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Immune-Stimulating Complexes Induce an IL-12-Dependent Cascade of Innate Immune Responses

Rosemary E. Smith,* Anne M. Donachie,* Dubravka Grdic,† Nils Lycke,† and Allan McI. Mowat*†

The development of subunit vaccines requires the use of adjuvants that act by stimulating components of the innate immune response. Immune-stimulating complexes (ISCOMS) containing the saponin adjuvant Quil A are potential vaccine vectors that induce a wide range of Ag-specific responses in vivo encompassing both humoral and CD4 and CD8 cell-mediated immune responses. ISCOMS are active by both parenteral and mucosal routes, but the basis for their adjuvant properties is unknown. Here we have investigated the ability of ISCOMS to recruit and activate innate immune responses as measured in peritoneal exudate cells. The i.p. injection of ISCOMS induced intense local inflammation, with early recruitment of neutrophils and mast cells followed by macrophages, dendritic cells, and lymphocytes. Many of the recruited cells had phenotypic evidence of activation and secreted a number of inflammatory mediators, including nitric oxide, reactive oxygen intermediates, IL-1, IL-6, IL-12, and IFN-γ. Of the factors that we investigated further only IL-12 appeared to be essential for the immunogenicity of ISCOMS, as IL-6- and inducible nitric oxide synthase knockout (KO) mice developed normal immune responses to OVA in ISCOMS, whereas these responses were markedly reduced in IL-12KO mice. The recruitment of peritoneal exudate cells following an injection of ISCOMS was impaired in IL-12KO mice, indicating a role for IL-12 in establishing the proinflammatory cascade. Thus, ISCOMS prime Ag-specific immune responses at least in part by activating IL-12-dependent aspects of the innate immune system. The Journal of Immunology, 1999, 162: 5536–5546.

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t is widely accepted that a single dose, orally administered, recombinant vaccine inducing long lasting mucosal and systemic immunity is an important goal of vaccine research (1). As recombinant proteins are poorly immunogenic, particularly for mucosal and MHC class I-restricted immune responses (2), their use in vaccines requires coadministration of an adjuvant (3). A variety of mucosally active adjuvants have been described, including live vectors such as mutant Salmonella (4) and nonviable agents such as cholera toxin (5) or the heat-labile enterotoxin of Escherichia coli (LT) (6). However, these often do not induce a full range of immune responses and can be associated with problems of toxicity or of being immunogenic themselves (7). Therefore, it would be beneficial to have a nonviable, nonimmunogenic vector that is capable of stimulating a full range of immune responses via a mucosal route.

We have used immune-stimulating complexes (ISCOMS) containing the saponin adjuvant Quil A for this purpose. ISCOMS are rigid, cage-like structures that form spontaneously in the presence of cholesterol, phosphatidylcholine, and Quil-A (8). We and others have shown that proteins incorporated in ISCOMS become highly immunogenic in vivo, inducing a wide range of immune effector responses, including delayed-type hypersensitivity (DTH), class I MHC-restricted CTL activity, serum Ab production, T cell proliferation, and secretion of both Th1- and Th2-dependent cytokines (2, 9–13). In addition, administration of ISCOMS by the oral route stimulates a full range of these systemic and immune responses as well as local secretory IgA- and cell-mediated immunity (14).

Little is known about the properties of ISCOMS that underlie their adjuvant effects. Most adjuvants appear to act by recruiting different components of the innate immune response, and previous studies have shown that neutrophil recruitment (15) as well as production of IL-1 from splenocytes cultured in vitro (16), serum IL-6 (17), and serum IL-12 (18), are all induced by ISCOMS. However, the evolution of these responses has not been studied in detail, and the effects of ISCOMS on other aspects of the innate immune system, such as macrophages, are not known. More significantly, it is not known whether activation of any of these innate responses is essential for the induction of a specific adaptive immune response. Here we detail the consequences of local administration of ISCOMS on the innate immune system and have examined these effects on the generation of Ag-specific immunity using gene-targeted knockout (KO) mice. Our results show that ISCOMS recruit a wide range of inflammatory cells and mediators and that IL-12 derived from adherent cells is of central importance for the immunogenicity of ISCOMS.

Materials and Methods

Mice

SPF female C57BL/6 mice were purchased from Harlan Olac (Bicester, U.K.) and female BALB/c mice were bred at the University of Glasgow (Glasgow, Scotland). The p40 IL-12-deficient (IL-12KO) BALB/c and C57BL/6 mice were obtained from Dr. J. Magram (Roche Pharmaceuticals, Piscataway, NJ). Inducible nitric oxide (NO) synthase deficient (iNOSKO) MF1 mice were a gift from Prof. F. Y. Liew, Department of Immunology.
University of Glasgow, Glasgow, Scotland. Female IL-6KO SV1 mice were obtained from Prof. J. Alexander (University of Strathclyde, Glasgow, Scotland). All these mice were maintained at the University of Glasgow except the IL-6KO mice, which were bred and maintained at the University of Strathclyde and were first used at 6–8 wk of age. Control mice were age-matched mice of the appropriate wild-type strain. Regular screening of the mouse strain showed these to be negative for all pathogens, including MHV.

Preparation and administration of OVA ISCOMS

ISCOMS containing palmitolized OVA were prepared as described previously (2) using phosphatidylcholine, cholesterol, and Quil-A (Sipikoside, a gift from Prof. B. Morein (Swedish University of Agricultural Sciences, Upsala, Sweden)). The integrity of the OVA ISCOMS was checked by electron microscopy and the protein content was assessed by Bradford staining (Bio-Rad, Hemel Hempstead, U.K.). The endotoxin content of ISCOMS was quantified using the E-Toxate Reagents Kit (Sigma) and was <0.5 µg/ml (equivalent to 2 ng/injection dose). The OVA ISCOMS used in this study were ~40 nm in diameter and contained protein and Quil-A at a ratio of 10:1. ISCOMS contained the equivalent of 0.5 µg of Quil A and 5 µg of protein and were administered either i.p. in a volume of 200 µl or s.c. in a volume of 50 µl. Control mice received saline alone, and peritoneal exudate cells (PEC) were removed 3.5 h later.

Isolation of PEC

PEC were obtained after sacrifice of mice by injection of 6 ml of prewarmed lavage medium consisting of RPMI 1640 (Life Technologies, Paisley, U.K.) containing 5% heat-inactivated FCS (Life Technologies). The abdomen was massaged gently, and the lavage fluid was aspirated with a 19-gauge needle. PEC were washed in RPMI, counted, and stored on ice until required. Individual cell counts were performed on PEC removed from three mice per group, but PEC phenotyping and culture were performed using pooled PEC from five animals.

Analysis of PEC by flow cytometry

Aliquots of 10⁶ PEC were washed twice in PBS containing 2% FCS (Life Technologies) and 0.02% sodium azide (FACS buffer) and stained for 45 min on ice in a total volume of 50 µl using PE-anti-CD4 Ab (PharMingen, San Diego, CA), FITC-anti-CD8 Ab (PharMingen), FITC-anti-CD25 Ab (PharMingen), biotinylated anti-B220 Ab (PharMingen), biotinylated anti-F4/80 Ab (Serotec, Kidlington, U.K.), FITC-anti-I-A<sup>ab</sup> Ab (PharMingen), PE-anti-CD11c Ab (PharMingen), FITC-anti-B7.1 Ab (PharMingen), FITC-anti-B7.2 Ab (PharMingen), and rat anti-mouse whole neutrophil Ab (Serotec). Second-step reagents were PE-rabbit anti-rat IgG (Serotec), PE-streptavidin (Vector, Oxford, U.K.), or FITC-streptavidin (Vector). Stained PEC were analyzed on a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Data from 10,000 cells were acquired and analyzed using LYSIS II software (Becton Dickinson).

Histological examination of PEC following cytospins

Cytospins were prepared from aliquots of 5 × 10⁵ PEC spun onto glass microscope slides at 200 rpm for 5 min using a cytocentrifuge (Shandon, Runcorn, U.K.). Cytospins were fixed in alcohol and stained with Giemsa (Sigma). The number of mast cells per field was counted at ×400 magnification using a Nikon Labophot microscope (Nikon, Melville, NY). Average mast cell numbers per field were calculated after counting 10 fields. The total mast cell number per mouse was calculated using the following formula: total mast cells = (average mast cells per field/PEC per field) × total PEC per mouse.

Measurement of inflammatory cytokine production in vitro

PEC (4 × 10⁶) were cultured in vitro in 24-well plates (Costar, Northumbria Biologicals, Cramlington, U.K.) in 1 ml of RPMI 1640 containing 10% heat-inactivated FCS, 100 µM penicillin, 100 µg/ml streptomycin, 50 µg/ml fungizone (all from Life Technologies), and 0.05 µM 2-ME (Sigma, Poole, U.K.) in the presence or the absence of 10 µg/ml LPS (Salmonella enteritidis, Sigma) plus 20 ng/ml recombinant murine IFN-γ (PharMingen) or with 10 µg/ml Con A. Supernatants were removed after 48 h of culture at 37°C in 5% CO<sub>2</sub> in air, centrifuged for 5 min at 13,000 × g, and frozen at −20°C until required. Cytokine levels in PEC culture supernatants were assayed by sandwich ELISA in 96-well Immunol 4 ELISA plates (Dynatech, Billingham, U.K.) using appropriate anti-cytokine Ab pairs for IL-6, IFN-γ (both from PharMingen), or IL-10 (Genzyme, Cambridge, MA). To measure immunoreactive p40 and p70 IL-12 levels, an mAb specific for the p40 subunit (C15.6, Genzyme) was used as the capture Ab, and a polyclonal rabbit anti-mouse IL-12 Ab raised against and specific for the mouse p70 heterodimer (a gift from Dr. F.-P. Huang, Department of Immunology, University of Glasgow) was used as the detecting Ab (19, 20). The concentration of cytokine present in supernatants was determined spectrophotometrically at 630 nm on an MR3000 microtiter plate reader (Dynatech) after incubation with the chromophore 3,3',5'-tetramethoxybenzidine (TMB) and H<sub>2</sub>O<sub>2</sub> in the Dynatech. Standards were assayed in triplicate, and the cytokine concentration was determined by extrapolation from a standard curve generated by serial dilution of the appropriate recombinant murine cytokine (PharMingen).

Measurement of NO and reactive oxygen intermediates

The concentration of NO present in PEC culture supernatants was determined by detection of nitrite, a stable metabolite of NO, by the Griess reaction. Briefly, the Griess reagent was made by mixing equal volumes of 5% orthophosphoric acid containing 1% sulfanilamide (Sigma) and 0.1% naphthylethylenediamine (Sigma) and was kept at 4°C until required. Triplicates of 50 µl of culture supernatant or a standard curve of serially diluted sodium nitrite were added to 96-well Immulon 4 microtiter plates (Dynatech), and 50 µl of the Griess reagent was then added. Any color generated was measured spectrophotometrically at 570 nm using a MR3000 microtiter plate reader (Dynatech). Reactive oxygen intermediates (ROI) released during the respiratory burst were measured by a chemiluminescence assay (21). Briefly, PEC were washed in HBSS (Life Technologies) without phenol red and warmed to 37°C in a water bath. One hundred microliters of 0.1 mM 5-amino-2,3-dihydro-1,4-phthalazinedione solution (Luminol; Sigma) was added to 400 µl of the warmed HBSS containing 2 × 10<sup>6</sup> PEC, and the cells were stimulated by addition of 1 µg/ml PMA (Sigma). The suspensions were placed at 37°C in the dark, and chemiluminescence was measured using a 1250 Luminometer (LKB Wallac, Turku, Finland) every 10 s for a total of 10 min. All measurements were expressed as millivolts.

Assessment of Ag-specific immune responses

OVA-specific immune responses were assessed in mice following s.c. immunization with OVA ISCOMS into a rear footpad or given i.p. as described previously (2). DTH responses were measured after s.c. challenge in the opposite rear footpad with 10 µg of heat-aggregated OVA 7 days after s.c. immunization. OVA-specific proliferative responses were assessed in parallel in the draining popliteal lymph nodes. Briefly, 2 × 10<sup>5</sup> splenocytes were cultured in 96-well microtiter plates (Costar) in the presence or the absence of 1 µg/ml OVA for 5 days at 37°C and 5% CO<sub>2</sub> in air. Cultures were pulsed with 1 µCi/well [³H]thymidine (West of Scotland Radiolucinatory Dispensary, Western Infirmary, Glasgow, U.K.) and harvested onto glass-filter fiber mats (Wallac) 16 h later, [³H]thymidine incorporation into DNA was measured using a Betaplate counter (Wallac). OVA-specific CTL activity was determined by the ability of spleen cells taken 7 days following i.p. immunization to lyse OVA-bearing EG7.OVA target cells as described previously (2). Briefly, effector spleen cells from immunized animals were restimulated in vitro with mitomycin C-treated EG7.OVA cells in culture medium containing 5% NCTC 135 (Life Technologies) and were cultured for 5 days at 37°C. After culture, the effector cells were washed thoroughly and incubated with [³C]-labeled EG7.OVA or non-OVA-expressing EL4 target cells at various E:T cell ratios in a volume of 200 µl of RPMI 1640 and 5% FCS in V-bottom microtiter plates (Costar) for 4 h at 37°C. One hundred microliters of supernatant was then removed and assayed for the presence of [³C]-OVA-specific CTL activity was calculated by the following formula:

\[
\% \text{cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100\%
\]

Spontaneous release was measured as the degree of [³C]-OVA released from unimmunized mice that had been restimulated in vitro with EG7.OVA cells, and total release was that obtained with 10% Triton X (Sigma).

Statistics

All results are expressed as the mean ± 1 SD and were compared using Student’s t test.

Results

Recruitment of inflammatory cells after local administration of ISCOMS

To study the ability of ISCOMS to stimulate the innate immune response, we examined the recruitment of inflammatory cells after i.p. injection of a dose of ISCOMS containing 5 µg of OVA and
0.5 µg of Quil A. We chose this approach because the peritoneum offers an anatomically defined site that is readily accessible and because our previous studies had used this protocol to induce Ag-specific immune responses in vivo. Within 3.5 h of administration of ISCOMS, there was a 50% decrease in the total number of PEC compared with that in saline-injected controls, and the numbers of PEC remained depressed until 48 h after ISCOMS injection, at which time there was a marked and significant increase in the number of PEC to levels 3 times those in control mice. The number of PEC was still significantly elevated by 72 h (Fig. 1a).

The nature of the inflammatory infiltrate was explored first by histological analysis of cytospins of PEC. PEC from control mice were constituted mostly of small resting lymphocytes and macrophages together with a small number of mast cells (Fig. 1b). The decrease in total PEC early after the injection of ISCOMS was accompanied by an increase in the number of polymorphonuclear leukocytes at 3.5 h (data not shown) and 7 h (Fig. 1c). Polymorphs were still present at 24 h (data not shown), but by 48 h polymorphonuclear cell numbers had returned to control levels, and there were now increased numbers of large mononuclear cells, many with the appearance of activated macrophages (Fig. 1d). In parallel, there were increased numbers of lymphoid cells, many of which were lymphoblastoid in appearance (Fig. 1d). One further aspect of the inflammatory infiltrates was an increase in the number of mast cells after the injection of ISCOMS. This was seen as early as 3.5 h, when a substantial number of these cells had degranulated (Fig. 2). A second rise in mast cell number was found at 48 h, although no degranulation had occurred at this time.

**Phenotypic analysis of PEC recruited by ISCOMS**

To analyze the inflammatory infiltrate in more detail, PEC were stained for expression of markers specific for neutrophils, macrophages (MΦ), dendritic cells (DC), and T and B lymphocytes. Consistent with the histological appearances described above, the decrease in PEC numbers early after injection of ISCOMS was accompanied by a large increase in the absolute numbers and proportions of neutrophils, which peaked at 3.5 h and declined rapidly thereafter (Fig. 3a). Only low numbers of MΦ, as identified by expression of the F4/80 marker, were found in control PEC, but their numbers began to increase by 7 h, reaching a peak at 48 h and then declining (Fig. 3a). Again this confirmed the microscopic analysis of cytospins. CD4+ lymphocytes were the predominant cell type in normal PEC, but their numbers declined rapidly over the first 7 h after ISCOMS injection before rising back toward control levels, reaching a peak at 48 h (Fig. 3b). The numbers of CD8+ T cells and B220+ B cells followed a similar pattern, with a rapid early decrease followed by recovery to levels that were eventually double the control values (Fig. 3, b and c). This peaked at 48 h for B cells and at 72 h for CD8+ T cells (Fig. 3b). The

**FIGURE 1.** ISCOMS recruit inflammatory cells following local injection. a, PEC recruitment following an i.p. injection of 5 µg of OVA ISCOMS. PEC were removed from three mice by lavage, and cell counts of PEC from the individual mice were performed. The results shown are mean PEC numbers for three mice ± 1 SD (*, p < 0.005 vs saline-injected mice). Cytospin appearances of 5 × 10^5 PEC were observed by microscopy at ×400 magnification after injection with saline (b) and 7 h (c) or 48 h (d) after i.p. ISCOMS. The presence of mast cells and small resting lymphocytes (b), polymorphonuclear cells and degranulating mast cells (c), and lymphoblastoid lymphocytes and activated macrophages (d) are indicated by arrows. The results shown are representative of three replicate experiments.
numbers increased dramatically from 7 until 48 h, when 25% of were virtually no class II cells (data not shown). By 7 h after the injection of ISCOMs, there come activated, as indicated by their increased expression of T and B lymphocytes. Once recruited, many of these cells be- in the numbers of B cells and MHC class II at this time. A rapid increase in MHC class II cells were found in control PEC, the majority of which were B cells (data not shown). By 7 h after the injection of ISCOMs, there were virtually no class II PEC, presumably reflecting the decline in the numbers of B cells and Mφ at this time. A rapid increase in MHC class II+ PEC then occurred, reaching a peak at 48 h (data not shown). This was paralleled by the reappearance of B cells and the de novo recruitment of MHC class II+ F4/80+ Mφ, whose numbers increased dramatically from 7 until 48 h, when 25% of Mφ were MHC class II+ (Fig. 3E). By 72 h few MHC class II+ Mφ remained. Very few CD25+ cells were found in control PEC, and their numbers decreased further immediately after injecting ISCOMS. At 48 h the numbers of CD25+ PEC were twice the control values (Fig. 3D), a pattern that was paralleled by the appearance of substantial numbers of CD25+expressing CD4+ T cells. At 48 h 26% of CD4+ T cells were CD25+ (Fig. 3F). Similar results were obtained for CD25 expression on CD8+ T cells, although the numbers were much smaller (data not shown). Thus, injection of ISCOMS induces a rapid, local recruitment of inflammatory cells, consisting initially of neutrophils and mast cells, followed by Mφ and DC, and later by both T and B lymphocytes. Once recruited, many of these cells become activated, as indicated by their increased expression of MHC class II and CD25.

Induction of local cytokine production by administration of ISCOMs

We next assessed the functional consequences of the cellular recruitment stimulated by an i.p. injection of ISCOMs. We chose to examine IL-1, IL-6, and IL-12 as products of activated Mφ and IFN-γ as a lymphocyte product. PEC from control mice showed some spontaneous production of IL-6, but no IL-1α and very low amounts of immunoreactive IL-12 (Fig. 4). The spontaneous production of IL-1α was significantly enhanced 48 h after ISCOMS injection, when PEC produced >20 times that of controls, and then fell to undetectable levels (Fig. 4A). The spontaneous production of IL-6 became undetectable early after the injection of ISCOMS, but increased rapidly after 24 h, reaching levels more than double the control values at 48 and 72 h (Fig. 4B). A similar pattern of ISCOMS-induced enhancement of IL-1α and IL-6 production was seen when PEC were stimulated with IFN-γ and LPS in vitro (data not shown). No TNF-α production was found in any PEC preparation, either spontaneously or after stimulation with IFN-γ and LPS (data not shown). Spontaneous IL-12 production rose gradually after 24 h after administration of ISCOMS, plateauing after IL-12 declined rapidly after injection of ISCOMS, but rose sharply to a large peak at 72 h, when levels were twice the control values (Fig. 4C).

There was no spontaneous production of IFN-γ from control PEC, but low levels were detected after stimulation with Con A (Fig. 4D). Significant levels of spontaneous IFN-γ production were detected 24 h after the injection of ISCOMS but not thereafter (data not shown). Con A-stimulated PEC showed a small, but significant, increase in IFN-γ production 3.5 h following injection with ISCOMS. This fell to the control level at 7 h, but increased again to a peak at 24 h, when IFN-γ production was twice that of control PEC (Fig. 4C). The production of all cytokines was dependent on the presence of Mφ, as removal of adherent cells from PEC cultures completely ablated IL-12, IL-1α, and IL-6 production (Table I) and significantly reduced IFN-γ production (data not shown). Thus, the local infiltration by Mφ and T cells with activated phenotypes is accompanied by the production of cytokines associated with these cells.

Stimulation of NO and ROI production by ISCOMS

In view of the marked recruitment of activated phagocytic cells such as Mφ and neutrophils, we next examined the production of two other characteristic mediators of such cells, NO and ROI. There was very little spontaneous NO production by control PEC, but NO production was stimulated by culture with IFN-γ and LPS (Fig. 5A). Immediately after injection with ISCOMS, IFN-γ-, and LPS-stimulated production of NO fell, but recovered from 7 h onward, reaching a peak at 48 h that was significantly above control levels (Fig. 5A). In addition, spontaneous production of NO became detectable 24 h after the injection of ISCOMS, and substantial amounts were still present at 48 h (Fig. 5A).

ROI production was measured by chemiluminescence after stimulation of PEC with PMA. Control PEC produced little or no ROI, but within 6 h of injection of ISCOMS, ROI production from PEC was greatly increased. PEC taken 24 h after ISCOMS injection produced even more ROI, with levels more than 20 times those in control PEC (Fig. 5B). Thereafter, there was a decline in ROI production, and by 72 h after injection of ISCOMS, ROI production had returned to control levels. Thus, the recruitment of
neutrophils and Mφ by ISCOMS is accompanied by increased production of ROI and NO, respectively.

Role of ISCOMS-induced inflammatory mediators in Ag-specific immunity

The experiments described above show that ISCOMS recruit a wide range of inflammatory cells and mediators to the site of injection. We therefore went on to explore whether any of these played an essential role in the induction of Ag-specific T cell responses by ISCOMS-associated Ag. To do this we immunized appropriate gene-targeted KO mice using immunization regimens that we had previously shown to be optimal (11). Systemic DTH and OVA-specific CTL activity were used as measures of CD4+ and CD8+ T cell function in vivo, respectively, while Ag-specific proliferation was used to determine immune responsiveness in vitro.

IL-6KO mice (Fig. 6) and iNOSKO mice (Fig. 7) generated normal immune responses after immunization with OVA ISCOMS, with DTH responses (Figs. 6A and 7A), OVA-specific proliferation (Figs. 6B and 7B), and CTL activity (Fig. 6C) identical with those in appropriate wild-type control animals. However,
p40 IL-12KO mice had impaired induction of Ag-specific immune responses both in vivo and in vitro (Fig. 8). These mice had a dramatic reduction in their DTH responses after challenge with Ag, with virtually no response above the background (Fig. 8A). In addition, both Ag-specific proliferative responses (Fig. 8B) and OVA-specific CTL activity (Fig. 8C) were significantly reduced compared with those in wild-type mice, with responses on the order of 50–60% those of normal animals. Therefore, we propose that IL-6 and inducible NO play no essential role in the generation of specific immunity induced by ISCOMS, but that IL-12 plays a critical role in this phenomenon.

Absence of innate responses in IL-12KO mice

To investigate the relationship between the decreased specific immunity in IL-12KO mice and the innate immune response, we assessed the ability of an i.p. injection of ISCOMS to stimulate peritoneal infiltration in these animals.

As we found previously, i.p. injection of ISCOMS into normal B6 mice lead to recruitment of PEC, with a peak in PEC numbers occurring at 48 h and returning to control levels by 72 h after injection (Fig. 9). In contrast to the decrease in PEC numbers that occurred at early times in wild-type mice, there was a small increase in the numbers of PEC in ISCOMS-injected IL-12KO mice at 3.5 h after injection. However, PEC numbers then decreased and remained around those seen in control mice at all subsequent time points (Fig. 9). In addition, PEC from IL-12KO mice did not show the increase in production of inflammatory mediators observed in wild-type mice, with IL-1α, IL-6, IFN-γ, and NO levels all remaining at control levels throughout the experiment (data not shown.)

We conclude that immunization with OVA ISCOMS induces Ag-specific immunity through the establishment of an IL-12-dependent innate response.

Discussion

The results presented here indicate that ISCOMS induce intense local activation of the innate immune response, recruiting a wide variety of inflammatory cells, including neutrophils, mast cells, DC, Mφ, and lymphocytes. Many of these cells are activated, as evidenced by the expression of surface activation markers and the
production of cytokines and other mediators. However, the majority of these factors were not essential for the immunogenicity of ISCOMS in vivo, with only the production of immunoreactive IL-12 appearing to be of major importance.

The local inflammatory response induced by ISCOMS consisted of an initial emigration of neutrophils, followed by Mφ and DC, and eventually lymphoid cells. Neutrophils are not normally present in the peritoneum, but following the administration of ISCOMS, a large increase in the numbers of polymorphonuclear cells in PEC was observed histologically, and phenotypic studies confirmed that these were mostly neutrophils. Our current studies extend previous observations that neutrophils are recruited to a site of immunization with ISCOMS (15, 22) by showing that these cells are probably activated in situ. This was shown by the fact that

FIGURE 5. ISCOMS induce local production of NO and ROI. A. Production of NO from PEC pooled from five mice following the injection of 5 μg of ISCOMS or saline. The results shown are the mean concentration of nitrite measured in triplicate after culture for 48 h in the presence or the absence of IFN-γ and LPS ± 1 SD (*, p < 0.01 vs saline-injected mice). B. Production of ROI from PEC pooled from five animals after the injection of 5 μg of ISCOMS or saline. The results show the release of ROI from PEC as measured by the PMA-stimulated chemiluminescence of Luminol in millivolts. The results are representative of two experiments.

FIGURE 6. OVA ISCOMS induce Ag-specific immune responses in IL-6KO mice. OVA-specific immune responses in IL-6KO mice following a single parenteral immunization of 5 μg of OVA ISCOMS. A. OVA-specific DTH responses measured 7 days after immunization. The results shown are the mean increments in footpad thickness ± 1 SD for five mice per group. B. OVA-specific proliferative responses in vitro assessed in spleens 14 days after immunization. The results shown are the mean counts per minute of quadruplicate cultures of cells pooled from five spleens stimulated for 5 days with 1 mg/ml OVA ± 1 SD. Background proliferations in medium alone were ~4000 cpm for all groups. C. OVA-specific CTL activity assessed 7 days after immunization. The results shown are the percent specific lysis of 51Cr-labeled EG7.OVA cells measured in spleen cells pooled from five mice per group after restimulation with EG7.OVA cells in vitro. There was no lysis of EG7.OVA cells by nonimmune cells or of non-OVA-expressing EL4 cells. Naive animals were wild-type mice that were immunized with saline alone.
Background proliferation in the presence of medium alone was from five spleens restimulated in vitro for 5 days with 1 mg/ml OVA showing mean counts per minute of quadruplicate cultures of cells pooled moved from immunized animals 14 days after immunization. The results show the mean increment in footpad thickness DTH responses measured 7 days after immunization. The results shown are 1 SD for five mice per group. OVA-specific immune responses in iNOSKO mice following a single sc. immunization of 5 μg of OVA ISCOMS. A. OVA-specific DTH responses measured 7 days after immunization. The results shown are the mean increment in footpad thickness ± 1 SD for five mice per group. B. OVA-specific proliferative responses in vitro assessed in spleens removed from immunized animals 14 days after immunization. The results show mean counts per minute of quadruplicate cultures of cells pooled from five spleens restimulated in vitro for 5 days with 1 mg/ml OVA ± 1 SD. Background proliferation in the presence of medium alone was ~3000 cpm for all groups. Naive animals were wild-type mice that received saline alone. The results are representative of three repeat experiments.

FIGURE 7. OVA ISCOMS induce OVA-specific immune responses in iNOSKO mice. OVA-specific immune responses in iNOSKO mice following a single sc. immunization of 5 μg of OVA ISCOMS. A. OVA-specific DTH responses measured 7 days after immunization. The results shown are the mean increment in footpad thickness ± 1 SD for five mice per group. B. OVA-specific proliferative responses in vitro assessed in spleens removed from immunized animals 14 days after immunization. The results show mean counts per minute of quadruplicate cultures of cells pooled from five spleens restimulated in vitro for 5 days with 1 mg/ml OVA ± 1 SD. Background proliferation in the presence of medium alone was ~3000 cpm for all groups. Naive animals were wild-type mice that received saline alone. The results are representative of three repeat experiments.

by ISCOMS are also unknown, but the rapidity of the response in naive animals clearly precludes a role for Ag-specific IgE-mediated effects.

Mφ and DC were next to appear in the recruited PEC, with many of the new Mφ being activated, as judged by their morphological appearance and their expression of MHC class II Ags. In addition, there was increased production of a number of inflammatory mediators characteristic of activated Mφ, including IL-1, IL-6, immunoreactive IL-12, and NO. The recruited DC were also class II MHC+, confirming their maturity, but their activation status remains to be determined. Around the time these potential APCs appeared in PEC, there was infiltration by B and T cells of both CD4+ and CD8+ subsets. Substantial numbers of the T cells were activated, as shown by their expression of the IL-2Rα molecule, and the recruitment of CD8+ T cells in response to ISCOMS is especially interesting in the light of the unusual ability of ISCOMS to prime MHC class I-restricted Ag-specific CTL in vivo (2, 11–14). Together, these results show that ISCOMS recruit many components of the innate and adaptive immune responses, including several populations of potential APC, such as DC, B cells, and activated Mφ. In preliminary studies we have found that phagocytic Mφ are important for induction of class I MHC-restricted CTL responses by ISCOMS (25), a finding consistent with recent evidence that the phagocytic uptake of Ag may be selective for the class I processing pathway (26). In addition, DC have recently been associated with class I MHC-restricted presentation of Ag, particularly after ingestion of apoptosed Mφ (27). It will be interesting to determine the interaction among all these cell types in vivo. The production of cytokines and other inflammatory mediators by inflammatory cells is now recognized as being critical for the induction of an effective primary immune response, and the presence of these mediators can determine the type of Ag-specific effector response that is generated (28). ISCOMS stimulated the production of a wide range of inflammatory mediators, including IL-1, IL-6, IL-12, and IFN-γ. The appearance of the proinflammatory cytokines IL-1 and IL-6 is consistent with the acute inflammatory reaction provoked by ISCOMS and confirms previous reports of stimulation of both IL-1 and IL-6 production by ISCOMS (16, 17). However, previous studies were unable to detect IL-1 production in vivo (29), and the IL-6 production demonstrated in vivo was not examined at the cellular level (17). Both these cytokines are important components of the innate immune response and are often found together with TNF-α during the early stages of inflammatory reactions to, for example, bacterial infection (30). However, as reported by others (17), we have been unable to detect the presence of TNF-α in supernatants of ISCOMS-stimulated PEC. This is somewhat surprising given the high levels of the other mediators, and we are currently examining TNF-α production at even earlier time points using more sensitive molecular techniques.

IL-6KO and iNOSKO mice had normal T cell-mediated immune responses to OVA ISCOMS despite the known role of these mediators in other models of immunity (31–33) and the increased levels of their production after injection of ISCOMS. However, IL-12 appeared to be critically important for the adjuvant effects of ISCOMS. The production of IL-12 by ISCOMS-recruited PEC was greatly enhanced, extending recent findings of elevated serum IL-12 levels after the injection of ISCOMS (18). In addition, Ag-specific immune responses to ISCOMS were greatly reduced in IL-12KO mice, and there was no recruitment of PEC or induced secretion of inflammatory mediators by PEC from these mice. This complete lack of cellular recruitment indicates a critical role for
IL-12 in initiating the inflammatory cascade, perhaps reflecting an absence of chemotactic factors such as IL-8, whose production may be dependent on IL-12 (34). The defective DTH and CTL responses to ISCOMs in IL-12KO mice were not particularly surprising in view of previous studies using these animals and of the known effects of IL-12 on the production of IFN-γ (35) and differentiation of CTL (36). Ag-specific proliferative responses were also reduced in ISCOMs-immunized IL-12KO mice, but as IL-12 is known to regulate the expression of the IL-2Rα and β subunits (37, 38), and we have found that IL-12KO mice do not develop CD25+CD4+ lymphocytes following administration of ISCOMs (data not shown), the reduced Ag-specific proliferation in IL-12KO mice could simply be due in part to IL-2 unresponsiveness in OVA-specific CD4+ lymphocytes. More recent work indicates that most other aspects of mucosal and systemic immunity are also defective in these animals (D.G., et al., manuscript in preparation). These findings extend recent studies showing diminished Ag-specific serum IgG Ab responses following administration of neutralizing anti-IL-12 p70 Ab (18), indicating that IL-12 plays a more generalized role in the adjuvant effects of ISCOMs. The basis of this remains to be determined, but it does not appear to reflect its role in directing IFN-γ production, as we have shown previously that IFN-γ receptor KO mice respond normally to immunization with OVA ISCOMs (39). Although this seems paradoxical in view of our findings that IFN-γ production by PEC is increased by ISCOMs, it is consistent with other systems in which undefined IFN-γ-independent effects of IL-12 have been described (40). The source of ISCOMs-induced IL-12 remains to be determined, but its production required the presence of adherent cells and correlated with the recruitment of Mφ and DC, cell types that both can produce this cytokine (35). It would be of interest to determine whether this is a direct effect of ISCOMs on the relevant accessory

**FIGURE 8.** Reduced immune responses to OVA ISCOMs in p40 IL-12KO mice. OVA-specific immune responses in IL-12KO mice following a single parenteral immunization with 5 μg of OVA ISCOMs. A, OVA-specific DTH responses measured 7 days after immunization. The results shown are the mean OVA-specific footpad increment after challenge for five mice per group ± 1 SD (*, p < 0.05 vs wild-type animals). B, OVA-specific proliferative responses in BALB/c mice assessed in spleens 14 days after immunization. The results shown are mean counts per minute of quadruplicate cultures of cells pooled from five spleens stimulated in vitro with 1 mg/ml OVA for 5 days ± 1 SD (*, p < 0.05 vs wild-type animals). Background proliferation from medium alone cultures was ~3500 cpm for all groups. C, CTL activity in B6 mice was assessed 7 days after immunization. The results shown are the percent lysis of 51 Cr-labeled EG7.OVA cells measured in spleen cells pooled from five mice per group after restimulation with EG7.OVA cells in vitro. There was no lysis of EG7.OVA cells by nonimmune cells or of non-OVA-expressing EL4 cells. Naive animals were wild-type mice that were immunized with saline alone. The results shown here are representative of three experiments.

**FIGURE 9.** Absence of PEC recruitment in ISCOMs-injected IL-12KO mice. PEC recruitment following an i.p. injection of 5 μg of OVA ISCOMs into wild-type IL-12KO B6 mice. PEC were removed from five wild-type mice and five p40 IL-12KO mice by lavage and pooled, and cell counts of PEC were performed. The results shown are mean PEC numbers for samples pooled form five mice per group. Control animals were immunized with saline. The data are representative of three replicate experiments.
cells or if it is secondary to CD40-CD40 ligand interactions known to be important for the induction of IL-12 in other systems. It should be noted that ISCOMs adjuvant shows a selective association with and dependence on IL-12 production. It is generally accepted that administration of Ag alone in vivo does not stimulate IL-12 production, while cholera toxin, an agent with mucosal and systemic adjuvant properties similar to those of ISCOMs, does not induce IL-12 readily, and immune responses to OVA and cholera toxin are entirely normal in IL-12KO mice (D.G. et al., manuscript in preparation). The unusual propensity for ISCOMs to stimulate IL-12-dependent immune responses is not limited to predictable responses such as IFN-γ production and CTL activity, as our current studies indicate a more global defect in Ab and cytokine production in IL-12KO mice (unpublished observations). The basis for these remains to be determined. Interestingly, the peak of IL-12 production was proceeded by an early spike in IFN-γ levels, supporting other recent evidence that IFN-γ may be required for initiation of significant levels of IL-12 production (41). Nevertheless, it is important to note that immune responses were only partially reduced in IL-12KO mice. The innate responses measured in these mice were absent, potentially indicating a role for additional factors in generating the residual immunity. Whether this reflects an ability of some of the factors investigated here to compensate for the absence of IL-12 or a role for novel factors such as IL-18 (42, 43) remains to be determined.

The ways in which ISCOMs can stimulate the innate immune system are currently being explored, but we consider that this reflects delivery of the adjuvant effects of the adjuvant Quil A to proinflammatory cells in the form of a lipopholic particle. Free Quil A reproduces patterns of specific and nonspecific effects similar to those found with intact ISCOMs, including the induction of mucosal and systemic responses as well as the recruitment and activation of local inflammatory cells (44). However, we and others have shown that 10- to 20-fold more free Quil A is required to induce equivalent responses to those found using Quil A in ISCOMs (45, 46). Thus, we propose that the particulate nature of ISCOMs targets Quil efficiently to cells of the immune system. The chemical basis of these effects of Quil A and ISCOMs is unclear, but should now be examined using individual fractions of Quil A that differ in their ability to stimulate inflammation and CTL activity, as our current studies indicate a more global defect in Ab and cytokine production in IL-12KO mice (unpublished observations). The basis for these remains to be determined. Interestingly, the peak of IL-12 production was proceeded by an early spike in IFN-γ levels, supporting other recent evidence that IFN-γ may be required for initiation of significant levels of IL-12 production (41). Nevertheless, it is important to note that immune responses were only partially reduced in IL-12KO mice. The innate responses measured in these mice were absent, potentially indicating a role for additional factors in generating the residual immunity. Whether this reflects an ability of some of the factors investigated here to compensate for the absence of IL-12 or a role for novel factors such as IL-18 (42, 43) remains to be determined.

In conclusion, our results show that the ability of ISCOMs to induce a wide range of Ag-specific immune responses is paralleled by the activation of a cascade of innate immune responses. This is consistent with other evidence that the best adjuvants are those that mimic the ability of pathogens to activate the innate immune system. However, our study also reveals the complexity of the resulting nonspecific signals that are generated, with many overlapping and redundant mechanisms employed, only a few of which may play an essential role in the development of an Ag-specific immune response. Elucidating and targeting these mechanisms will be important in the design of a successful vaccine.

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References


