hematopoietic cells, in addition to direct immune activations, may contribute to overall potency [21].

The activation of appropriate T helper responses is critical to ensure efficient induction of immune effector mechanisms. Secondary to the TCR-antigen epitope engagement, receptor/ligand interactions via co-stimulatory molecules on T cells and APCs are of equal significance in the initiation and maintenance of immune responses [23]. The B7 (B7-1/B7-2 or CD80/CD86) and CD28 family of co-stimulatory molecules have been clearly demonstrated to have critical roles in the regulation of T and B cell responses [24–26]. In addition, adhesion molecules such as ICAM-1/LFA-1 provide important cell-cell interactions between T cells and APCs, which influence the development of immune responses [27–29]. Infections with a number of microbial agents have been shown to alter the expression of B7-1/B7-2 and ICAM-1, which have negative impact on the development of immunity [30–38]. Although some adjuvants such as the TLR ligands have been shown to activate immune cells resulting in increased expression of these co-stimulatory molecules/ligands (reviewed in [39]), it is not known if their individual expression is critical to the ability of these adjuvants to enhance the immunogenicity of vaccines. To begin addressing this question, we performed parallel immunization studies in knockout mice deficient in CD80 or CD86, or both; and also in mice deficient in ICAM-1. Eight adjuvant formulations based on MPL (MPL-SE, MPL-AF), a saponin derivative (QS21), and Montanide ISA720 were used in adjuvant-assisted immunizations with the *P. falciparum* Merozoite Surface Protein, MSP1-19, using regimens similar to previous studies with other immune gene knockout mouse models [20,21]. Results on the study of vaccine immunogenicity, as measured by antibody and cellular responses, suggested that for several adjuvants co-stimulation via either CD80 or CD86 were equally efficient and a deficiency in either one B7 molecule could be compensated by the other. On the other hand, a few adjuvant formulations showed greater dependency for CD86 co-stimulation than for CD80. The results also suggested that the potency of MPL adjuvants was generally more sensitive to deficiencies in the co-stimulatory molecules. Similar analyses of the adhesion molecule, ICAM-1 revealed that adjuvant formulations devoid of immunomodulators (ie. MPL, QS21) were sensitive to ICAM-1 KO in terms of their ability to potentiate vaccine immunogenicity. The significance of these findings is further discussed.

MATERIALS AND METHODS

Mouse Strains

Female mice (C57Bl/6 background, 8–12 weeks old) of the following strains were purchased from Jackson Lab. (Bar Harbor, Maine): CD80, CD86, and CD80/86 double deficient mice (CD80^{-/-}; CD86^{-/-}; CD80/86^{-/-}), and ICAM-1 deficient (ICAM-1^{-/-}). Normal, age and sex matched C57Bl/6 mice were used as Wild-Type (WT) controls.

Malaria vaccine antigen

The C-terminal 19 kDa fragment of *Plasmodium falciparum* Merozoite Surface Protein 1, MSP1-19 was used as the immunogen. The recombinant protein was expressed in *Pichia pastoris* as a fusion protein with the P30 and P2 universal T epitopes [22]. The production and purification of this vaccine antigen was described in detail in a previous study [22]. The utilization of the recombinant antigen with universal T helper epitopes was to help insure that any observed differences in immunogenicity is not due to preferential recognition of T epitopes regulated by immune response (IR) genes [40]. This immunogen was a kind gift from Dr. Anthony Stowers.

Adjuvant formulations

The following adjuvants were used. Montanide ISA720, a metabolizable oil adjuvant (Seppic Inc. Fairfield, NJ) [41]; MF59, squalene/oil emulsion (Chiron Corp. Emeryville, CA) [42]; QS21, a saponin derivative (Antigenics Inc. Lexington, MA) [43]; MPL (from *E. coli* F583 Rd mutant, Sigma-Aldrich, St Louis, MO); MPL-AF, monophosphoryl lipid A in aqueous formulation (Corixa Inc. Seattle, WA) [44]; and MPL-SE, monophosphoryl lipid A in squalene emulsion (Corixa Inc. Seattle WA) [45]. Additional formulations comprised of combinations of above adjuvants were also used, ISA720/MPL, ISA720/QS21, ISA720/QS21/MPL. This is based on combining the carrier-type adjuvant (ISA720) with the immunomodulators. Due to stringent MTA (Material Transfer Agreement) restrictions, we were not able to study the combined formulations of QS21 with MPL-AF or with MPL-SE, or ISA720 with MPL-AF; instead, commercially available MPL (from *E. coli* F583 Rd mutant, Sigma-Aldrich, St Louis, MO) was used in the combined formulations. The MPL-AF (MPL in aqueous solution) was used as the “MPL alone” equivalent. Similarly, since MTA prohibits combining QS21 with MF59, only the ISA720 was used.

Formulation of antigen and adjuvants, and dosing

Each dose of the MSP1-19 antigen (P30P2MSP1-19) is 10 ug. The antigen was diluted in PBS (pH 7.0 or pH 6.8 for QS21 preparations). For MSP1-19/QS21, 10 ug of antigen was diluted in PBS (pH 6.8) and reconstituted with 5 ug QS21 to 100 ul. For MSP1-19/MF59, 10 ug MSP1-19 was vortexed with MF59 at a volume ratio of 1:1 in a final volume of 100 ul. For MSP1-19/MPL-SE, 10 ug of MSP1-19 in 150 ul PBS was vortexed with 50 ul MPL-SE (MPL content at 1000ug/ml). For MSP1-19/MPL-AF, 10 ug of MSP1-19 was mixed with 50 ug MPL-AF to a final volume of 100 ul. For MSP1-19/ISA720, 10 ug MSP1-19 was emulsified with ISA720 at a volume ratio of 7:3 (oil:water) in a total volume of 100 ul. The ISA720/MPL, ISA720/QS21, and ISA720/QS21/MPL were similarly emulsified with MSP1-19 except that 50 ug of MPL and/or 5 ug of QS21 were reconstituted with the antigen before mixing with ISA720. Thus, the amount of MPL (or QS21) given to each mouse is kept the same irrespective of the formulations.

Immunization regimen

Mice (5 per group) were immunized intraperitoneally with a dose of 10 ug MSP1-19 (P30P3MSP1-19) in different adjuvant formulations as described above. A total of three immunizations were given at 4 weeks interval. Mice were bled one week before the first and three weeks after the last immunization. The use of mice in this study has the full approval from the University of Hawaii Institutional Animal Care and Use Committee.

Serum antibody assays

The development of antibody responses to MSP1-19 in the immunized mice were evaluated by ELISAs according to established methods [20,46]. Briefly, ELISA plates (Costar/Corning, Acton, MA) were coated with the test antigen, MSP1-19 [47] at 0.4ug/mL and blocked with

1% Bovine Serum Albumin (BSA) in Borate Buffered Saline (BBS). Test sera were serially diluted in 1% BSA/0.5% yeast extract (DIFCO/BD Biosciences, San Jose, CA) in BBS. Diluted sera were incubated in ELISA wells for 60 min. and washed 7 times with BBS. Washed wells were incubated for 60 min with horseradish peroxidase (HRP) conjugated, goat anti-mouse antibodies at a 1/1000 dilution (H & L chain specific, Kirkgaard and Perry Laboratories; ie. assay for total Ig). Peroxidase substrates, H₂O₂ and 2,2'-azinobis (3-ethylbenzthiazolinesulfonic acid)/ABTS (Kirkgaard and Perry Laboratories, Gaithersburg, MD) were used for color development and the optical density (O.D.) determined at 410nm. Endpoint titers were calculated as the reciprocal serum dilutions giving an O.D. of 0.2, which is over four fold the mean O.D. value of baseline sera. The immunoglobulin isotypes of the anti-MSP1-19 antibodies were determined by ELISA as described [20]. Mouse sera were diluted to 1/500 for the assays, and HRP-conjugated, goat anti-mouse IgG1, IgG2a, IgG3, and IgM (SouthernBiotech Inc., Birmingham, AL) at a 1/4000 dilution were used as secondary antibodies. The O.D. was determined as above.

Antigen stimulated ELISPOT assays

IFN- γ and IL-4 ELISPOT assays were performed using splenocytes from immunized mice according to described methods [48–51]. Briefly, 96 wells multiscreen filter plates (Milipore Inc., Bedford, MA) were coated with monoclonal antibodies to mouse IFN- γ (R4-6A2, 10 μ g/ml) and IL-4 (11B11, 5 μ g/l) (BD Biosciences, San Diego, CA) overnight at room temp. Plates were washed and further incubated with DMEM/10% Fetal Bovine Serum at 37°C for 1 hr. Spleens from immunized mice were removed 5 days after the last booster immunization and single cell suspensions of splenocytes were prepared. A total of 2.5×10^5 splenocytes were added to each antibody-coated well. The test assays were done in triplicate wells and P30P2MSP1-19 was added to each well at a final concentration of 2 μ g/ml. This antigen concentration was previous determined to be optimal from a dose range of 1 μ g to 10 μ g. Positive Control wells were incubated with phorbol myristate acetate (PMA, 5 ng/ml) and ionomycin (1 ng/ml). Negative Control wells were incubated with growth medium alone. After a 48 hr. incubation at 37°C in 5% CO₂, wells were washed, incubated with biotinylated monoclonal anti- IFN- γ (XMG1.2, 2 μ g/ml) and anti-IL-4 (BVD6-24G2, 1 μ g/ml) antibodies (BD Biosciences, San Diego, CA). Wells were then washed, incubated in a 1/800 dilution of peroxidase labeled streptavidin (Kirkgaard and Perry Laboratories, Gaithersburg, MD), and spots were developed in a solution of 3'3'-diaminobenzidine tetrahydrochloride, DAB and 30% H₂O₂ (Sigma-Aldrich, St. Louis, MO). Spots were enumerated microscopically.

RESULTS

Immunogenicity of the MSP1-19 in CD80, CD86, and CD80/86 deficient mice immunized with different adjuvant formulations

Figure 1 shows the ELISA antibody titers of CD80^{-/-} and CD86^{-/-} mice (Panel A), as well as the CD80/86^{-/-} double knockout mice (Panel B) immunized with P30P2MSP1-19 in different adjuvant formulations, as compared with Wild-Type (WT) controls. Formulations, MF59, ISA720, MPF-AF and ISA720/MPL induced significantly lower anti-MSP1-19 antibodies in CD86^{-/-} mice as compared with WT mice. No effects were observed with CD80^{-/-} mice. On the other hand, anti-MSP1-19 antibodies induced by MPL-SE were lower in both CD80^{-/-} and CD86^{-/-} mice. The immunogenicity of MSP1-19 was not significantly affected by either CD80 or CD86 knockout when the vaccine was adjuvanted in QS21, ISA720/QS21, and ISA720/QS21/MPL. Furthermore, none of the adjuvant formulations were able to induce significant levels of anti-MSP1-19 antibodies in CD80/CD86^{-/-} double knockout mice (Figure 1, Panel B). Thus, the effects we observed with the single CD80 or CD86 knockout mice (Figure 1, Panel A) were most likely due to the remaining intact CD86 or CD80 molecule in the respective deficient mice.

Figure 2 shows the profiles of IgG1/IgG2a (ie. TH2/TH1 type) anti-MSP1-19 antibodies in the CD80 and CD86 KO mouse strains immunized with the adjuvant formulation, MPL-SE (Panel A) and with ISA720/QS21 (Panel B), as compared with WT controls. The MPL-SE formulation was sensitive to CD80 or CD86 KO; whereas ISA720/QS21 was not (see Figure 1). In short, there were no significant changes in the immunoglobulin isotype profiles in the CD80^{-/-} and CD86^{-/-} mice as compared to WT mice, irrespective of the adjuvant's sensitivity to CD80 or CD86 deficiency ($p > 0.05$, Student t test, for either the absolute OD values or OD ratios of IgG1/IgG2a). Similar results were obtained with the remaining six adjuvant formulations (data not shown). The low levels of IgG2a seen with ISA720/QS21 is likely due to the specific adjuvant combinations and not because of the C57Bl/6 background, since the C57Bl/6 mice immunized with MPL-SE (Figure 2, Panel A) produced high levels of IgG2a.

Figure 3 shows the results of ELISPOT assays for IFN- γ and IL-4 production of antigen stimulated splenocytes from CD80^{-/-} and CD86^{-/-} mice immunized with two different adjuvant formulations, ie. ISA720/QS21 which showed no preference for either CD80 or CD86, and ISA720/MPL which showed a preference for CD86 (see Figure 1). There were no significant differences observed between the KOs and WT mice. The results were similar for the remaining six adjuvant formulations (data not shown).

Immunogenicity of MSP1-19 in ICAM-1 deficient mice immunized with different adjuvant formulations

Figure 4 shows the ELISA antibody titers of ICAM-1 ^{-/-} mice immunized with P30P2MSP1-19 in different adjuvant formulations, as compared with WT controls. ICAM-1 KO mice receiving ISA720 or MF59 had significantly lower antibody titers as compared with WT mice; while KO mice receiving the remaining six formulations were unaffected. It is noteworthy that both ISA720 and MF59 do not contain immunomodulators. ELISPOT analyses of ICAM-1 ^{-/-} mice immunized with P30P2MSP1-19 in ISA720 and in MF59 showed significant decreases in IFN- γ producing cells, but not IL-4 producing cells, as compared with WT controls (Figure 5). No significant changes in IFN- γ and IL-4 production were observed with the remaining adjuvant formulations (data not shown). Despite a decrease in IFN- γ production in ICAM-1 ^{-/-} mice immunized with MF59 and with ISA720, the profiles of immunoglobulin isotypes of the anti-MSP1-19 antibodies in these mice did not differ significantly from that in WT mice (data not shown).

DISCUSSION

It has been clearly established that co-stimulation via CD80 or CD86 is important in the activation and initiation of T cell responses [25,26]. Much less is known regarding the relative importance of CD80 versus CD86 during adjuvant-assisted potentiation of immune responses, although some adjuvants have been shown to activate APC with subsequent increased expression of these co-stimulatory ligands [39]. Our results indicated that while none of the adjuvant formulations that we studied can bypass the requirement for CD80 and CD86 mediated signaling (ie. CD80/86 double KO mice) to potentiate antibody responses, several formulations demonstrated a preference for CD86 or CD80 co-stimulation to exert adjuvant activity. The results further suggest that emulsion-type adjuvants (ie. MF59 and ISA720) as well as MPL, or formulations based on the two types of adjuvant, are more sensitive to a requirement of specific B7 interactions. Although the number of formulations we examined was small, it appears that more adjuvants showed reliance on CD86 than on CD80. This is in line with the established evidence that CD86 plays additional roles in the development of antibody responses [52–54]. Accordingly, CD86 on B cells provides bi-directional signaling in the activation of T cells via CD28, which in turn transduce enhancing signals to B cells to increase antibody production. The well known ability of MPLs to stimulate B cells would

corroborate with such mechanisms leading to a greater preference for CD86 to potentiate antibodies. However, a direct effect or activity of ISA720 or MF59 on B cells, or the ability of the two emulsion-type adjuvants to up-regulate CD80/86 expression remain to be demonstrated.

It is of significance that all adjuvant formulations containing QS21 were unaffected by CD80 or CD86 KO in potentiating anti-MSP1-19 antibody responses. Although the mode of action of saponin adjuvants such as QS21 is unknown, and have shown not to related to TLR binding [55], our data suggest that it may efficiently enhance both B7-1 and B7-2 mediated signaling and thus most likely to have an effect at the T cell:APC level. A direct up-regulation of CD80/CD86 expression by QS21 remains to be demonstrated. Another finding is that the addition of QS21 to the ISA720/MPL formulation (ie. ISA720/MPL/QS21) abolished the effects of CD86 KO. A similar observation was made in the immunogenicity of MSP1-19 formulated with ISA720/MPL in IL-6 KO mice [21] in which inclusion of QS21 abrogated the effects of IL-6 deficiency on the production of IgG2a antibodies. Thus, it appears that the immunological properties of QS21 can dominate over other adjuvant components, at least within the confines of the formulations that we studied.

It is also of interest that CD80 or CD86 knockout had no significant effects on the production of IFN- γ and IL-4, or the relative distribution of IgG1/IgG2a profiles in all the adjuvant formulations tested; a result that is different from another KO mouse model (IL-6 $-/-$) we previously examined[21]. Co-stimulation via CD86 has been associated with TH2-type responses; whereas a TH1-biased response has been attributed to CD80 [56]. The apparent lack of changes in the TH1/TH2 profiles as measured by IFN- γ /IL-4 production or by the IgG1/IgG2a responses suggests that compensatory pathways and/or cell types stimulated by these adjuvants are sufficient to maintain the immunological balance that are characteristics of the adjuvant-induced immune responses in “wild-type” or normal hosts.

Our results with the ICAM-1 $-/-$ mice suggest that the requirement for interactions via this adhesion molecule to induce antibodies in adjuvant-assisted immunizations is restricted to formulations not containing immunomodulators, ie. ISA720 and MF59. The accompanied reduction of IFN- γ responses in the ICAM-1 $-/-$ mice receiving ISA720 or MF59 suggest a lowering of activation and/or degree of APC:T cell interactions leading to the decreased antibody responses. Thus, formulations that contain immunomodulators may be able to provide additional stimuli, eg. cytokines such as IFN- γ , IL-6; which serve to circumvent the requirement for the adhesion molecule to initiate/stimulate a more robust T cell response. It is also possible that the immunomodulators may up-regulate other adhesion ligands such as ICAM-2 [57,58] that can functionally compensate for the ICAM-1 knockout. The lack of changes in the TH1/TH2 profiles in the KO mice for both the ICAM-1 $-/-$ sensitive and insensitive adjuvants is in line with the established roles of ICAM-1, which have been limited to the activation of T cells and the development of CD8+ CTL responses [28]. Taken together, these results suggest that depending on the nature of the adjuvant constituents, the role of ICAM-1/LFA-1 interactions in adjuvant-assisted immunizations may be complementary rather than obligatory.

Although the data presented here primarily address the immunological properties of adjuvant formulations, the ability of anti-MSP1 antibodies to inhibit parasite growth in vitro is an excellent correlate of the protective efficacy of the MSP1 vaccine/adjuvant formulations [59–61]. Thus, we are currently investigating if different adjuvant formulations can induce parasite inhibitory antibodies in the various knock-out mice.

Our present study, though limited in scope, provides another angle of view on the role of co-stimulatory and adhesion molecules on adjuvants' efficacy. The key question that our present

study addresses is whether the adjuvants can fully function in the absence of these immune molecules, irrespective of the adjuvants' ability to up-regulate their cellular expression. Our results showed that while many adjuvants or immunomodulators can activate the expression of co-stimulatory and/or adhesion ligands on immune cells; at least some of these molecules may not be essential to fully potentiate vaccine immunogenicity. Functional redundancy or similarities of some of these molecules may have partly contributed to the observed effects. However, the ability (or inability) of adjuvant formulations to activate immune cells via other pathways may also account for the differential dependency on the co-stimulatory and adhesion ligands. The data from the present studies should allow us to perform more focused and in-depth analyses on the most interesting adjuvants, ie. ISA720 and saponin (QS21), using related KO models such as CD28 $-/-$ or LFA-1 $-/-$, and an expanded cytokine profiling to better define the pathways that these adjuvants utilize under the selective immunodeficient environment.

It has been clearly documented that infections by a variety of parasitic, viral and bacterial agents can lead to alteration in the expression of co-stimulatory and adhesion molecules, with CD80, CD86 and ICAM-1 among the most prominent; and can result in the dysregulation of immune responses [30–38]. Immunization of hosts with prior or concurrent infections with these agents, a feature not unusual in many developing countries, may significantly compromise vaccine effectiveness. An in-depth understanding on the adjuvants' activities under the selected immune deficient environment will aid in the design of vaccine formulations that can maintain efficacy.

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Figure 1.

ELISA antibody titers to MSP1-19 of mice (Panel A: CD80 KO, CD86 KO and WT; Panel B: CD80/86 KO and WT), immunized with P30P2MSP1-19 in different adjuvant formulations. Mouse sera were obtained at 21 days after tertiary immunizations. Significant differences (Student t test) between WT and KO mice of an adjuvant group are indicated by the asterisks or the pound symbol, “#”.

A: MPL-SE **B: ISA720/QS21** **Figure 2.**

Immunoglobulin isotypes profiles, IgG1 and IgG2a, of anti-MSP1-19 antibodies from CD80 KO, CD86 KO and WT mice immunized with P30P2MSP1-19 in the adjuvant formulation, MPL-SE (Panel A) and in ISA720/QS21 (Panel B). No significant differences ($p > 0.05$, Student t test, by OD values or by IgG1/IgG2a OD ratios) were found in the Ig profiles between WT and KO mice for both adjuvant groups.

Figure 3. ELISPOT (IFN- γ and IL-4) of antigen stimulated splenocytes from WT, CD80 KO, and CD86 KO mice immunized with P30P2MSP1-19 in ISA720/QS21, and in ISA720/MPL. No significant differences (Student t test) were found in the number of IFN- γ or IL-4 spots between WT and KO mice for both adjuvant groups.

Figure 4.

ELISA antibody titers to MSP1-19 of mice (ICAM-1 KO and WT) immunized with P30P2MSP1-19 in different adjuvant formulations. Mouse sera were obtained at 21 days after tertiary immunizations. Significant differences (Student t test) between WT and KO mice of an adjuvant group are indicated by the asterisks.

Figure 5. ELISPOT (IFN- γ and IL-4) of antigen stimulated splenocytes from WT and ICAM-1 KO mice immunized with P30P2MSP1-19 in ISA720, or in MF59. Significant differences (Student t test) between WT and KO mice of an adjuvant group are indicated by the asterisks.