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Laurye van Maele, Delphine Fougeron, Delphine Cayet, Aurélie Chalon, Diego Piccioli, et al.. Toll-like receptor 4 signaling in hematopoietic-lineage cells contributes to the enhanced activity of the human vaccine adjuvant AS01. *European Journal of Immunology*, 2019, 49 (12), pp.2134-2145. 10.1002/eji.201948234 . inserm-02398403

HAL Id: inserm-02398403

<https://inserm.hal.science/inserm-02398403v1>

Submitted on 7 Dec 2019

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Research Article

Toll-like receptor 4 signaling in hematopoietic-lineage cells contributes to the enhanced activity of the human vaccine adjuvant AS01

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The 3-O-desacyl-4'-monophosphoryl lipid A (MPL) activates immunity through Toll-like receptor 4 (TLR4) signaling. The Adjuvant System AS01 contains MPL and is used in the candidate malaria vaccine and the licensed zoster vaccine. Recent studies reported that AS01 adjuvant activity depends on a transient inflammation at the site of vaccination, but the role of stromal or structural cells in the adjuvant effect is unknown. We investigated this question in mouse models by assessing the role of TLR4 on hematopoietic versus resident structural cells during immunization with AS01-adjuvanted vaccines. We first established that TLR4-deficient animals had a reduced immune response to an AS01-adjuvanted vaccine. Using bone marrow chimera, we consistently found that *Tlr4* expression in radio-sensitive cells, i.e., hematopoietic cells, was required for an optimal adjuvant effect on antibody and T-cell responses. At day 1 after injection, the pro-inflammatory reaction at the site of injection was strongly dependent on TLR4 signaling in hematopoietic cells. Similarly, activation of dendritic cells in muscle-draining lymph nodes was strictly associated with the radio-sensitive cells expressing *Tlr4*. Altogether, these data suggest that MPL-mediated TLR4-signaling in hematopoietic cells is critical in the mode of action of AS01.

Keywords: adjuvant · dendritic cells · hematopoietic cells · TLR4 · vaccine



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Adjuvants are used in vaccines to enhance the immunogenicity of the vaccine antigens, especially with subunit vaccines in which recombinant antigens often lack sufficient immunogenicity. Common to all adjuvanted vaccines, an early activation of the

innate immune system conditions the level and/or quality of the immune response specific to the co-administered antigens, which usually translates into improved efficacy [1, 2]. A recent study demonstrated that the ability of different adjuvants to trigger an early innate response in humans was associated with higher T-cell and antibody response [3]. The innate response after intramuscular vaccination with adjuvanted vaccines is generally characterized by local cytokine production and cell recruitment in muscle and draining lymph nodes (dLN), as well as by an increase in the

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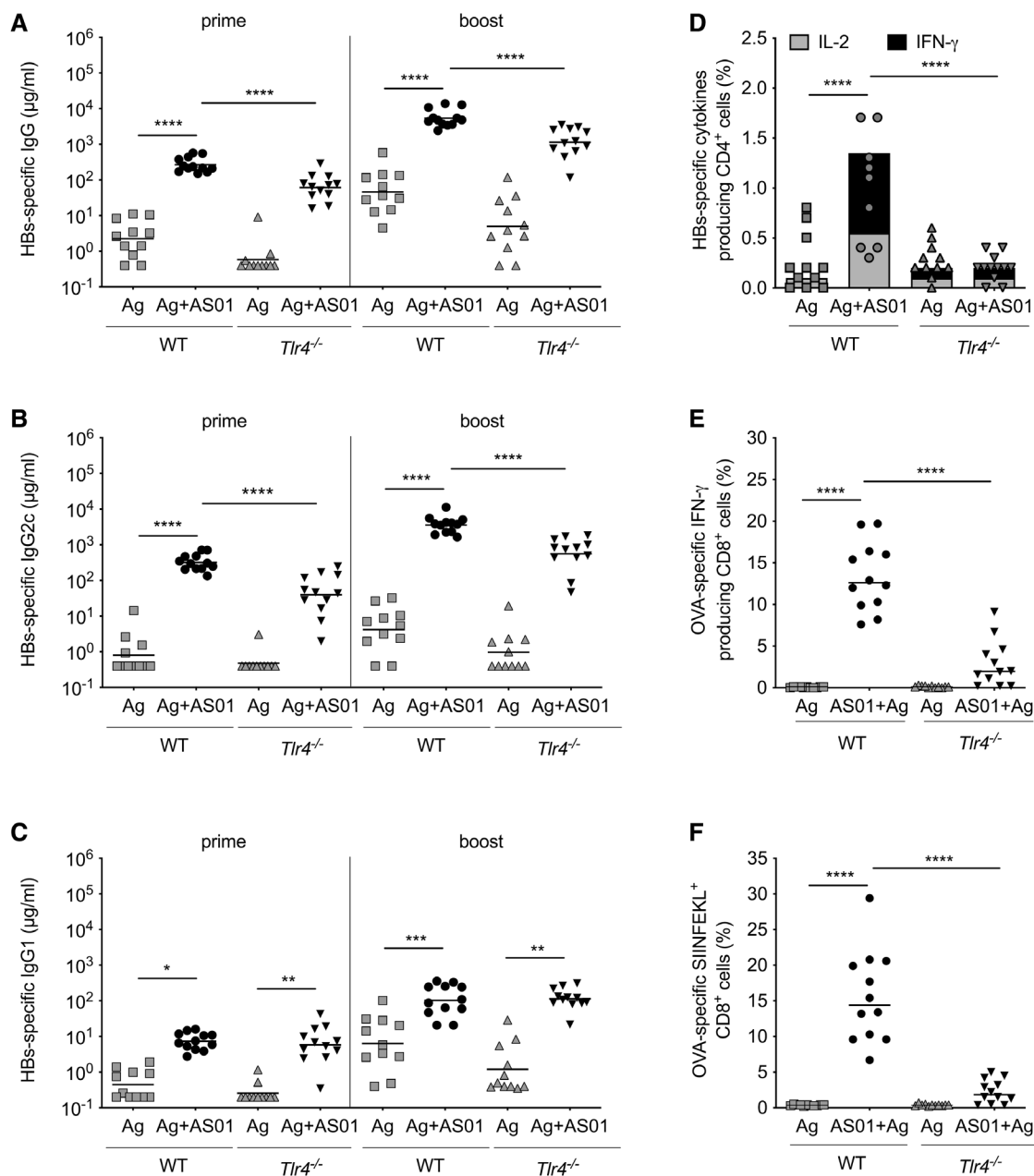
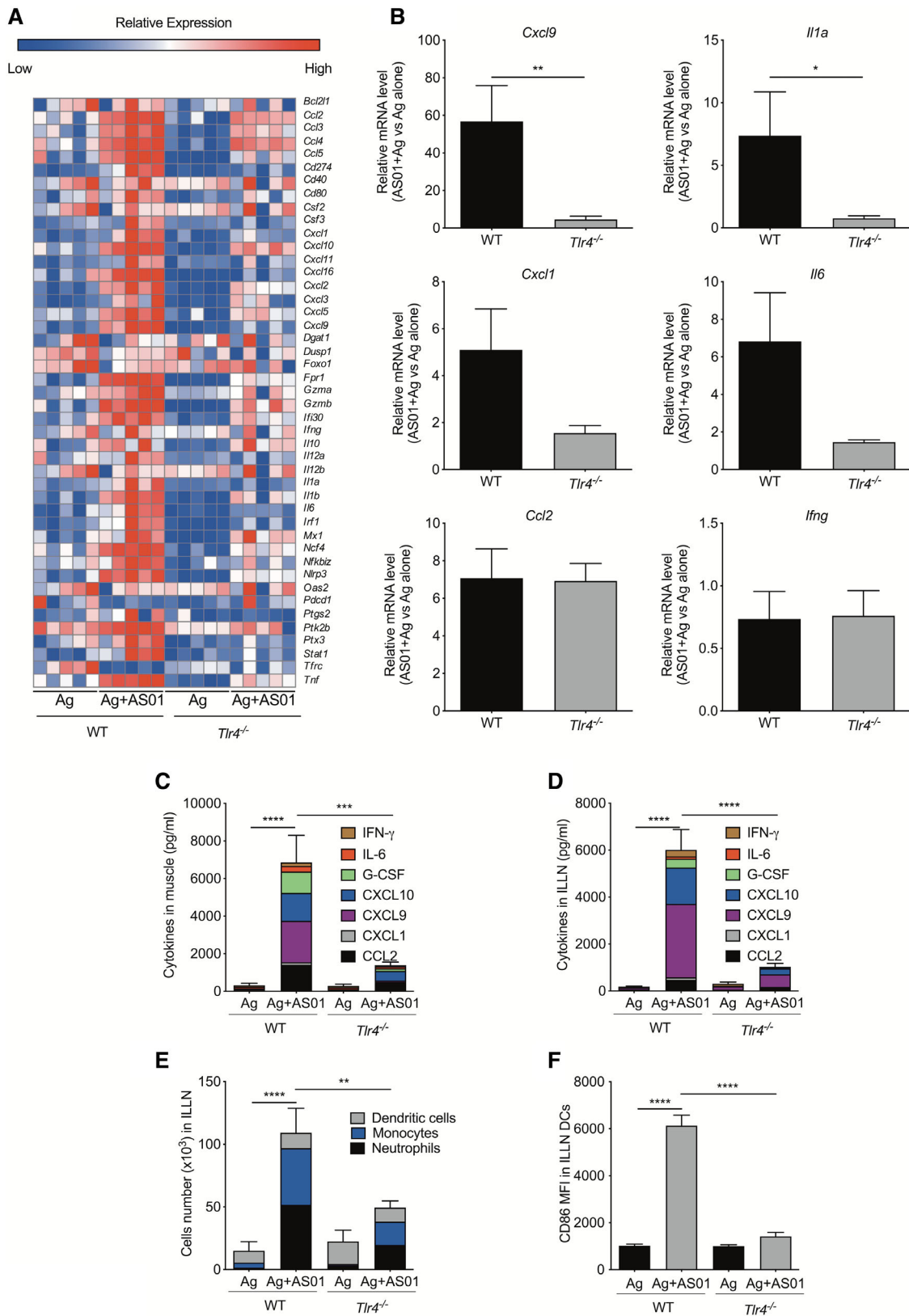


Figure 1. Adaptive immune responses induced by AS01-adjuvanted vaccines involves TLR4 signaling. C57BL/6J (WT) or *Tlr4*-deficient mice (*Tlr4*^{-/-}) (*n* = 11–12 biological replicates per group) were vaccinated i.m. at day 1 and day 14 either with antigens (Ag: OVA and HBs) or AS01+Ag. (A–C) HBs-specific IgG (A), IgG2c (B) and IgG1 (C) concentrations were determined in the serum at day 15 (prime, left panel) and day 21 (boost, right panel). Plots represent values for individual mice as well as geometric means. (D–F) The HBs- and OVA-specific T-cell responses were monitored at day 21 in splenocyte cultures by intracellular cytokine staining or tetramer staining using flow cytometry. All responses are expressed as percentage of Ag-specific CD4⁺ (or CD8⁺) T cells with respect to all CD4⁺ (or CD8⁺) T cells. (D) Intracellular cytokine staining on CD4⁺ T cells for IFN- γ and IL-2 after in vitro stimulation with HBs-specific peptide pool. (E–F) Frequency of OVA-specific CD8⁺ T cells as measured by intracellular staining for IFN- γ after in vitro stimulation with ova-specific pool (E) or OVA_{257–264}(SIINFEKL)/H-2K^b tetramer staining on freshly isolated lymphocytes (F). Results are expressed as the median. Each dot represents total cytokine production in an individual mouse. Results are representative of one out of two experiments. Statistical significance (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001) was assessed by two-way ANOVA test with Tukey correction for evaluation of vaccine effect and genotype effect.

number of activated antigen-presenting cells (APCs) loaded with antigen, which eventually leads to improved T-cell priming [4–9]. In mice, depletion of CD11c⁺ cells, which comprise dendritic cells (DCs), abrogates the adjuvant effect of several adjuvants [4, 10]. It has been postulated that direct DC activation is optimal for

efficient T-cell priming [11]. Although most of the adjuvants can directly activate DCs in vitro, it is unclear whether it also happens in vivo on resident or recruited DCs or whether DC activation can occur through a trans-activation by the inflammatory milieu. Resident macrophages in the dLN have also been recently shown to be



the target cells of some adjuvants and to play a critical role in initiating the innate response [12–14]. More generally, the role of the direct activation by adjuvants of structural cells versus resident or recruited hematopoietic cells, such as monocytes, DCs, and neutrophils is unknown. Factors that may be derived from structural cells, such as IL-6, a key cytokine involved in B-cell differentiation, or chemokines such as CCL2 that are needed to recruit innate cells, can participate to the response. For example, TLR4 is expressed on muscle cells and other structural cells at site of injection ([15] and www.immgen.org) so they may be rapidly activated after administration of MPL-containing adjuvants such as AS04 and AS01, thereby contributing to the immune response [4, 5]. Therefore, it is plausible that the expression of innate receptors on structural cells plays a role in the mode of action of adjuvants.

Here we used vaccine adjuvanted with the Adjuvant System AS01 to address this question. AS01 is used in the malaria candidate vaccine RTS,S and in the recently approved herpes zoster vaccine as well as other vaccines in development [16]. AS01 is an example of a new generation of adjuvants based on the combination of immunostimulants, namely the saponin QS-21 and MPL formulated in liposomes. While TLR4 is the only receptor for MPL, liposomal QS-21 appears to activate the innate immunity through the destabilization of the lysosomal membrane upon cholesterol-dependent endocytosis, resulting in the activation of the caspase-1 and Syk pathways [14, 17]. Overall, the nature and kinetics of the innate response to MPL and AS01 have been well characterized in mice [4, 5, 13] as well as in humans when combined with Hepatitis B surface Ag (HBs) [3, 18]. The mouse model established the crucial role of DC and IFN-related pathway in the mode of action of AS01 thanks to a synergistic effect between MPL and QS-21 [13]. In human, IFN-related pathways have been shown to contribute to the association between the innate response and the impact on antigen-specific adaptive response, in particular for CD4⁺ T cells [3].

In this study, we took advantage of TLR4 being the single pattern-recognition receptor to MPL to analyze the role of structural cells versus cells derived from the hematopoietic compartment in the TLR4-mediated adjuvant effect of AS01. We first established the different pro-inflammatory and immune responses that were strictly TLR4-dependent after vaccination with an AS01-adjuvanted vaccine by comparing the response in TLR4-deficient animals (*Tlr4*^{-/-}) and wild-type (WT) animals. Using bone marrow chimera, we then analyzed whether those responses were

dependent on the expression of *Tlr4* on hematopoietic cells and how this correlated with the outcome of the antigen-specific responses.

Results

TLR4 contributes significantly to the adjuvant effect of AS01

Because the response to AS01 is not only driven by the TLR4 agonist MPL but also by QS-21, it is expected that a significant immune response is triggered by AS01-adjuvanted vaccines in *Tlr4*-deficient animals. Consequently, we first defined the baseline response to AS01-adjuvanted vaccines in *Tlr4*-deficient animals before evaluating the TLR4-specific contribution of structural versus hematopoietic cells in the model. C57BL/6J wild-type mice (WT) and congenic *Tlr4*^{-/-} mice were vaccinated twice (at days 1 and 14) by intramuscular injection of ovalbumin (OVA), and the hepatitis B virus surface antigen (HBs) formulated or not with AS01, a model previously established in Detienne *et al.* [17]. HBs-specific antibodies were measured in serum two weeks after the first immunization (day 15) and one week after the second immunization (day 21), whereas HBs-specific CD4⁺ T-cells frequencies and OVA-specific CD8⁺ T-cells frequencies were measured 1 week after the second dose (day 21) (Fig. 1).

In response to the AS01-adjuvanted vaccine, *Tlr4*^{-/-} mice had a 4.3 to 4.8 fold lower total IgG and 6.5 to 8.1 fold lower IgG2c than WT mice after the first (prime) and second (boost) dose, respectively (Fig. 1A and B). No impact was seen on IgG1 (Fig. 1C). Therefore, TLR4 partially contributed to the Th1-biased IgG2c response with AS01-adjuvanted vaccines, while the remaining response was indicative of the contribution of the MPL-independent signals in AS01. The absence of TLR4 had a more marked effect on the AS01-dependent increase in antigen-specific T-cell responses in spleen (Fig. 1D–F). In the *Tlr4*^{-/-} mice, the HBs-specific CD4⁺ T-cell level after the AS01-adjuvanted vaccine was reduced to levels induced by the non-adjuvanted vaccine, showing that the effect of AS01 on CD4 activation was strongly dependent on TLR4 signaling (Fig. 1D). TLR4 deficiency significantly impaired the CD8⁺ T-cell response as the frequencies of IFN- γ -producing CD8⁺ T cells (as monitored by intracellular staining) or OVA-specific CD8⁺ T cells (as detected by tetramer) were lower compared to those in the WT mice (up to 6.5 and 7.8-fold,

Figure 2. Innate immune responses induced by AS01-adjuvanted vaccines involves TLR4 signaling. C57BL/6J (WT) or *Tlr4*^{-/-} mice ($n = 5$ biological replicates per group) were vaccinated i.m. by injections either with antigens (Ag: OVA and HBs) or AS01+Ag, in the *2 gastrocnemius* muscles. The muscles were then sampled 24 h post-vaccination to prepare total RNA (A and B) or protein extract (C). The draining iliac lymph nodes (ILLN) were also sampled to prepare total protein extracts for cytokine analysis or cell suspensions for flow cytometry (D–F). (A) Expression of immune-related genes in muscle are presented as heat map of $\Delta C_T + C_T$ mean values, i.e. the relative expression of the gene of interest normalized to the calibrator gene *Gusb* (ΔC_T) in individual sample and mean C_T value of *Gusb*. Heat maps are based on average linkage method and Pearson correlation. (B) Transcriptional response of immune-related genes in muscle. Levels of mRNA in Ag alone-vaccinated animals were arbitrarily set to 1 and used to calculate relative gene expression in animals that received AS01+Ag. (C) Innate cytokines/chemokines in muscles. (D) Cytokine/chemokine signature in ILLN. (E) Absolute counts of total neutrophils, monocytes, and dendritic cells were measured by flow cytometry. (F) Activation of dendritic cells as defined by CD86 staining (median fluorescence intensity, MFI) was measured by flow cytometry. Results are expressed as the mean \pm SEM. Data are representative of one to two experiments. Statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) was assessed by Mann–Whitney test compared to WT group (B) or two-way ANOVA test with Tukey correction for evaluation of vaccine effect and genotype effect (C–F).

respectively) (Fig. 1E and F). Overall, the results suggested that the TLR4-mediated effect of AS01 was mainly in enhancing Th1 response and very likely as a consequence, IgG2c levels.

We next evaluated the contribution of TLR4 signaling in the innate-immune response driven by AS01-adjuvanted vaccines, which is key in shaping the subsequent adaptive response. Because TLR4 is known to be expressed on several innate cells, we expected to observe an impact on AS01-induced cytokines or pro-inflammatory gene expression as well as on innate cell recruitment and activation at the injection site (*gastrocnemius* muscle) and draining iliac lymph nodes (ILLN). In WT mice, a majority of pro-inflammatory genes analyzed were specifically upregulated by AS01 in the muscle 24h after injection (Fig. 2A and B). This time point was chosen as the peak innate response, as reported previously [4]. In the *Tlr4*^{-/-} animals, upregulation of gene expression was generally reduced. Relative to the non-adjuvanted group, gene expression after AS01-adjuvanted vaccine injection was significantly altered in the absence of TLR4, demonstrating that activation of the TLR4 pathway had a pronounced impact on local innate activation induced by AS01. Specifically, in the muscle, 24 hours after injection, several genes were found to be strictly TLR4-dependent since their relative expression in response to the AS01-adjuvanted vaccine was significantly different between WT and *Tlr4*-deficient mice like *Cxcl9* or *Il1a* (Fig. 2B). The second group of genes displayed a higher but not significantly different expression in WT compared to *Tlr4*^{-/-} animals. Those genes included *Cxcl1* or *Il6*. Finally, we observed genes whose expression was similarly increased by the vaccine in WT and *Tlr4*^{-/-} included *Ccl2* or *Ifng*. Consistent with gene expression, the levels of pro-inflammatory cytokines in the AS01 group were significantly lower in *Tlr4*-deficient mice as compared to WT mice, in both muscle and ILLN (Fig. 2C and D). The increase in numbers of ILLN monocytes and neutrophils in response to AS01-adjuvanted vaccine was partially impaired in *Tlr4*-deficient mice (Fig. 2E). While the number of ILLN DCs were similar in response to the vaccine in WT or *Tlr4*^{-/-} mice, the magnitude of DC activation (represented by CD86 expression) in response to the AS01-adjuvanted vaccine was reduced in the *Tlr4*^{-/-} mice to the same level as in the non-adjuvanted group (Fig. 2F). These data suggest that DC maturation is mainly mediated by MPL in AS01 formulation. Given that DCs play a key role in T-cell priming by AS01-adjuvanted vaccines, this result, together with the reduced expression of CXCL9 [19], is in line with the major effect of TLR4 deficiency on CD4 T-cell response (Fig. 1D).

Overall, the first set of experiments define the key markers that are modulated by TLR4 in AS01 response. It does then allow us to assess the contribution of stromal and hematopoietic cells in the response to AS01, in particular in DC activation.

Adaptive immune responses to AS01-adjuvanted vaccine requires *Tlr4* signaling in hematopoietic cells

WT and *Tlr4*^{-/-} mouse chimeras were generated in which the recipient mice were irradiated to eliminate the hematopoietic stem

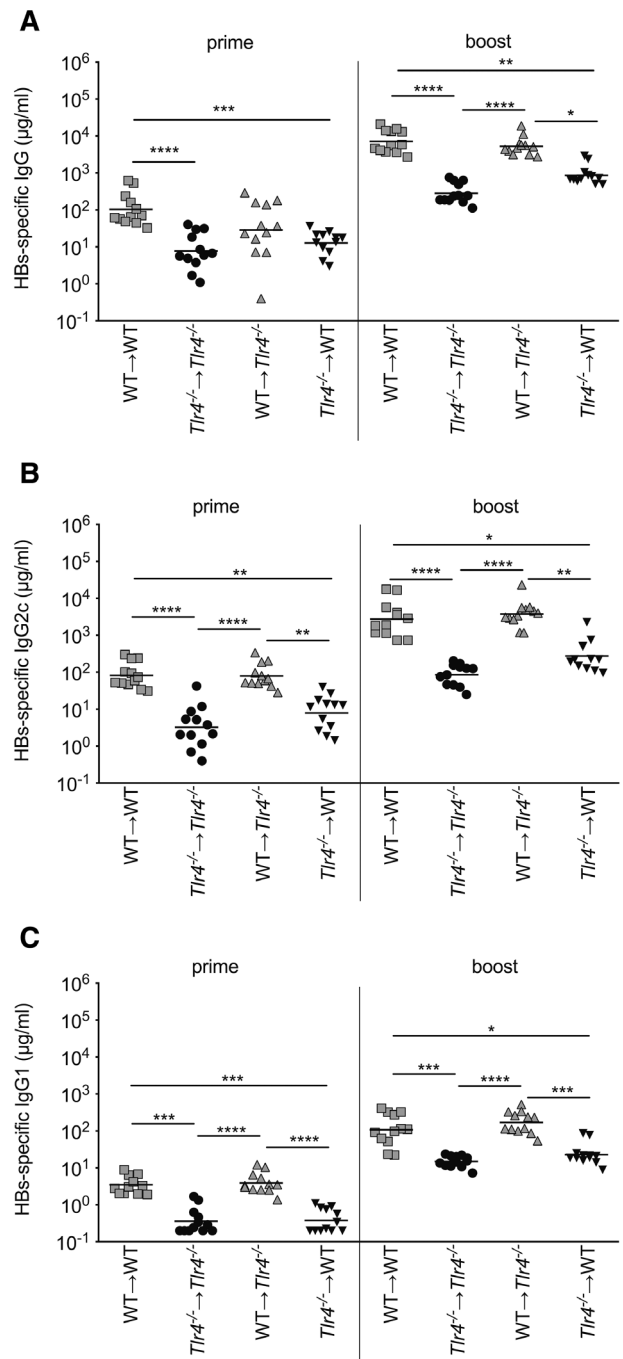


Figure 3. Antibody response induced by AS01-adjuvanted vaccines is influenced by TLR4 signaling in hematopoietic cells. Bone marrow chimeras: C57BL/6J (WT)→WT, *Tlr4*^{-/-}→*Tlr4*^{-/-}, WT→*Tlr4*^{-/-}, *Tlr4*^{-/-}→WT (n = 11–12 biological replicates per group) were vaccinated i.m. at day 1 and day 14 with a vaccine containing antigens (Ag: OVA and HBs) and AS01. HBs-specific IgG (A), IgG2c (B), or IgG1 (C) concentrations were determined in the serum. Antibody response was analyzed at day 15 (prime) and day 21 (boost). Plots represent values for individual mice as well as geometric means. Results are representative of one out of two experiments. Statistical significance (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001) was assessed by one-way ANOVA Kruskal–Wallis test with Dunn's correction.

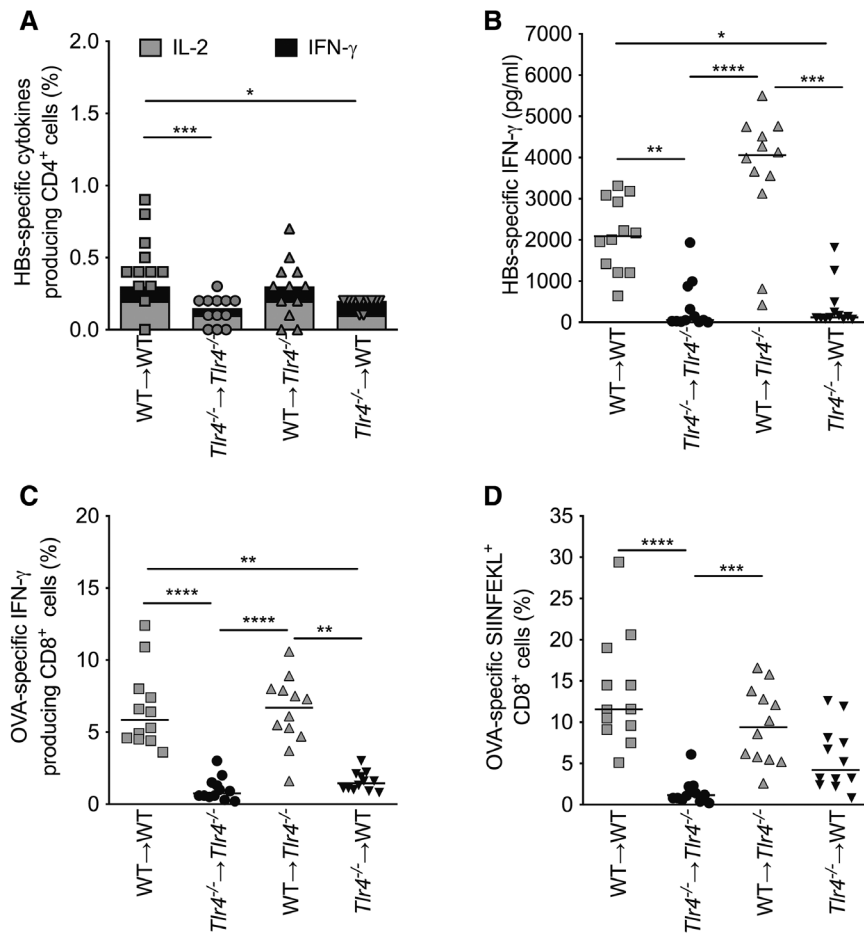


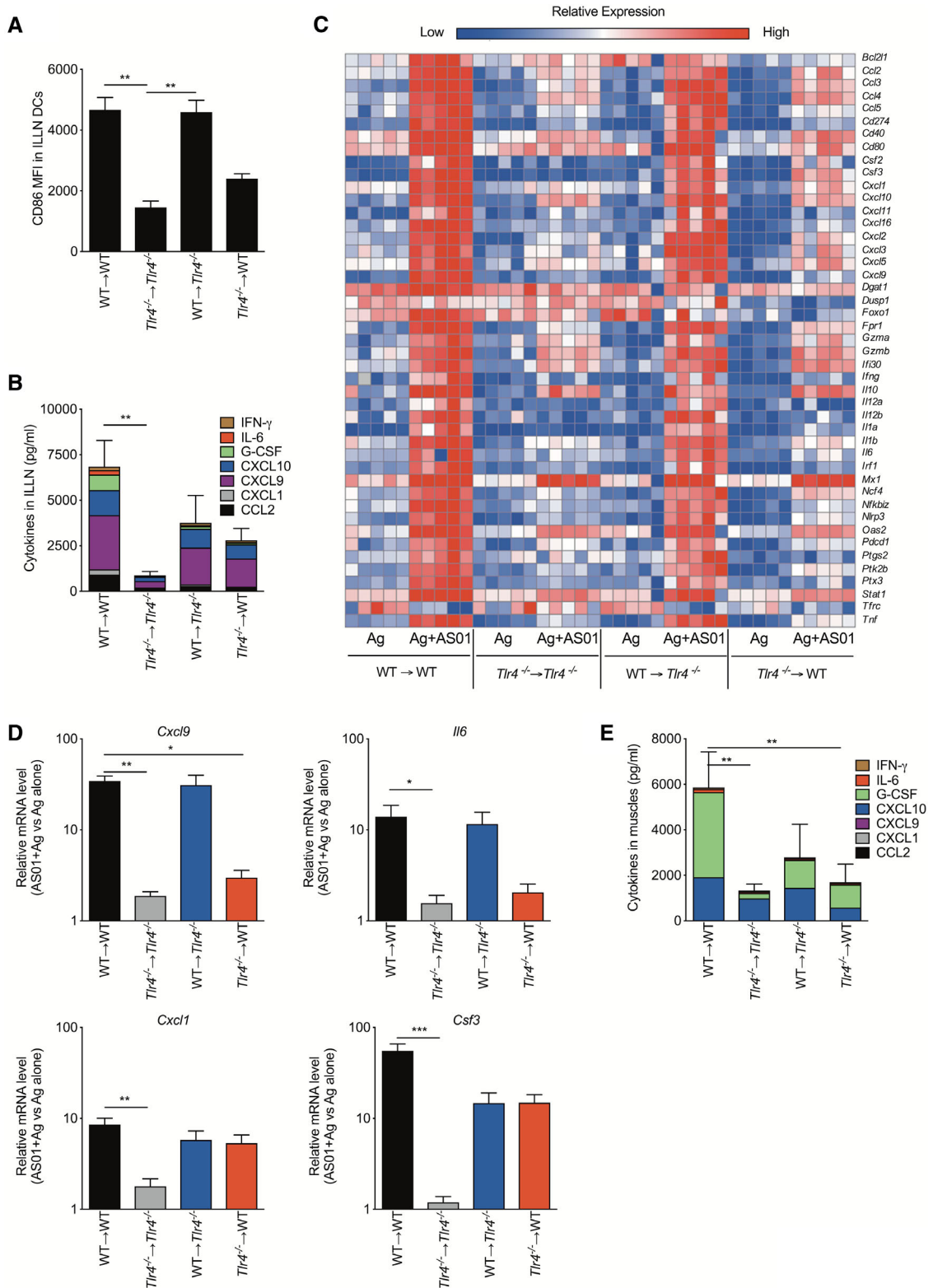
Figure 4. Hematopoietic TLR4 signaling modulates the T-cell responses induced by AS01-adjuvanted vaccines. Bone marrow chimeras: C57BL/6J (WT)→WT, *Tlr4*^{-/-}→*Tlr4*^{-/-}, WT→*Tlr4*^{-/-}, *Tlr4*^{-/-}→WT ($n = 11=12$ biological replicates per group) were vaccinated i.m. at day 1 and day 14 with a vaccine containing antigens (Ag: OVA and HBs) and AS01. The HBs- and OVA-specific CD4⁺ and CD8⁺ T-cell responses were monitored at day 21 in splenocyte cultures by cytokine secretion assays, intracellular cytokine staining or tetramer staining. Each dot represents total cytokine production in an individual mouse. (A) Intracellular cytokine staining for IFN- γ , and IL-2 after in vitro stimulation with HBs-specific peptide pools was measured by flow cytometry. (B) IFN- γ secretion by HBs-stimulated splenocytes. (C) Frequency of OVA-specific CD8⁺ T cells as measured by IFN- γ intracellular expression after in vitro stimulation with OVA-specific peptide pools. (D) OVA₂₅₇₋₂₆₄(SIINFEKL)/H-2K^b tetramer staining on freshly isolated lymphocytes. All responses are expressed as percentage of Ag-specific CD4⁺ (or CD8⁺) T cells with respect to all CD4⁺ (or CD8⁺) T cells. Plots represent values for individual mice. Results are given as median. Results are representative of one out of two experiments. Statistical significance ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$) was assessed by one-way ANOVA Kruskal-Wallis test with Dunn's correction.

cells, and the hematopoietic compartment was reconstituted by bone marrow transplant from a donor. This approach allowed to investigate the contribution of TLR4 signaling in the radio-sensitive cells, i.e., mainly the hematopoietic cells determined by the donor (*Tlr4*^{-/-}→WT) and conversely, the contribution of TLR4 signaling in the radio-resistant compartment, i.e., the structural cell defined by the recipient (WT→*Tlr4*^{-/-}), compared to their respective controls (*Tlr4*^{-/-}→*Tlr4*^{-/-} and WT→WT). Importantly, the reconstitution of the hematopoietic compartment was fully effective 12 weeks post-transplantation as verified by flow cytometry analysis on blood and lymph nodes (see Materials and methods).

In line with data in *Tlr4*^{-/-} mice (Fig. 1), HBs-specific antibody concentrations were lower in the *Tlr4*^{-/-}→*Tlr4*^{-/-} chimeras than in the WT→WT chimeras, by 10.6 and 23.9 fold for total IgG and by 22.4 and 22.6 fold for IgG2c, after 1 dose (prime) or 2 doses (boost), respectively (Fig. 3). The levels of HBs-specific IgG and IgG2c antibody response was unaffected when the hematopoietic cells express *Tlr4* (WT→*Tlr4*^{-/-}) compared to WT→WT group. By contrast, when the hematopoietic cells were deficient for *Tlr4* (*Tlr4*^{-/-}→WT), the levels of HBs-specific antibodies were in the same range, if not slightly higher, to that of animals deficient for TLR4 in all compartments (*Tlr4*^{-/-}→*Tlr4*^{-/-}). In contrast to WT and *Tlr4*^{-/-} animals, the

IgG1 response of bone marrow chimera to AS01-adjuvanted vaccine was also found to be modulated by TLR4 signaling, which may be due to intrinsic factors related to irradiation and reconstitution. These data suggest that the response of structural cells to MPL could not compensate for the absence of TLR4 on hematopoietic cells to support antibody response to an AS01-adjuvanted vaccine.

Regarding T-cell response, the antigen-specific CD4⁺ and CD8⁺ T-cell frequencies in spleen were significantly lower in the *Tlr4*^{-/-}→*Tlr4*^{-/-} chimeras than in the WT→WT chimeras (Fig. 4) as expected from experiments in *Tlr4*^{-/-} mice (Fig. 1D–F). Moreover, the mean concentration of IFN- γ secreted by HBs-specific peptide-stimulated splenocytes in the *Tlr4*^{-/-}→*Tlr4*^{-/-} chimeras was lower than that in the WT→WT chimeras (Fig. 4B). Immunization of WT→*Tlr4*^{-/-} mice with AS01-adjuvanted vaccines elicited similar antigen-specific CD4 and CD8 responses than in WT→WT. In contrast, the *Tlr4*^{-/-}→WT group was poorly responsive to vaccination with regards to CD4⁺ and CD8⁺ T cells, as poorly as *Tlr4*^{-/-}→*Tlr4*^{-/-} controls (Fig. 4). The results were consistent with the observations on antibodies, i.e., the response was equivalent to WT when *Tlr4* expression was maintained on hematopoietic cells, suggesting a minimal contribution of TLR4 signaling in structural cells for T-cell response after AS01-adjuvanted immunizations.



TLR4 trigger by AS01-in hematopoietic cells is sufficient for optimal DC activation

Given the established direct role of DCs in T-cell response after dosing with the AS01-containing vaccines and the impact of TLR4 signaling on DC activation, we then looked how DC activation in the draining lymph nodes (ILLN) was impacted in the chimeric mice (Fig. 5A). As observed for the T-cell response, the degree of DC activation (measured by MFI of CD86) was similar in WT→WT and WT→*Tlr4*^{-/-} chimeras, whereas DC activation in *Tlr4*^{-/-}→WT was reduced but remained slightly higher than in the complete absence of TLR4 (*Tlr4*^{-/-}→*Tlr4*^{-/-}). This observation suggests that full DC activation upon injection with AS01-adjuvanted vaccine required *Tlr4* expression on hematopoietic cells, including DCs themselves. However, DCs can be stimulated by the inflammatory environment, which can be modulated by structural cells expressing *Tlr4* and may explain the residual higher DC activation in the *Tlr4*^{-/-}→WT chimeras. This prompted us to look at the level of inflammatory response in those mice that could impact DC maturation indirectly. In *Tlr4*^{-/-}→WT chimeras, a pro-inflammatory response to AS01-adjuvanted vaccine was indeed induced through activation of radio-resistant cells, which was however reduced compared to WT→WT chimeras, consistent with the difference in DC activation observed in the two groups (Fig. 5B). A limited response was found in the *Tlr4*^{-/-}→*Tlr4*^{-/-} chimeras, in agreement with data obtained in *Tlr4*-deficient mice (Fig. 2C and D). Interestingly, the level of cytokines was similar between *Tlr4*^{-/-}→WT and WT→*Tlr4*^{-/-} chimeras, showing that the presence of TLR4 on both hematopoietic cells and structural cells contributed to the pro-inflammatory effect of AS01 in the ILLN.

TLR4 on hematopoietic cells is essential to AS01-driven innate activation at the site of injection

A similar gene expression analysis as described in Fig. 2A was performed in the muscle of the chimeric mice. The overall pro-inflammatory gene expression levels in response to the AS01-adjuvanted vaccine were relatively high in WT→WT chimeras and WT→*Tlr4*^{-/-} chimeras and relatively low in the *Tlr4*^{-/-}→*Tlr4*^{-/-} and *Tlr4*^{-/-}→WT chimeras (Fig. 5C and D). We observed that the two compartments (hematopoietic and structural) were involved in the expression of genes like *Csf3* or *Cxcl1*. For most of the other pro-inflammatory genes tested like *Cxcl9* or *Il6* a major contri-

bution of TLR4 on hematopoietic cells was observed (Fig. 5D). The overall patterns of pro-inflammatory gene expression in the control *Tlr4*^{-/-}→*Tlr4*^{-/-} chimeras and WT→WT chimeras were generally similar to those observed in the *Tlr4*^{-/-} mice and WT mice (Fig. 2A), excluding an effect of irradiation on the results. Consistent with the gene expression profile, the data on cytokine levels at the injection site showed that hematopoietic cells contributed the most to the TLR4-dependent changes observed at the time point studied (Fig. 5E). Indeed, the overall cytokine concentrations in the *Tlr4*^{-/-}→WT chimeras were relatively low, similar to the respective concentrations in *Tlr4*^{-/-}→*Tlr4*^{-/-} chimeras, whereas in the WT→*Tlr4*^{-/-} chimeras, the overall cytokine levels were higher, but yet, not reaching the levels observed in WT→WT group. The cytokine data were also consistent with gene expression level, as shown for CXCL9, G-CSF (*Csf-3*) and CXCL1. Altogether, the results suggested that while certain aspects of the innate immune response to the AS01-adjuvanted vaccine, such as DC activation, were dependent primarily on TLR4 expression on donor hematopoietic cells, some others were partially due to TLR4 expression on structural recipient cell populations or requires a concerted stimulation of both compartments. However, because irradiation does not eliminate 100% hematopoietic cells, we cannot completely exclude that in *Tlr4*^{-/-}→WT chimeras, some of the response may be driven by TLR4-proficient hematopoietic cells remaining after irradiation.

Discussion

The present study demonstrates how the property of a clinically relevant adjuvant hinges preferentially on its ability to stimulate hematopoietic cells rather than structural resident cells. Specifically, we show that the TLR4-mediated effect of AS01 on promoting adaptive immunity appeared to be mainly dependent on TLR4 signaling of cells within the hematopoietic lineage but not on non-hematopoietic cells, such as those at the injection site or dLN (e.g. muscle fibers, stromal cells, and endothelial cells). In the bone marrow chimeras, antigen-specific T cells were indeed dramatically reduced in the absence of *Tlr4* expression on donor cells and this was associated with both reduced DC activation and local inflammatory signals. In contrast, lack of TLR4 expression in recipient cells only partially impacted cytokine expression in muscle and lymph nodes and had a limited effect on vaccine response.

Figure 5. Hematopoietic TLR4 signaling modulates local innate immunity and dendritic cell activation in response to AS01-adjuvanted vaccines. Bone marrow chimeras: C57BL/6J (WT)→WT, *Tlr4*^{-/-}→*Tlr4*^{-/-}, WT→*Tlr4*^{-/-}, *Tlr4*^{-/-}→WT (n=5 biological replicates per group) were vaccinated i.m. by injections of the same vaccine preparation (either antigens [Ag: OVA and HBS] or AS01+Ag), in the 2 *gastrocnemius* muscles. The draining iliac lymph nodes (A–B) and the muscles (C–E) were then sampled 24 hours post-vaccination to prepare cell suspensions for flow cytometry analysis in ILLN (A), total protein extracts for cytokine analysis in ILLN (B) and one muscle (E) or total RNA on the contralateral muscle (C–D). (A) Activation of dendritic cells as defined by CD86 staining (median fluorescence intensity, MFI) was measured by flow cytometry. (B) Cytokine/chemokine signature. (C) Expression of immune-related genes in muscle are presented as heat map of $\Delta C_T + C_T$ mean values, i.e. the relative expression of the gene of interest normalized to the calibrator gene *Gusb* (ΔC_T) in individual sample and mean C_T value of *Gusb*. Heat maps are based on average linkage method and Pearson Correlation as distance metric. (D) Transcriptional response of immune-related genes in muscle. Levels of mRNA in Ag alone-vaccinated animals were arbitrarily set to 1 and used to calculate relative gene expression in animals that received AS01+Ag. (E) Cytokine production in AS01-injected muscle. Results are given as mean \pm SEM. Results are representative of one to two experiments. Statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was assessed by one-way ANOVA Kruskal–Wallis test with Dunn's correction.

Because both innate and adaptive parameters were monitored in the different chimeras, the study provides an opportunity to assess which TLR4-dependent parameters of AS01-induced innate immune response are associated with the enhancement of the adaptive response. In the *Tlr4*^{-/-}→WT chimeras, AS01-mediated TLR4 signaling was associated with DC activation in the dLN (Fig. 5A). These data not only confirm the previously reported role of DC in AS01 adjuvant effect [4] but also extend this finding by suggesting a requirement for a direct TLR4 engagement for full activation of DC. MPL can directly activate DCs and other human hematopoietic cells as reported using *ex vivo* stimulated PBMC [4, 20, 21]. In addition, MPL in AS01 is likely to be rapidly drained to the LN to target resident DC. The requirement of direct DC activation for optimal induction of adaptive immunity is in line with the model proposed by Reis e Sousa and colleagues [11, 22]. However, this study also shows that DC can also be indirectly activated by the inflammatory milieu remaining in the *Tlr4*^{-/-}→WT mice, which may be important for other properties of DC not measured here. It is also possible that monocytes may contribute to the AS01 adjuvant effect through differentiation into DC or by providing cytokines to support DC-activation or T-cell priming [23–27]. A more detailed analysis of the monocyte/DC profile will be required to address the role of TLR4 engagement in this response. Finally, in the *Tlr4*^{-/-}→WT mice where the adaptive response was reduced, the observed increased levels of cytokines in dLN at 24h were found to be dispensable for the TLR4-dependent development of the adaptive immune response. However, one cannot exclude that other parameters of the adaptive immune response, such as avidity and persistence of the antibody response, may be impacted by those effectors.

Beyond innate effectors, TLR4 expression may also be required for optimal antigen-specific response, at least in mice. Indeed, direct activation of B cells by TLR4 signaling has been shown to improve antibodies response and could potentially account for the difference in the level of HBS-specific IgG observed here [28]. However, the observation that IgG2c but not IgG1 are impacted suggests that the absence of Th1 cells (due to reduced DC activation) rather than a defect in B cells may be responsible for this effect.

We show that the absence of *Tlr4* gene unequally impacted adaptive response with only a partial inhibition of antigen-specific IgG, while the impact on antigen-specific T cells was more substantial. This is consistent with previous observations that the added value of combining MPL and QS-21 in AS01 is more pronounced for the induction of cell-mediated immunity than for antibody response [13, 29]. The synergistic effect between MPL and QS-21 is mediated by an early IFN- γ production by resident cells in the dLN and is controlled by IL-18 and IL-12 [13]. Because this synergy depends on the presence of MPL response, as expected, the IFN signature, i.e. IFN- γ , CXCL9, and CXCL10 levels in the dLN was significantly reduced in *Tlr4*^{-/-} mice. TLR4 signaling in both resident cells and cells derived from the hematopoietic lineage seems to contribute to this signature as equal levels of CXCL9 and CXCL10 were found in WT→*Tlr4*^{-/-} and *Tlr4*^{-/-}→WT in the dLN, yet not reaching level of WT animals. As macrophages have

been shown to be critical for this early IFN- γ , it is possible that macrophages are partially depleted by irradiation or that, alternatively, stromal cells and DCs both contribute to MPL-driven IL-12 production shortly after AS01 injection.

Although our data is consistent with MPL mediating the main TLR4-dependent adjuvant effect of AS01, it is also plausible that TLR4 could also be triggered indirectly by QS-21 through induction of the high-mobility group protein B1 (HMGB1) as recently reported [17]. Inhibition of HMGB1 binding to MD-2 lowered antigen-specific CD4⁺ and CD8⁺ T-cell responses (but not antibody responses) in response to a QS-21- adjuvanted vaccine and therefore a contribution of QS-21 to the difference between WT and *Tlr4*^{-/-} cannot be excluded.

The TLR4-mediated inflammatory response induced by AS01 in the muscle appeared to occur primarily in cells within the hematopoietic lineage. Given that the muscle is constituted mainly by structural cells with limited number of macrophages or DCs, this result was somewhat surprising to us. *In vitro*, TLR4 engagement induces the production of cytokines by primary muscle cells and endothelial cells [30, 31], therefore it is likely that those cells do respond to MPL *in vivo*. It is possible that the cytokine and gene expression measured at 24h was largely biased towards detecting the response of hematopoietic cells, such as neutrophils or monocytes, which recruitment peaks at that time point [4]. Gene expression analysis in WT mice shows that change in gene expression occurs as early as 2h after AS01 injection [13], with a significant part being triggered by MPL specifically. This would suggest that a TLR4-dependent effect of AS01 on structural cells may be detectable earlier than 24h. In addition, QS-21 on its own is able to recruit cells in the muscle [17], although at a lower level than AS01 and this could therefore explain why the inflammatory signal, likely coming from recruited cells, was detected in *Tlr4*^{-/-} mice reconstituted with *Tlr4*-expressing hematopoietic cells.

The contribution of structural cells to TLR activation was stressed out in several studies using mucosal route of administration where cell recruitment and activation was compartmentalized in lumen and parenchyma [32–34]. Indeed, respiratory epithelial cells were shown to respond to TLR activation and participate directly to activation of DCs. In muscle, the threshold or the pathway of activation of TLR4 in the structural cells may be different from the cells coming from the hematopoietic lineage; for instance, TLR-specific signaling of structural cell may mobilized cytokines, chemokines, and immune effectors that are different from those induced in hematopoietic cells and in a different kinetic.

The results of this study provide insights into how adjuvant may rely on cells from different origin for optimal effect. It suggests that approaches aimed at targeting DC might be sufficient to induce a good T-cell response, while for antibody response, this requirement may be less important. In fact, little is known about the type of innate pathways or cells that are involved in the generation of sustained antibody response by adjuvanted vaccines. The study also indicates that at least some of the inflammatory responses induced by the adjuvant in stromal cells locally may not be necessarily needed to promote T-cell response. Based on this knowledge, one could postulate that an adjuvant designed

not to activate tissue-resident cells but local hematopoietic cells would remain potent while having a reduced inflammatory effect. This may therefore serve as the basis for the rational design of improved adjuvants with a better tolerability profile.

Materials and methods

Vaccine formulations

The hepatitis B surface antigen (HBs) was provided by GSK, Rixensart, Belgium. Ovalbumin (OVA) was from Calbiochem and confirmed to be endotoxin depleted (below 1.08 EU/mg); AS01 (GSK) was used at the following concentration: 1 µg of MPL and 1 µg of QS-21 (*Quillaja saponaria* Molina, fraction 21; licensed by GSK from Antigenics LLC, a wholly owned subsidiary of Agenus Inc, a Delaware, USA corporation) formulated in liposomes in 20 µL/injection

Animal husbandry and vaccination

Animals were maintained in a specific pathogen-free facility (#A59-350009, Pasteur Institute, Lille, France). Animal husbandry and experiments were ethically reviewed at the Pasteur Institute (CEEA 052011) and carried out in accordance with European Directive 2010/63/EU and the GSK's Policy on the Care, Welfare and Treatment of Animals. Six- to 12-week-old male and female mice (C57BL/6J, congenic C57BL/6J-CD45.1 [B6.SJL-PtprcaPepcb/BoyCr], and *Tlr4*^{-/-}) were obtained from Janvier, Charles River or bred in house. Mice ($n = 5$ –12/group) were vaccinated by the intramuscular (i.m.) route at Day 1 (for innate response evaluation) or at Days 1 and 14 (for adaptive response evaluation). A single vaccine dose of 40 µL per animal contained 2 µg OVA and 8 µg of HBs alone or in combination with AS01 and was administered as two i.m. injections of 20 µL in both left and right *gastrocnemius* muscles.

To generate bone marrow (BM) chimeric mice, wild type (WT), *i.e.*, C57BL/6J or C57BL/6J-CD45.1 or *Tlr4*^{-/-} mice were sublethally irradiated (11 Gray) and intravenously (i.v.) injected with 3–5 × 10⁶ BM cells. The degree of chimerism was assessed by measuring CD45.1 and CD45.2 expression in leukocytes using flow cytometry 12–20 weeks after bone marrow transplantation. In the iliac lymph node of the recipient, the proportions of cells from the donor were: >92% T cells, >98% B cells, 96% monocytes, and >87% DCs. Vaccinations were performed at 22–28 weeks post-irradiation.

ELISA determination of antigen-specific antibody concentrations

ELISA plates were prepared by absorption of HBs (50 µg/mL in PBS), or anti-mouse IgG (5 µg/mL in PBS; 1031-01 from South-

ernBiotech) overnight at 4 °C on Maxisorp microplates (Nunc), and 1 h blocking at room temperature with 1% dried milk in PBS. ELISA plates were incubated for 1 h at room temperature with serial dilutions (on antigen coated plates) of the serum samples or (on anti-mouse IgG-coated plates) of IgG and IgG1 standards (IgG, I5381, Sigma; and IgG1 derived from MM17F3 hybridoma, a gift from Dr. Catherine Uttenhove and Dr. Jacques Van Snick, Ludwig Institute for Cancer Research, Brussels). Primary antibody binding was revealed with subsequent incubations with biotin-conjugated goat anti-mouse IgG-, IgG1-, or IgG2c antibodies (Southern-Biotech, Birmingham, AL, USA); peroxidase-streptavidin complex (RPN1231 GE Healthcare); and TMB (555214, BD Bioscience); and measured using a microplate reader at 450/570 nm wavelength. Absolute quantities of antibodies were calculated according to the standard curves and expressed as µg/mL.

Antigen-specific T-cell frequencies

Splenocytes from vaccinated mice (2 × 10⁷ cells/mL; 96-wells microplate) were stimulated with anti-CD49d (9C10) and anti-CD28 (37.51) antibodies (1 µg/mL each, BD Biosciences); and HBs peptides (1 µg/mL; encompassing the whole antigen, 15-mers with 11 amino-residue overlap; GSK) or OVA peptides (pool consists of 17 peptides selected for H2-Kb epitope content, all are 15-mers with 11 amino-residue overlap; GSK) in complete RPMI supplemented with 5% fetal calf serum (FCS, Lonza). Antigen-specific stimulation of T cells was monitored by intracellular cytokine staining after 18 h incubation at 37 °C in which brefeldin A (1 µg/mL, BD Biosciences) was added for the last 16 h. For intracellular cytokine staining, cells were suspended with PBS containing 2% FCS and 2 mM EDTA, incubated first with anti-FcγRII/III (2.4G2) antibody for 5 min; then incubated with the following anti-mouse antibodies: CD4-APC-Cy7 (GK1.5, BD Biosciences) and CD8-PE or CD8-APC (53-6.7, BD Biosciences) for 30 min at 4 °C. Cells were then fixed and permeabilized using Cytofix-Cytoperm and Perm/Wash (BD Biosciences); and then incubated with IFN-γ-APC (XMG1.2, BioLegend) and IL-2-AlexaFluor 488 (JES6-5H4, BD Biosciences) for 30 min at 4 °C. Data were collected on LSR Fortessa or Canto II flow cytometers (BD Biosciences) and analyzed with FACS Diva or FlowJo software. The frequency of SIINFEKL (OVA_{257–264})-specific CD8⁺ T cells was determined in peripheral blood or splenocytes by staining with PE-conjugated H-2K^b-OVA_{257–264} tetramers (Beckman Coulter), CD45-BV510, CD4-APC-Cy7 and CD8-APC (BD Biosciences). Antigen-specific T-cell frequency was expressed as a percentage within the CD4⁺ or CD8⁺ T-cell subset. Experiments and analysis were conducted according to the flow cytometry guidelines [35] and the gating strategy as described in Supplementary Figures 1 and 2.

Gene expression

Total RNA from *gastrocnemius* muscles was extracted with Nucleospin RNA kits (Macherey-Nagel). RNA was reverse-transcribed

with the High-Capacity cDNA Archive Kit (Applied Biosystems). High throughput analysis of gene expression was performed using Taqman Low Density Arrays on a ViiA7 Real-Time PCR System (Applied Biosystems). Data were processed out using Thermo Fisher Cloud qPCR RQ analysis module. Relative mRNA levels ($2^{-\Delta\Delta Ct}$ or Rq) were determined by comparing (i) the cycle thresholds (Ct) for the gene of interest and the calibrator gene *Gusb* (ΔCt) and (ii) ΔCt values for AS01+Ag and Ag standalone groups ($\Delta\Delta Ct$). Ct upper limit was fixed to 33 cycles.

Innate cell phenotyping

Cells were isolated from muscle draining iliac lymph node (ILLN) after type IA collagenase (Sigma Aldrich) and DNase treatment (Roche). Cells were stained as described above with the following anti-mouse antibodies: CD45-BV510, FITC-lineage staining (CD3 ϵ -FITC (145-2C11, BioLegend), CD19-FITC (1D3, BD Biosciences), and Ly6G-FITC (1A8, BioLegend)), CD11b-PerCP-Cy5.5 (M1/70, eBioscience), CD11c-PE-Cy7 (N418, BD Biosciences), MHCII-eFluor 450 (I-A/I-E-specific, M5/114.15.2, eBioscience), CD86-AlexaFluor 700 (GL-1, BioLegend), and Ly6C-APC-Cy7 (AL-21, BD Biosciences). Monocytes were defined as CD45⁺ Lin^{neg} CD11b⁺ Ly6C⁺, DCs as CD45⁺ Lin^{neg} CD11c⁺ MHCII⁺ and neutrophils as CD45⁺ Lin⁺ (Ly6G⁺) CD11b⁺. Experiments and analysis were conducted according to the flow cytometry guidelines [35] and the gating strategy as described in Supporting Information Fig. 3.

Cytokine detection in cell supernatants, sera and tissues

Tissues (muscle or ILLN) were homogenized in an anti-protease solution (Halt Protease & Phosphatase inhibitor cocktail (Thermo scientific)). The homogenates were cleared by centrifugation and stored at -70°C until analysis. Protein levels were determined in cleared homogenates or sera by cytokine-specific beads (Millipore, IL-6, CXCL1, G-CSF, CCL2, IFN γ , CXCL9, and CXCL10) using the Luminex platform.

Statistical analyses

Results are expressed as geometric means for antibody response, median for T-cells response or means \pm SEM for gene expression, cytokine production and innate cell recruitment or activation (CD86) as indicated in Fig. legends. No logarithm transformation has been applied. Intergroup differences were analyzed using the two-tail Mann-Whitney test (when two group), the one-way ANOVA Kruskal-Wallis test with Dunn's correction (when more than two group) or the two-way ANOVA with Tukey correction with "vaccine" and "genotype" as Factor (GraphPad Prism 6.0). Results were considered significant for $p < 0.05$ indicated by "*", $p < 0.01$ indicated by "**", $p < 0.001$ indicated by "***" and $p < 0.0001$ indicated by "****".

Acknowledgments: We thank Matthew Morgan and Ulrike Krause for editorial support. This work was funded by GlaxoSmithKline Biologicals S.A. (Rixensart, Belgium) under a collaboration agreement with Institut Pasteur de Lille (Lille, France). GSK took responsibility for all costs incurred in publishing.

Author contributions: LVM and DF performed all animal, and flow cytometry experiments. DC and AC provided technical assistance for RT-qPCR and cytokine analysis by Luminex, respectively. AMD, CC, LVM, DF, and JCS designed experiments and wrote the manuscript. AMD and JCS supervised the experimental work. All authors were involved in critically revising the manuscript for important intellectual content. All authors had full access to the data.

Conflict of interest: All authors have declared the following interests: AC, CC, and AMD are employees of the GSK group of companies. AMD reports ownership of GSK shares and/or restricted GSK shares. The other authors report no financial or commercial conflict of interest.

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Abbreviations: AS: Adjuvant System · DC: dendritic cell · dLN: draining lymph node · HBs: Hepatitis B surface antigen · iLLN: draining iliac lymph node · MPL: 3-O-desacyl-4'-monophosphoryl lipid A

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The peer review history for this article is available at <https://publons.com/publon/10.1002/eji.201948234>

Received: 2/5/2019

Revised: 28/6/2019

Accepted: 24/7/2019

Accepted article online: 5/9/2019