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The Lymphatic Immune Response Induced by the Adjuvant AS01: A Comparison of Intramuscular and Subcutaneous Immunization Routes

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The liposome-based adjuvant AS01 incorporates two immune stimulants, 3-*O*-desacyl-4′-monophosphoryl lipid A and the saponin QS-21. AS01 is under investigation for use in several vaccines in clinical development. i.m. injection of AS01 enhances immune cell activation and dendritic cell (DC) Ag presentation in the local muscle-draining lymph node. However, cellular and Ag trafficking in the lymphatic vessels that connect an i.m. injection site with the local lymph node has not been investigated. The objectives of this study were: 1) to quantify the in vivo cellular immune response induced by AS01 in an outbred ovine model, 2) to develop a lymphatic cannulation model that directly collects lymphatic fluid draining the muscle, and 3) to investigate the function of immune cells entering and exiting the lymphatic compartments after s.c. or i.m. vaccination with AS01 administered with hepatitis B surface Ag (HBsAg). We show that HBsAg-AS01 induces a distinct immunogenic cellular signature within the blood and draining lymphatics following both immunization routes. We reveal that MHCII^{high} migratory DCs, neutrophils, and monocytes can acquire Ag within muscle and s.c. afferent lymph, and that HBsAg-AS01 uniquely induces the selective migration of Ag-positive neutrophils, monocytes, and an MHCII^{high} DC-like cell type out of the lymph node via the efferent lymphatics that may enhance Ag-specific immunity. We report the characterization of the immune response in the lymphatic network after i.m. and s.c. injection of a clinically relevant vaccine, all in real time using a dose and volume comparable with that administered in humans. ***The Journal of Immunology*****, 2016, 197: 2704–2714.**

Adjuvants are added to vaccine formulations to enhance the immunogenicity of vaccine Ags and improve adaptive immune outcomes. Adjuvants stimulate the immune system by enhancing innate cell recruitment, activation, and Ag presentation via improved Ag uptake by APCs or increased Ag bioavailability. The liposome-based Adjuvant System AS01 incorporates two immune stimulants, 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) and the saponin QS-21. AS01 recently received a positive evaluation by the European Medicines Agency as the adjuvant for the malarial vaccine RTS,S/AS01 and is under investigation for use in several candidate vaccines in clinical development, including those targeting HIV, herpes zoster, and tuberculosis (1–6). It was recently shown that AS01 drives adaptive immunity via innate immune cell activation and enhancement of dendritic cell (DC) Ag presentation in the local draining lymph node (7).

After vaccination, cells that have encountered the formulation at the injection site migrate to the local lymph node via the afferent lymphatics (AL). The immunological signals received by the local lymph node from cells migrating in AL shape the adaptive immune response, which is conveyed to the periphery by Ag-specific lymphocytes emigrating the lymph node via the efferent lymphatics (EL) (8). Examination of the afferent and EL compartments therefore permits the investigation of cells and soluble factors transmitting signals to and from the local lymph node, which are responsible for vaccine immunity.

Human vaccines are typically administered either i.m. or s.c.; however, few studies directly compare immunogenicity and reactogenicity of the same vaccine injected via both routes. Subcutaneous injection of adjuvanted vaccines has been associated with increased rates of site reactions, whereas i.m. vaccination is associated with similar immune outcomes and a lower rate of adverse events (9, 10). There are marked differences in the cellular composition of muscle and skin tissues that may influence these vaccination outcomes. The s.c. fat beds contain few immune cells; however, they are adjacent to the skin dermal layers, which contain large numbers of lymphocytes, macrophages, and specialized dermal DCs that drain into the local lymph node, whereas muscle tissue contains few immune cells and very low DC numbers (7, 11). In murine studies, it was noted that the DC populations in lymph nodes draining the two sites are different, which may lead to altered Ag-specific immune responses (12). However, little is known regarding the trafficking of cells within the lymphatic

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Abbreviations used in this article: AL, afferent lymphatics; DC, dendritic cell; EL, efferent lymphatics; HBsAg, hepatitis B surface Ag; MPL, 3-*O*-desacyl-4′-monophosphoryl lipid A.

vessels that connect the muscle injection site with the local lymph node and whether this may contribute to altered immune responses observed between the vaccine administration routes.

In this study, we investigate and compare the phenotype and function of immune cells entering the lymphatic compartments after s.c. or i.m. vaccination with the human Adjuvant System AS01 administered with hepatitis B surface Ag (HBsAg). To do this, we use existing s.c. lymphatic cannulation techniques (13) and develop a new cannulation model that directly collects lymphatic fluid draining the muscle after i.m. vaccination. We quantify the in vivo innate and adaptive immune response induced by s.c. or i.m. injection of HBsAg-AS01 in the lymphatic network draining both sites of vaccination and examine the systemic immune response after primary and boost vaccination in outbred sheep, all using a dose and volume of AS01 comparable with that administered in humans.

Materials and Methods

Vaccine formulations

AS01 and the HBsAg were obtained from GlaxoSmithKline Vaccines (Rixensart, Belgium). HBsAg was produced from genetically engineered yeast (*Saccharomyces cerevisiae*) and was formulated in AS01, an Adjuvant System containing QS-21 (*Quillaja saponaria* Molina, fraction 21; licensed by GlaxoSmithKline from Antigenics, a wholly owned subsidiary of Agenus, a Delaware) and MPL in a liposome-based formulation. For Ag labeling, HBsAg was resuspended at 1 mg/ml in PBS, and a 15-M excess of A647 succinimidyl ester (Invitrogen) resuspended in DMSO at 5 mg/ml was added and incubated at room temperature for 1 h. Unconjugated fluorophore was removed from solution using an Amicon Ultracel 3K centrifugal filter (Millipore) by centrifuging at 2000 × *g* for 30 min and washed with PBS five times. We injected 500 μl of HBsAg-AS01, the equivalent of a standard adult human dose of $AS01_B$ comprising 50 μg of MPL and 50 μg of QS-21 in a liposome-based formulation with 40 μg of Ag (35 μg of HBsAg + 5 μg of A647-HBsAg). Injections were performed with a 25G needle s.c. in the area drained by the prefemoral lymph node or i.m. in the quadriceps muscle, which drains directly to the iliac lymph node.

Animals and immunization strategy

All sheep were obtained from a Monash University–approved supplier, and written approval for all animal experiments was obtained from the Monash University Ethics Committee (approval no. MARP/2013/054). Animals were maintained and cared for in purpose-built facilities. Food and water were available ad libitum at all times, excluding the 18 h before surgery. To initially establish immune responses in peripheral blood, we randomly assigned 1-y-old merino sheep into two vaccination groups: six sheep were immunized s.c. in the prefemoral drainage area, and six sheep were injected i.m. in the iliac draining area with HBsAg-AS01. Secondary injections were performed 4 wk after primary injection, and blood was collected before each injection and 1–3, 5, and 7 d after each injection. Four sheep with cannulated prefemoral pseudo-AL and four sheep with cannulated iliac pseudo-AL received a single s.c. injection or a single i.m. injection of HBsAg-AS01, respectively, to monitor the immune response in AL. Five animals with cannulated prefemoral EL vessels and four animals with cannulated iliac EL vessels received a single s.c. injection or a single i.m. injection of HBsAg-AS01, respectively, to monitor the immune response in EL.

Prefemoral pseudo-AL and EL cannulation surgery

Ovine prefemoral pseudo-AL and EL cannulations were performed as previously described (13). In brief, for prefemoral pseudo-AL cannulation, the prefemoral lymph nodes of merino sheep were removed at 1 y of age and at least 2 mo was allowed for reanastomosis of the AL with the larger EL vessel. A secondary surgery was performed to insert a 0.96 × 0.58 mm heparin-coated polyvinyl chloride cannula into the pseudo-AL vessel. For prefemoral EL cannulation, a 0.96 × 0.58 mm heparin-coated polyvinyl chloride cannula was inserted into the EL vessel of the prefemoral lymph node.

Iliac pseudo-AL and EL cannulation surgery

In preliminary experiments, to determine i.m. lymphatic drainage patterns, we injected sheep i.m. in the quadriceps muscle with 100 μl of 5% carbon-based India ink diluted in PBS. At 24 h postinjection, sheep were euthanized and all rear draining lymph nodes were excised and visually analyzed for the presence of black dye. In all sheep, only the rear iliac lymph nodes contained the dye. Before collection of iliac AL, the iliac nodes on the right side of 1-y-old merino wethers were surgically excised through a 10-cm abdominal surgical incision. The omentum was gently punctured and the surgical field cleared using sterile sponges and a surgical spreader. The lymph nodes were located and excised using blunt dissection. The midline of the peritoneum and skin were sutured and the sheep allowed to recover for a minimum of 3 mo. To cannulate the iliac lymphatic vessel, we reaccessed the abdominal cavity via a larger, more rostral incision along the midline and the large lymphatic vessels running along the vena cava were located. Smaller lymphatic vessels were ligated and a 5 French heparin-coated polyurethane cannula (Solomon Scientific, San Antonio, TX) was inserted into the largest lymphatic vessel on the right-hand side of the vena cava. The cannula was anchored in place with two sutures and exteriorized to the right side of the sheep's flank. The incisions in the peritoneum and the skin were closed and the sheep were allowed to recover. Further information detailing the iliac lymph node anatomy in the sheep can be found in a publication by Heath and colleagues (14), and we have also included a stylized diagram displaying the anatomy and cannulae insertion site in Supplemental Fig. 1.

For all cannulation models, the cannulae were exteriorized and placed in a sterile collection flask attached to the side of the sheep. Immunizations were performed once the lymph was clear of erythrocytes and contained <1% neutrophils (resting values), which typically required 5–7 d. All collection tubes were surrounded by ice during the entire lymph collection experiment.

Sample collection and flow cytometry

Heparinized blood was collected by venipuncture from the jugular vein. Immediately after collection, 100 μl of whole blood was lysed with 800 μl of 155 mM ammonium chloride. After 3-min incubation, 2 ml of ice-cold FACS buffer (5% horse serum, 2 mM EDTA in PBS) was added and the cells centrifuged at 400 × *g* at 4°C, washed twice with FACS buffer, and used for flow cytometry analysis.

Prefemoral and iliac AL and EL were collected on ice in sterile 50-ml tubes containing 0.05 IU of heparin (Pfizer) and 20 μl of 100× cell culture penicillin-streptomycin (Invitrogen). AL was collected before primary injection, 0–4, 4–8, 8–12, 24–28, 48–50, and 72–73 h after injection. EL was collected before primary injection, and 0–4, 4–8, 8–12, 24–28, 48–50, and 72–73 h, and 5 and 7 d after injection. Immediately after collection, lymph cells and supernatant were separated by centrifugation at 400 × *g* at 4°C. To prepare lymph cells for flow cytometry, we added 800 μl of 155 mM ammonium chloride to 100 μl of lymph and incubated for 3 min; then 2 ml of ice-cold FACS buffer was added and the cells centrifuged at 400 × *g* at 4°C. After two washes with 2 ml of FACS buffer, the cell pellet was resuspended in 25 μl of Fc block (2% BSA, 2 mM EDTA, 0.05% azide, 5% sheep serum in PBS) for Ab staining.

The surface marker Abs used were anti-MHCII, CD14, CD172a (SIRPα), CD16, and CD4, CD8, γδ TCR, NKp46. Twenty-five microliters of Ab cocktails made up at 2× were added to the resuspended cells and incubated on ice for 30 min. For intracellular cytokine staining, EL cells were resuspended in 150 μl of 1% PFA for 20 min on ice, centrifuged at 400 × *g*, and washed twice in 500 μl saponin buffer (1% horse serum, 0.05% azide, 0.1% saponin in PBS). The intracellular Ab (anti–IFN-γ) was then added for 30 min on ice. All samples for flow cytometry were performed on an LSR-Fortessa machine (Becton Dickinson) and analyzed using the FlowJo software. Flow cytometry gating strategies are outlined in the respective figures of the *Results* section. DCs ($FSC^{high}MHCII^{high}SIRP\alpha^{high/low}$), monocytes ($MHCII^{int}$, $SIRP\alpha^{+}$, $CD14^{+}CD16^{+/-}$), and neutrophils ($MHCII^{low}SIRP\alpha^{+}SSC^{high}$) were gated based on our previous work and phenotypes in the literature (13, 15, 16).

ELISA for the identification of HBsAg-specific Abs

An indirect ELISA was used to measure relative HBsAg-specific Ab levels in each EL and serum sample. In brief, 100 μl of HBsAg (0.5 μg/ml in PBS) was added to a flat-bottom, 96-well, high-binding plate (Corning) and left to bind overnight at 4°C. The plate was washed with 0.05% PBST and blocked with 5% skim milk in PBS at 200 μl/well for at least 1 h at room temperature. Sera and EL samples (diluted 1/1000) were added at 100 μl/well for 1 h at room temperature. The wells were washed five times before 100 μl of rabbit anti-sheep Igs/HRP Ab (P0163; diluted 1/1000 in PBS; DAKO) was added and allowed to incubate for 1 h. After five washes, reactions were developed using TMB substrate (Invitrogen) at 100 μl/well, stopped by the addition of 50 μl/well of 2 M H_2SO_4, and read at 450 nm on a spectrophotometer (Molecular Devices, Spectramax Plus).

Statistical analysis

Results are presented as mean ± SEM. Differences between groups and within each group were calculated with a two-way repeated-measures

ANOVA using a Holm–Sidak posttest to correct for multiple comparisons. Significance was determined as the confidence interval >95% ($p < 0.05$). The statistical software used was GraphPad Prism, version 6.01.

Results

AS01-adjuvanted vaccine immunogenicity in outbred sheep

To determine whether AS01 was capable of exerting its observed adjuvant effect in sheep, we first measured the end-point Ab titer of HBsAg-specific Abs in the blood for 7 d after primary and secondary i.m. and s.c. injection of HBsAg-AS01. Total HBsAg-specific end-point Ab titers were significantly increased 7 d after primary vaccination in both groups (Fig. 1A). Secondary vaccination further enhanced the HBsAg-specific Ab response, with significant increases observed at 5 and 7 d after i.m. and s.c. injection of AS01 (Fig. 1A).

Evidence suggests that AS01 mediates its adjuvant effect via stimulation of innate immunity, as measured by the recruitment of neutrophils, monocytes, and DCs to the site of injection and the draining lymph node (7). To first determine the effects of AS01 in ovine peripheral blood, we measured immune cells after immunization with HBsAg-AS01. This revealed a significant increase in the percentage of neutrophils 24 h after primary and secondary vaccination, independent of the injection site (Fig. 1B, 1C). The percentage of monocytes significantly increased at 3 d after vaccination, 2 d later than the observed peak in neutrophils (Fig. 1D). As in humans, three subsets of monocytes were identified in blood: $CD14^+CD16^-$, $CD14^+CD16^+$, $CD14^{low}CD16^+$ (Fig. 1E).

The majority of monocytes were $CD14^+$, with $CD14^+CD16^+$ comprising ~50% of monocytes and $CD14^+CD16^{low}$ comprising ~30% of monocytes at baseline (Fig. 1E). Subcutaneous injection of AS01 increased the percentage of $CD14^+CD16^-$ monocytes 2–3 d after primary vaccination, with a corresponding decrease observed in $CD14^+CD16^+$ monocytes at these time points (Fig. 1E). Intramuscular injection of AS01 increased the percentage of $CD14^+CD16^-$ monocytes 7 d after primary vaccination, with a corresponding decrease observed in $CD14^+CD16^+$ monocytes at this time point (Fig. 1E). Collectively, these data demonstrate that the adjuvant effect of AS01 is conserved in the sheep model.

i.m. and s.c. vaccination with HBsAg-AS01 induces the recruitment of Ag-carrying monocytes and neutrophils from the site of injection into the AL vessels

It was shown in mice that AS01 induces the recruitment of innate cells at the muscle injection site and in the draining lymph node (7). However, the kinetics and trafficking of cells between the muscle and the local draining lymph node is unknown. As such, we developed a lymphatic cannulation model that directly collects lymphatic fluid draining the muscle after i.m. vaccination in the area draining the iliac lymph node. Immune cell trafficking, phenotype, and activation status in the iliac lymphatic vessels after i.m. vaccination was compared with s.c. injection using an established model that collects lymphatic fluid draining the s.c. space after s.c. vaccination in the area draining the prefemoral lymph node (8).

The total lymph volume and cell count per hour in AL draining the iliac i.m. injection site was significantly greater at baseline and at most time points after vaccination with AS01 than that observed in AL draining the prefemoral s.c. injection site (Fig. 2A). However, under homeostatic conditions, the percentage of DCs, monocytes, and neutrophils in iliac AL were dramatically less than that observed in prefemoral AL (Fig. 2B). Therefore, despite iliac AL being composed of a significantly lower percentage of innate cells, due to its significantly higher volume draining to the lymph node per hour, the numbers of DCs, monocytes, and neutrophils trafficking to the iliac lymph node under homeostatic conditions is remarkably similar to the prefemoral lymph node (Fig. 2C).

To characterize the immune response induced by i.m. and s.c. injection of AS01-adjuvanted vaccine in the local lymphatic network, we investigated the phenotype and Ag uptake capacity of innate cells in iliac and prefemoral AL over a period of 72 h. HBsAg was fluorescently labeled to A647 to allow investigation of Ag trafficking via flow cytometry.

Injection with HBsAg-AS01 induced a rapid increase in the number of neutrophils migrating in iliac and prefemoral AL per hour, respectively (Fig. 2C). This increase occurred predominantly between 4 and 12 h after vaccination, where the numbers of neutrophils in AL were ~10-fold greater than DCs or monocytes at this time. The neutrophil-associated response returned to baseline numbers before 48 h in both AL compartments, demonstrating the transient nature of this response. A significant increase in monocytes within iliac AL was observed 8–12 h after i.m. injection with AS01, resolving to baseline by 72 h after vaccination (Fig. 2C). This trend was also observed for monocyte recruitment in prefemoral AL after s.c. injection; however, this did not reach significance (Fig. 2C).

Neutrophils were responsible for the majority of AL cell-associated HBsAg transport during the first 28 h after vaccination in both compartments (Fig. 2D, 2E). Monocytes also carried Ag during the first 50 h after vaccination, with peak lymphatic cellular Ag transport occurring at 8–12 h (Fig. 2D, 2E). Interestingly, no change in the total number of DCs recruited into AL was observed after either vaccination with HBsAg-AS01; however, $HBsAg^+$ MHC-II^{high} DCs were observed in both AL compartments after vaccination and were the predominant cell type carrying Ag between 48 and 72 h (Fig. 2D, 2E). No change in the numbers of lymphocytes, Ag-positive lymphocytes, or lymphocyte subsets was observed in either AL compartment after vaccination (data not shown). Therefore, for both routes of immunization, the Ag coadministered with AS01 is initially transported from the site of injection to the draining lymph node by neutrophils, followed by monocytes and finally DCs.

$CD14^+$ monocytes are responsible for monocyte-associated Ag transport in muscle- and skin-draining AL after vaccination with HBsAg-AS01

To determine the phenotype of monocyte subsets recruited into AL and the subsets responsible for Ag transport after vaccination, we investigated expression of CD14 and CD16. Similar to that observed in peripheral blood, three subsets of monocytes were identified in both AL compartments: $CD14^+CD16^-$, $CD14^+CD16^+$, and $CD14^{low}CD16^+$ (Fig. 3A, 3B). HBsAg-AS01 injected into muscle increased the number of $CD14^+CD16^+$ monocytes in iliac AL between 4 and 50 h after vaccination, whereas s.c. injection of AS01 increased both $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes in prefemoral AL at these time points (Fig. 3A, 3B). Consistent with their recruitment in both AL compartments, the $CD14^+$ monocyte subsets were responsible for the majority of monocyte-associated HBsAg transport, with very few $CD14^{low}$ monocytes carrying Ag in AL (Fig. 3C, 3D).

A proportion of Ag-carrying neutrophils and monocytes exit muscle- and skin-draining lymph nodes via the EL after i.m. and s.c. vaccination with HBsAg-AS01

We next cannulated the EL vessels draining the respective lymph nodes to investigate whether i.m. or s.c. injection of AS01 differentially influences the cells exiting the lymph node. Unlike AL, no significant difference in total lymph volume and cell count per hour was observed between iliac EL and prefemoral EL at any time point after vaccination (Fig. 4A). No significant difference in the baseline percentage of monocytes or neutrophils was observed between EL compartments (Fig. 4B).

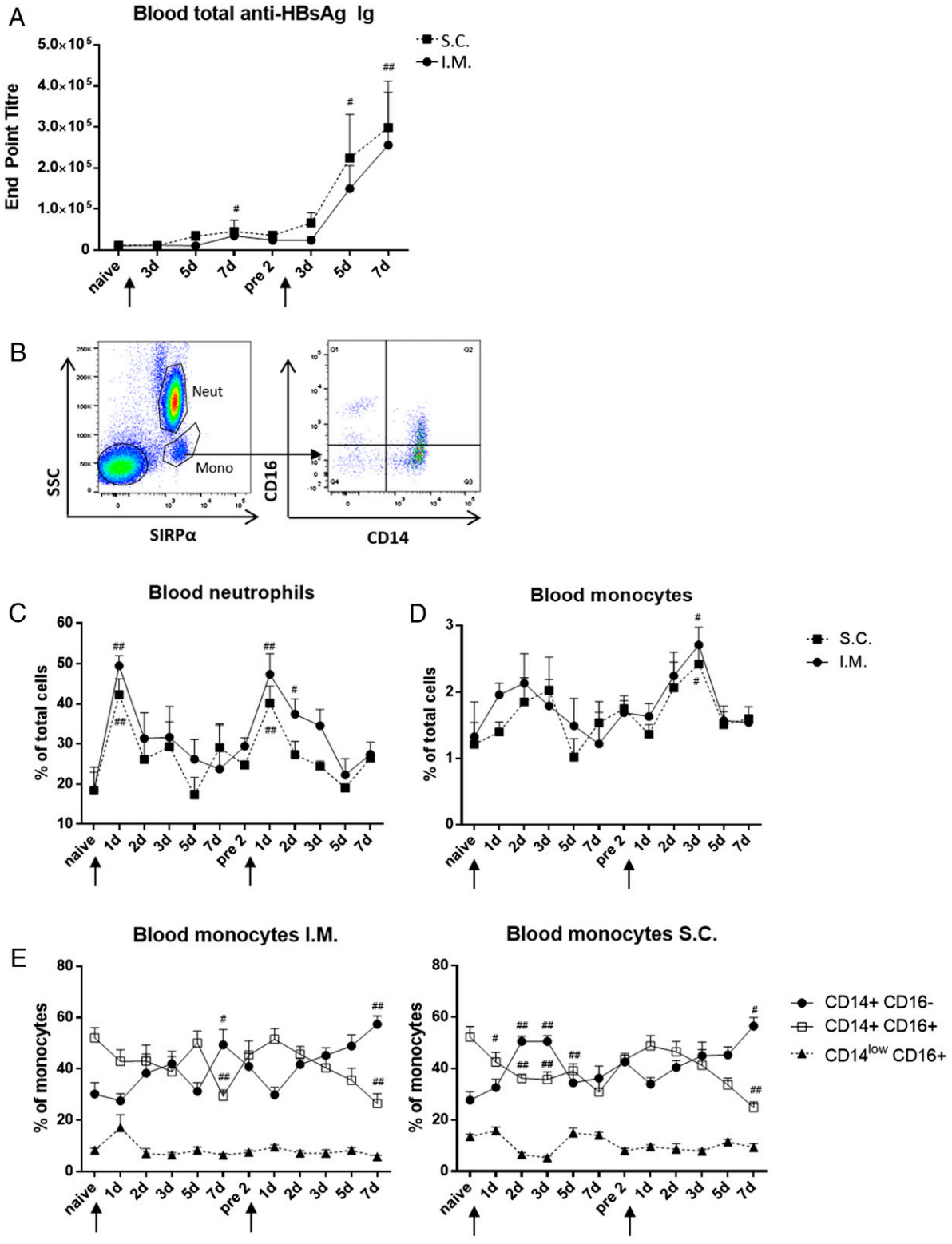

FIGURE 1. Immune response to primary and secondary i.m. and s.c. immunization of HBsAg-AS01 in peripheral blood of sheep. (**A**) HBsAg-specific total Ig mean end-point Ab titers before and after primary and secondary immunization. (**B**) Gating strategies used to investigate the innate cellular immune response in peripheral blood and monocytes were $SIRP\alpha^{+}CD14^{+}CD16^{+/-}$ and neutrophils were $SIRP\alpha^{+}$ SSC^{high}. (**C**) Percentage of systemic neutrophils after s.c. (square, dashed lines) and i.m. (circle, complete lines) primary and secondary immunization with HBsAg-AS01. (**D**) Percentage of systemic monocytes after s.c. (square, dashed lines) and i.m. (circle, complete lines) primary and secondary immunization with HBsAg-AS01. (**E**) The percentage of each monocyte subset ($CD14^{+}CD16^{-}$, $CD14^{+}CD16^{+}$, and $CD14^{low}CD16^{+}$) after s.c. and i.m. primary and secondary immunization with HBsAg-AS01. Arrows indicate immunization times. Data are expressed as mean ± SEM of six animals in each group. A repeated-measures two-way ANOVA with a Holm–Sidak multiple comparisons test was used to determine significance, where asterisks indicate differences between groups and number signs indicate differences from baseline (pre) within each group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

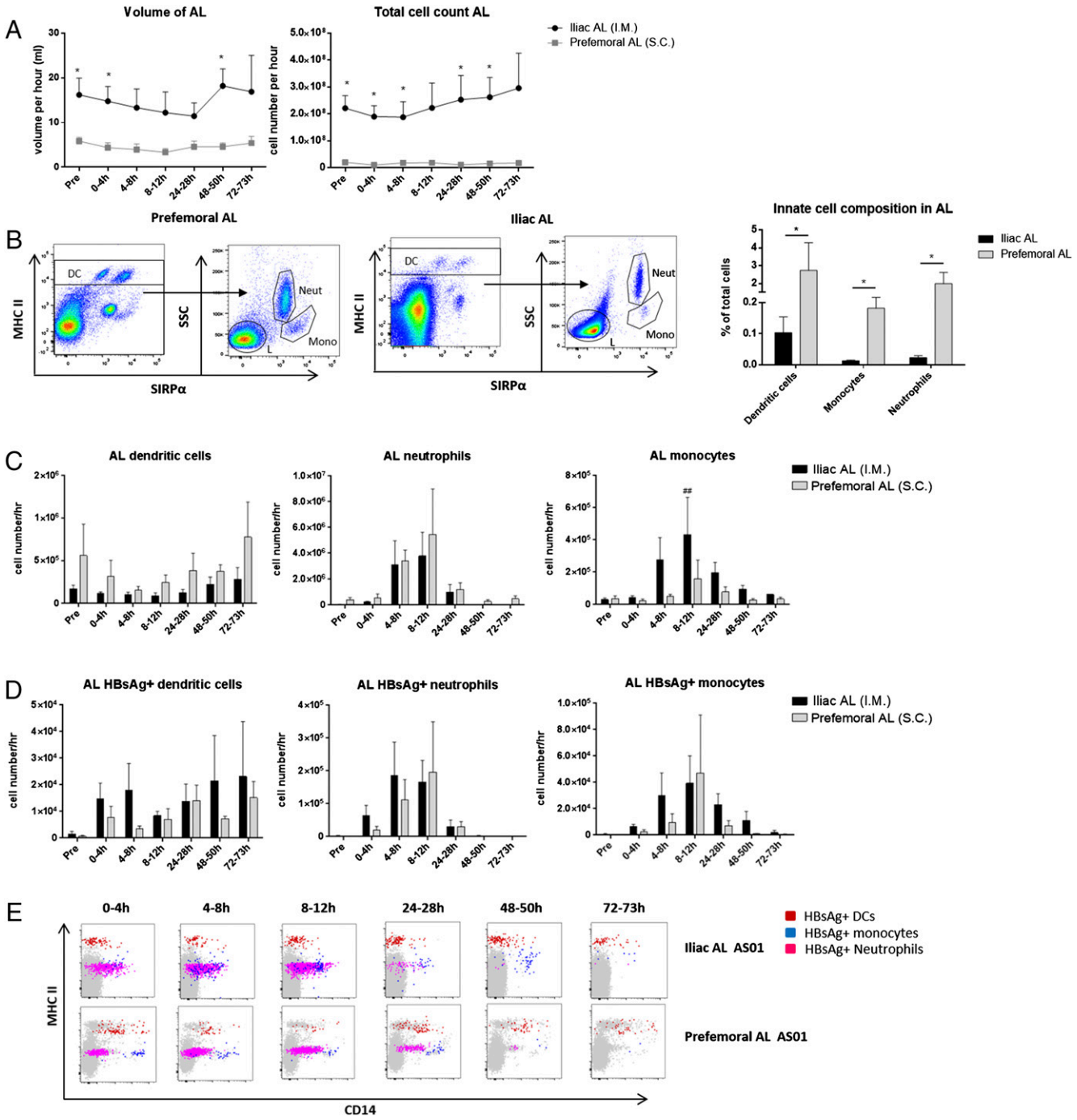

FIGURE 2. i.m. and s.c. primary immunization of HBsAg-AS01 induces the recruitment of neutrophils and monocytes into muscle- (iliac) and skin-draining (prefemoral) AL that carry HBsAg to the local lymph node. (**A**) Lymph volume and total cell count per hour in muscle (iliac) and skin (prefemoral) draining AL after s.c. and i.m. injection of HBsAg-AS01, respectively. AL was collected at baseline (pre) and 0–4, 4–8, 8–12, 24–28, 48–50, and 72–74 h after both immunization routes. (**B**) Gating strategies used to investigate the innate cellular immune response in prefemoral and iliac AL; DCs were MHC-II^{high} SIRPα$^{+/-}$; monocytes were MHCII^{int}, SIRPα^{+}, SSC^{int}, CD14^{+}, and CD16$^{+/-}$; and neutrophils were MHCII^{low}, SIRPα^{+}, and SSC^{high}. Flow cytometry plot from a representative animal at the 4-h time point to illustrate neutrophil phenotype. The homeostatic percentages of DCs, monocytes, and neutrophils were determined at baseline in both iliac (black bars) and prefemoral AL (gray bars). (**C**) DCs, neutrophils, and monocytes in iliac (black bars) and prefemoral (gray bars) AL are expressed as the number of cells migrating in AL per hour over the vaccination time course. (**D**) Number of HBsAg^{+} DCs, neutrophils, and monocytes migrating in iliac (black bars) and prefemoral (gray bars) AL per hour over the vaccination time course. (**E**) Flow cytometry plots of one representative animal from each group (iliac or prefemoral AL) illustrating the number of HBsAg^{+} DCs (red), HBsAg^{+} monocytes (blue), and HBsAg^{+} neutrophils (pink) overlaid onto a background of all cells in AL (gray) over the vaccination time course. Data expressed as mean ± SEM of four animals in both groups. A repeated-measures two-way ANOVA with a Holm–Sidak multiple comparisons test was used to determine significance, where asterisks indicate differences between groups and number signs indicate differences from baseline (pre) within each group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#\#}p < 0.01$.

Innate immune cells, including monocytes and neutrophils, were shown to exit the local lymph node after both injections of HBsAg-AS01. Neutrophils showed a transient increase in both EL compartments, with numbers peaking at 24–28 h after injection and declining to near-baseline levels by 48–50 h (Fig. 4C). At 24–28 h, the total number of neutrophils and HBsAg^{+} neutrophils

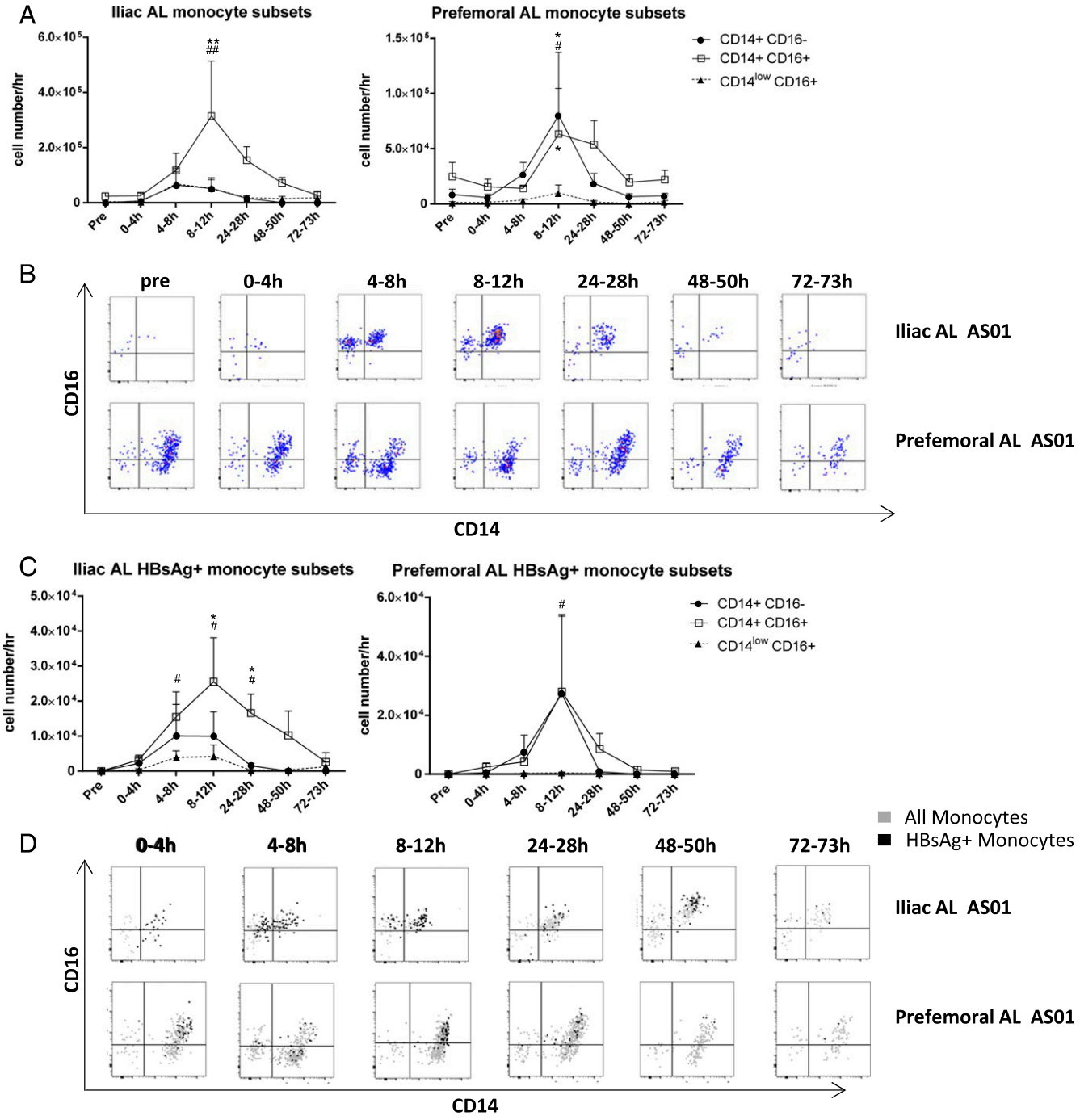

FIGURE 3. Phenotype and Ag uptake capacity of monocyte populations recruited in muscle- (iliac) and skin-draining (prefemoral) AL. (**A**) Monocyte populations in both AL compartments were first gated on SSC^{int}, $MHCII^{int}$, $SIRP\alpha^{+}$ expression and further characterized into three subsets: $CD14^{+}CD16^{-}$, $CD14^{+}CD16^{+}$, and $CD14^{low}CD16^{+}$, expressed as cell number per hour over the vaccination time course and (**B**) illustrated in flow cytometry plots of a representative animal from each group. (**C**) Number of $HBsAg^{+}$ $CD14^{+}CD16^{-}$, $CD14^{+}CD16^{+}$, and $CD14^{low}CD16^{+}$ monocytes per hour in both AL compartments. This is also illustrated in flow cytometry plots of a representative animal from each group (**D**), where $HBsAg^{+}$ monocytes (black) are overlaid onto a background of all monocytes (gray). Data are expressed as mean ± SEM of four animals in both groups. A repeated-measures two-way ANOVA with a Holm–Sidak multiple comparisons test was used to determine significance, where asterisks indicate differences between groups and number signs indicate differences from baseline (pre) within each group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

leaving the prefemoral lymph node was significantly greater than those leaving the iliac lymph node (Fig. 4D). Monocytes were also present in both EL compartments, with a significant increase observed at 8–12 h in prefemoral EL (Fig. 4C). This consistent increase in innate cells within prefemoral lymph nodes may be related to the smaller size of the prefemoral node, weighing on average 1 g less than the iliac lymph node in sheep (data not shown). The numbers of neutrophils leaving the iliac lymph node for the entire 24 h after injection were not sufficient to account for the increase in neutrophils observed in blood at this time, contributing 0.06% of neutrophils in blood after i.m. injection (Table I). This was also apparent for EL monocytes where the entire 24-h period contributed just 0.0155% of total blood monocytes at the 24-h time point. Both of the lymphatic 24-h values were significantly higher than the resting levels, indicating the lymph may contribute cells to the blood pool, but the increases

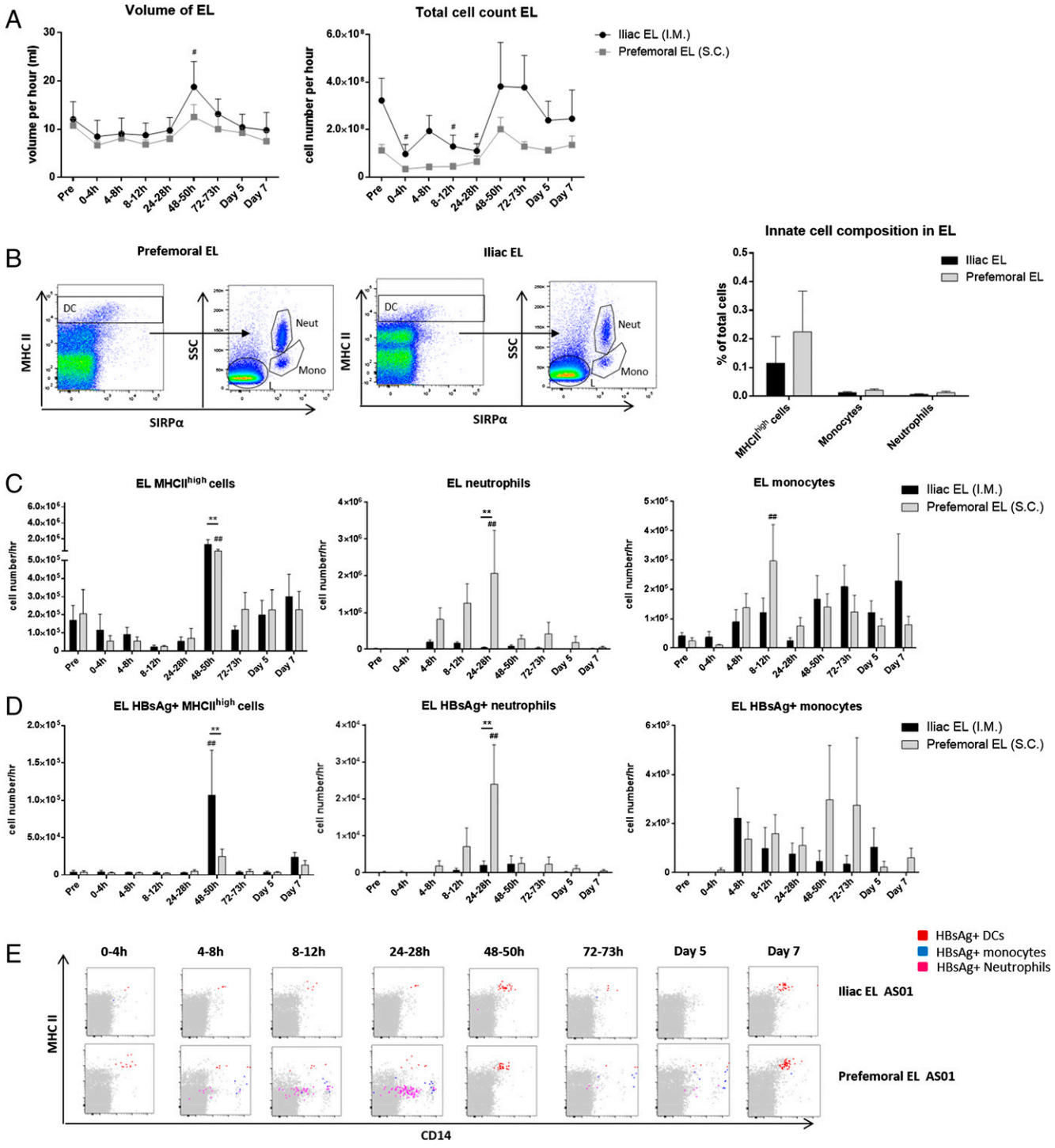

FIGURE 4. i.m. and s.c. primary immunization of HBsAg-AS01 induces the emigration of DCs, neutrophils, and monocytes out of the local muscle- (iliac) and skin-draining (prefemoral) lymph nodes via the EL. (**A**) Lymph volume and total cell count per hour in muscle- (iliac) and skin-draining (prefemoral) EL after s.c. and i.m. injection of HBsAg-AS01, respectively. EL was collected at baseline (pre) and 0–4, 4–8, 8–12, 24–28, 48–50, and 72–74 h, and 5 and 7 d after both immunization routes. (**B**) Gating strategies used to investigate the innate cellular immune response in prefemoral and iliac EL, a representative plot collected 50 h postvaccination showing MHC-II^{high} SIRPα^{int} (referred to as MHC-II ^{high} cells), is followed by a representative plot from cells collected 4 h after vaccination showing monocyte and neutrophil gates for both prefemoral and iliac lymph. All gates were set on live, single cells. The homeostatic percentages of MHC-II^{high} cells, monocytes, and neutrophils were determined at baseline in both iliac (black bars) and prefemoral EL (gray bars). (**C**) MHC-II^{high} cells, neutrophils, and monocytes in iliac (black bars) and prefemoral (gray bars) EL are expressed as the number of cells migrating in EL per hour over the vaccination time course. (**D**) The number of HBsAg^{+} MHC-II^{high} cells, neutrophils, and monocytes migrating in iliac (black bars) and prefemoral (gray bars) EL per hour over the vaccination time course. (**E**) Flow cytometry plots of one representative animal from each group illustrating the number of HBsAg^{+} DCs (red), HBsAg^{+} monocytes (blue), and HBsAg^{+} neutrophils (pink) overlaid onto a background of all cells in EL (gray) over the vaccination time course. Data expressed as mean ± SEM of $n = 4$ in iliac EL and $n = 5$ in prefemoral EL. A repeated-measures two-way ANOVA with a Holm–Sidak multiple comparisons test was used to determine significance, where asterisks indicate differences between groups and number signs indicate differences from baseline (pre) within each group: $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

seen within blood after vaccination must come from existing or newly synthesized cells (Table I). This was not evaluated for the prefemoral node because the EL travels to subsequent nodes before entering the circulation via the thoracic duct. There was a significant increase in the total number of efferent MHC^{high} cells (FSC^{high}, SSC^{int} $MHCII^{high}$ $SIRP\alpha^{int}$) in iliac and prefemoral EL 48–50 h after injection that were transporting Ag. These Ag-positive cells again increased at day 7, suggesting some periodicity; however, this was not significant (Fig. 4C–E). The numbers of these cells were higher in iliac EL than prefemoral EL after AS01 injection (Fig. 4C–E). Interestingly, there was no increase in DCs entering the lymph nodes before 48–50 h and no clear increase in $HBsAg^+$ DC migration at 48–50 h into either lymph node from the AL before this time point.

Direct comparison of innate cell trafficking into and out of the muscle- and skin-draining lymph nodes

The numbers of monocytes and neutrophils entering the lymph node via AL were directly compared with the numbers of these cells leaving the lymph node via EL over the vaccination time course. Significantly more neutrophils entered both the iliac and the prefemoral lymph nodes at 8–12 h compared with those leaving the respective lymph node at this time (Fig. 5A). No significant difference was observed in the number of monocytes entering and leaving either lymph node (Fig. 5B). This was not analyzed for DCs because further studies are required to confirm the nature of the DC-like $MHCII^{high}$ cells observed in EL.

Functional immune responses in EL after vaccination with HBsAg-AS01

To investigate the adaptive immune response induced after i.m. and s.c. AS01-HBsAg administration, the number, phenotype, and activation status of lymphocytes leaving the local draining lymph node via the EL was determined. There was a rapid and transient reduction in the number of CD4 T cells, CD8 T cells, B cells, and γδ T cells migrating in iliac and prefemoral EL after vaccination with AS01 (Fig. 6A). This is a phenomenon known as lymph node cell shutdown (17). No change in the relative proportion of each lymphocyte population was observed (data not shown). Interestingly, during this lymph node cell shutdown period at 4–8 h postvaccination, a small percentage of CD8 T cells, CD4 T cells, and γδ T cells in both EL compartments were expressing the IFN-γ cytokine (Fig. 6B). The percentage of IFN-γ^+-CD8 and -γδ T cells was significantly greater in prefemoral EL than iliac EL at this time point (Fig. 6B). Between 3 and 7 d after vaccination, a significant increase in the number of blast cells was observed in both EL compartments (Fig. 6C). A marked and significant increase in HBsAg-specific mean end-point Ab titer was also observed in both EL compartments at 7 d postvaccination (Fig. 6D).

Discussion

AS01 is a liposome-based human Adjuvant System that contains the TLR-ligand MPL and the saponin adjuvant QS-21. AS01 enhances T cell and Ab-mediated immune responses against a range of pathogens, and clinical trials have demonstrated that vaccine formulations incorporating AS01 induce protective immune responses (6, 18). To evaluate sheep as an outbred model for AS01-induced immunity and route of administration effects, we injected sheep with AS01-adjuvanted HBsAg vaccine s.c. or i.m. and evaluated clinical blood parameters. AS01 induced a significant increase in anti-HBsAg IgG titer and transiently increased the percentage of neutrophils and monocytes within the blood of vaccinated animals, independent of the route of administration. Similar effects have been described in humans (19) and other models, including mice (7), demonstrating that the sheep model is relevant for further study investigating the mode of action of AS01.

The enhanced adaptive immune response observed with AS01 requires activation of migratory $MHCII^{high}$ DCs in mice, which are rare in normal muscle tissue and typically migrate to the lymph node from other peripheral tissues. To address how i.m. injection of AS01 enhances the acquisition of Ag by DCs and to determine whether cells migrating through the draining lymph node contribute to the increase in innate cells within peripheral blood, we developed an ovine cannulation model that directly collects the lymphatic fluid draining an i.m. vaccination site. We have used this model to detail and compare the in vivo phenotype and activation status of immune cells migrating to and from the local lymph node after i.m. or s.c. administration of AS01. This is the first account to our knowledge that measures lymphatic drainage parameters after an i.m. injection of a clinically relevant vaccine.

Consistent with previous studies investigating local responses to innate stimulating adjuvants (13, 20–22), HBsAg-AS01 induced the rapid and transient recruitment of neutrophils and monocytes into both prefemoral and iliac AL within the first 12 h of administration. The response is consistent with i.m. injection of AS01 in mice, where monocytes and neutrophils were present within the muscle 3–6 h after vaccination, after which they rapidly cleared the injection site (7). Our results indicate these cells are responsible for the majority of cell-associated Ag transport in AL during the first 28 h after vaccination with AS01. This is consistent with previous work, showing that monocytes and granulocytes from AL carry the majority of Ag in the early hours after vaccination (16),

Table I. Contribution of iliac EL neutrophils and monocytes to circulating neutrophils and monocytes 24 h postvaccination with AS01

Parameter (30-kg Sheep)	Blood	Iliac EL	Lymphatic Contribution
Total fluid volume, l	1.686		
Total cell number	8.34×10^9		
Resting mean % neutrophils	18%	0.1%	
Resting neutrophils, EL/h	1.5×10^9 (0 h)	3.2×10^4	0.00002%
Mean % neutrophils (24 h after AS01)	49%	Variable	
Total neutrophils (24 h after AS01)	4.13×10^9 (24 h)	2.4×10^6 (0–24 h)	0.06%
Resting mean % monocytes	1.3% (0 h)	0.17% (0 h)	
Resting monocytes (EL/h)	1.08×10^8	5.44×10^4	0.0005%
Mean % monocytes (24 h after AS01)	1.95% (24 h)	Variable	
Total monocytes (EL 0–24 h)	1.62×10^8 (24 h)	2.52×10^6(0–24 h)	0.0155%

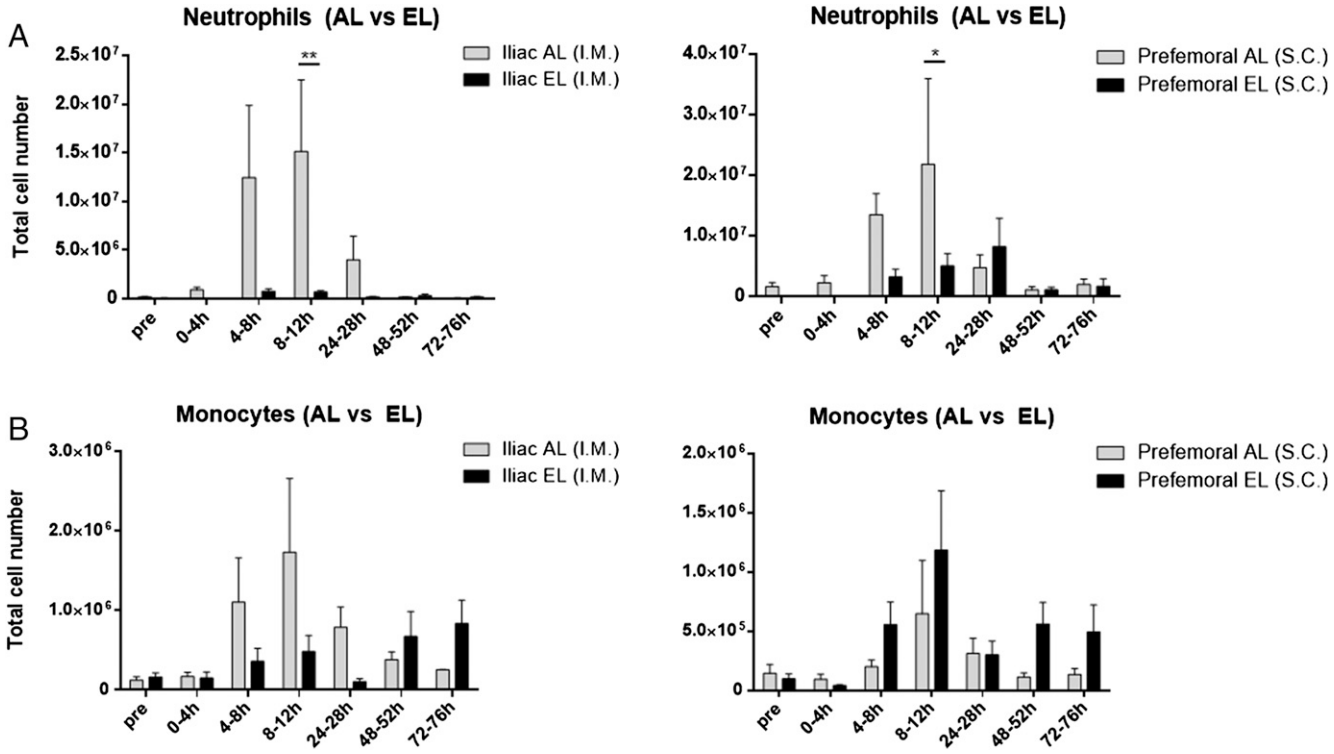

FIGURE 5. Direct comparison of innate cell trafficking into and out of the muscle- (iliac) and skin-draining (prefemoral) lymph nodes. (**A**) Total number of neutrophils within each 4-h collection period compared between iliac AL and EL, and prefemoral AL and EL. (**B**) Total number of monocytes within each 4-h collection period compared between iliac AL and EL, and prefemoral AL and EL. This analysis was not performed for DCs because further studies are required to confirm the DC-like MHCII^{high} cells observed in EL. Data expressed as mean ± SEM for all animals in both groups. A repeated-measures two-way ANOVA with a Holm-Sidak multiple comparisons test was used to determine significance, where asterisks indicate differences between groups: $*p < 0.05$, $**p < 0.01$.

further supporting that these cells may play a crucial role in the rapid clearance of Ag from the injection site. This is also supported by our previous work, showing that depot adjuvants that induce little cellular Ag transport result in granuloma formation at the injection site (21). This work, however, does not exclude rapid free non–cellular-associated flowing of Ag to the lymph node, as demonstrated in mice (7).

In humans and ruminants, three major subsets of peripheral blood monocytes have been identified: CD14^{2+} CD16^{-} monocytes (classical), CD14^{2+}CD16^{+} (intermediate), and CD14^{+} CD16^{2+} (nonclassical) (23, 24). Both classical and nonclassical subsets produce inflammatory cytokines and display phagocytic activity; however, the CD14^{+}CD16^{2+} subset has been shown to express higher levels of activation markers and demonstrate superior Ag presentation to T cells (25, 26). We show that vaccination with HBsAg-AS01 induces the recruitment of Ag carrying CD14^{+} CD16^{+} monocytes from the injection site into the local lymph node via AL. Interestingly, in muscle AL, this subset migrated in greater numbers and carried the majority of Ag when compared with the CD16^{-} subset. In s.c. AL, however, both subsets migrated in similar numbers and carried Ag to the local lymph node.

We observed no change in the total number of AL MHCII^{high} DCs migrating after vaccination after either s.c. or i.m. injection of AS01-adjuvanted vaccine, consistent with our previous work and studies in cattle using other adjuvants (13, 20–22, 27). Together with monocytes in muscle AL, DCs remained the only cell type carrying Ag at 48 and 72 h after injection. It was recently shown that activated MHCII^{high} CD11c^{+} Ag-loaded DCs were the only cell type capable of efficiently presenting Ag to T cells in the draining lymph node after vaccination with AS01-adjuvanted vaccine (7). It was unclear, however, whether these DCs obtain Ag at the site of injection before their migration to the lymph node or encountered the Ag in the lymph node itself. We show that Ag-positive mature MHCII^{high} DCs present within the AL are Ag-positive at all time points after either s.c. or i.m. injection with AS01. Muscle-resident DCs are present in extremely low numbers; however, up to 10% of migratory DCs present within the collected lymph are Ag-positive, suggesting they are very efficient at acquiring Ag at the site of injection or within the draining lymphatic vessels. A portion of MHCII^{high} DCs in iliac lymph may originate from the muscle, because others have shown muscle DCs are present in mice and drain to the local node (28). The role and proportion of these migratory DCs and DCs directly activated by the adjuvant in the lymph node remain to be determined.

To further characterize the migration of innate cells and measure adaptive immune responses, we examined the cell composition within the EL that drains the lymph node and ultimately traffics immune cells back into the circulation. Injection of AS01 induced a transient and selective increase in HBsAg^{+} monocytes, neutrophils, and DCs out of the lymph node in the first 48 h after vaccination. We could not detect MHCII^{high} SIRP$\alpha^{+/-}$ DCs within EL, suggesting the release of innate cells from the lymph node is selective for neutrophils and monocytes during the first 48 h. At 48 h, a small but clear increase in FSC^{high} SSC^{int} MHCII^{high} SIRPα^{int} population of cells was observed. After 50 h, these cells were the primary cell type transporting cell-associated HBsAg out of the lymph node and may represent a rare and newly identified cDC2 subset isolated in sheep blood (29) or a novel EL cell population.

The migration of innate cells out of the lymph node appears to be dependent on the incoming immune stimuli because this effect was not observed for injection of liposomes incorporating the TLR9 agonist CpG (13). This selective migration of cells from that

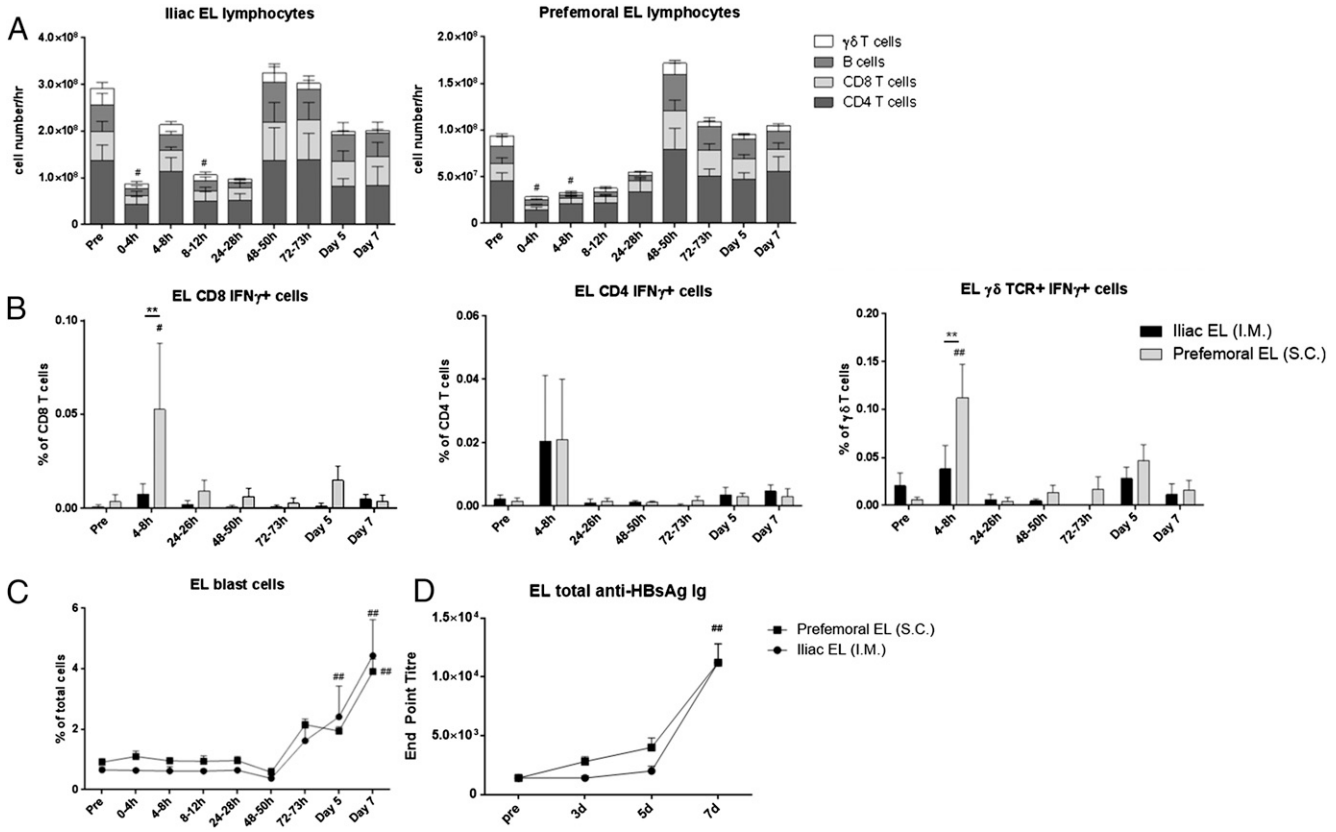

FIGURE 6. i.m. and s.c. injection of HBsAg-AS01 induces lymph node cell shutdown, increases the percentage of T cells producing IFN-γ, and induces HBsAg-specific Abs in muscle- (iliac) and skin-draining (prefemoral) EL. (**A**) The number of γδ T cells, B cells, CD8 T cells, and CD4 T cells in iliac and prefemoral EL per hour over the vaccination time course. (**B**) Percentage of CD8, CD4, and γδ T cells expressing intracellular IFN-γ in iliac (black bars) and prefemoral (gray bars) EL over the vaccination time course. (**C**) The percentage of blast cells in iliac (circle) and prefemoral (square) EL over the vaccination time course. (**D**) HBsAg-specific total Ig end-point Ab titers for iliac (circle) and prefemoral (square) EL over a selected vaccination time course. Data expressed as mean ± SEM of $n = 4$ in iliac EL and $n = 5$ in prefemoral EL. A repeated-measures two-way ANOVA with a Holm–Sidak multiple comparisons test was used to determine significance, where asterisks indicate differences between groups and number signs indicate differences from baseline (pre) within each group: $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

draining lymph node may enhance the ability of AS01 to amplify the Ag-specific immune response by transporting Ag to subsequent lymph nodes within the drainage chain. Further studies are required to investigate this hypothesis. Notably, however, at no point did we observe any side effects, including site reactions, fever, or pain, after AS01 administration in sheep. This suggests that the presence of innate inflammatory cells within the EL and peripheral blood does not appear to promote vaccine side effects.

We also observed that the number of neutrophils exiting the prefemoral lymph node after s.c. injection of AS01 was higher than the number leaving the iliac lymph node after i.m. injection, suggesting there was a higher level of inflammation within the prefemoral lymph node. The size of the skin-draining lymph node is significantly smaller than the muscle-draining lymph node, perhaps indicating that the release of neutrophils via EL is lymph node size dependent. Interestingly, the total numbers of neutrophils leaving the lymph node via the EL for the entire 24 h after injection were not sufficient to account for the increase in neutrophils observed in blood 24 h postvaccination with AS01, contributing only 0.06% of neutrophils in blood after i.m. injection. This suggests that AS01 induces the mobilization of neutrophils from the bone marrow or a resident reservoir (30), observed previously in nonhuman primates after injection of MPL (31).

In contrast with innate cell populations, a marked and significant reduction in the number of lymphocytes leaving both the muscle- and the skin-draining lymph node via EL was observed within the first 28 h of vaccination with AS01. This suggests there is some selective control of cells exiting the lymph node during inflammation. The reduction in the number of lymphocytes exiting the lymph node via EL has been observed previously under several inflammatory conditions and is a process referred to as lymph node cell shutdown (17, 32). It is hypothesized that lymph node shutdown occurs to extend the time for APC–T cell interactions and is likely dependent on the production of PGE_2 (33). The depletion or inactivation of neutrophils in mice increases the immune response within distal lymph nodes in a thromboxane A_2–dependent manner (34), consistent with neutrophils playing a key role in limiting the spread of immune cell trafficking by inducing local lymph node shutdown. In our study, lymph node shutdown occurred during the period of peak neutrophil migration within AL. Interestingly, during this period, a small percentage of T cells in EL were producing IFN-γ, most evident in CD8 and γδ T cells in prefemoral EL. This likely reflects an early lymphocyte response that has been observed previously in EL after injection with nanoparticles (35). It would be interesting to determine the contribution of these early, activated T cells to the Ag-specific response.

We report the development of a muscle lymphatic cannulation model to investigate in vivo immune responses to the human Adjuvant System AS01 in outbred sheep. This model quantified immune cell recruitment to the lymph node after vaccination in real time, demonstrating that $MHCII^{high}$ DCs, neutrophils, and monocytes can acquire Ag within AL. AS01 also induces a distinct and transient

cellular signature within the blood and draining lymphatics, which may be useful for the development of clinical biomarkers in target species. We show that AS01 induces the selective migration of Ag-positive neutrophils, monocytes, and a MHCII^{high} cell type out of the lymph node that may enhance immunity in subsequent lymph nodes. In outbred sheep, AS01 is an effective adjuvant with no apparent side effects and represents a promising candidate for inclusion in both human and veterinary vaccine formulations.

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Disclosures

A.M.D. and C.C. are employees of the GlaxoSmithKline group of companies. A.M.D. owns GlaxoSmithKline stocks. The other authors have no financial conflicts of interest.

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