Drug-Inducible, Dendritic Cell-Based Genetic Immunization

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Drug-Inducible, Dendritic Cell-Based Genetic Immunization

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Determining the mechanism of Ag loading of Langerhans cells (LC) for genetic immunization (GI) is complicated by the inability to distinguish between the response generated by direct transfection of LC from that due to exogenous uptake. To unravel this mechanism, we examined the impact of gene gun treatment on LC with respect to their activation and migration from skin, transgene expression, and ability to initiate humoral and cellular immune responses upon transfer to naive mice. To assess responses generated by direct LC transfection, an RU486-inducible expression system was used as a GI vector. In vitro skin organ cultures were developed from gene gun immunized mouse ear specimens to obtain LC. Gene gun treatment markedly augmented (3-fold) LC migration from ear skin, and these LC expressed the transgene at RNA and protein levels. Transfer of 2 x 10⁶ migratory cells resulted in identical cellular responses to, but 10-fold lower humoral responses than, standard GI. Using an RU486-inducible system, we were able to measure responses generated by directly transfected LC. Our results indicate that direct transfection is a predominant pathway for LC Ag loading. The ability to regulate transgene expression with inducible DC-based vaccines demonstrates a new level of immunological control. The Journal of Immunology, 2003, 170: 5483–5490.

Genetic immunization (GI) is a new form of immunization that occurs upon the introduction of plasmid DNA, encoding foreign Ag genes, into target tissues of the skin or muscle (1–5). The expression of these foreign proteins in cells of the recipient elicits potent humoral and cellular immune responses, with the latter characteristic being a hallmark advantage of GI over protein subunit vaccines. By demonstrating that bone marrow-derived cells are responsible for generating GI-mediated immune responses, it is thought that dendritic cells (DC) are key mediators (6, 7). The skin as a target tissue for GI has multiple advantages over muscle, the other target tissue, with one obviously being its accessibility. More importantly, because it functions as a first line defense against the environment, the skin is a specialized immune organ containing high densities of skin-specific DCs, termed Langerhans cells (LC). LC are immature dendritic cells making up 1–3% of the cells in the skin epidermis (8). These specialized cells constantly monitor the surrounding milieu by sensing inflammatory cytokines elaborated by neighboring keratinocytes and through active macro and pinocytotic uptake of substances in the environment. LC become activated upon uptake of exogenous foreign Ags in particulate forms or by cross-priming, in which apoptotic/necrotic cell debris is taken-up for presentation of cell-associated Ags. Activation of LC by this process results in their migration from the epidermis to the dermis and ultimately to draining lymph nodes where Ag presentation takes place (9). Although the vast majority of cells that are transfected by gene gun are non-APCs, such as keratinocytes or fibroblasts (or myotubes when muscle is targeted), transgene expression has been confirmed in significant, albeit relatively small, numbers of LC (10, 11). Therefore, LC may obtain transgene-encoded Ag via two pathways: 1) through exogenous uptake of Ag secreted by or contained in cell bodies derived from neighboring transfected cells or 2) through direct in situ transfection of LC in which transgene expression acts as an endogenous Ag source. Thus, the relevant Ag-loading pathway(s) for effective Ag presentation remains unclear.

To address the mechanism of LC activation and Ag loading by GI, we characterized the impact of gene-gun treatment on LC function. First, LC migratory capacity from skin and LC activation state were examined. Second, we examined transgene expression in migratory LC and their potential to initiate Ag-specific responses in recipient animals. Third, the relative contribution of direct vs indirect Ag loading was investigated through the use of an inducible vector system that permits exogenous control of transgene expression.

Materials and Methods

Animals and cell lines

Specific pathogen-free, age-matched (4–8 wk), female A/J mice were obtained from Harlan Laboratories (Indianapolis, IN) and maintained under specific pathogen-free conditions at University of Texas Southwestern Medical Center (Dallas, TX). Both the XS106 LC line and NF46 fibroblasts were established from the epidermis of newborn A/J mice and maintained in vitro, as described previously (12–14).

Gene gun immunization

Standard GI vectors used in this study have been described previously (12). The standard vectors pCMVi-Luc and pCMVi-AAT encode the reporter gene luciferase (Luc) and the model secreted Ag human α-1-antitrypsin (AAT), respectively (1, 12). The pTracer-GFP reporter vector, used for visualizing transfected LC, used the SV40 promoter to drive green fluorescent protein (GFP) expression (Clontech Laboratories, Palo Alto, CA). Plasmid DNA, purified with Qiagen Endo-free columns (Valencia, CA), was precipitated onto gold particles (2 ± 1 μm diameter; Metz Metallurgical, South Plainfield, NJ) at 4 μg/mg. A biologic gene gun (Rumsey-Loomis, Ithaca, NY) was used to deliver a total of 1 μg of DNA at the pressure of 1200 pounds per square inch into the skin of mouse ears and into cell lines (15, 16). In some experiments, the Helios gene gun (Bio-Rad,
Hercules, CA) was compared with the Rumsey-Loomis model. Helios bullet preparation and delivery at 900 pounds per square inch was done following the manufacturer’s instructions. XS106 cells were transfected by gene gun as previously described (12). The level of gene expression achieved by gene gun delivery of pCMV-Luc DNA was determined by assaying for luciferase (Luc) activity 24 h after bombardment, as described previously (12).

Phenotypic analysis

Migratory cells (5 × 10³ cells per sample) were washed and incubated in 10 μl with a mixture of Fc-Block (anti-CD16/CD32, clone 2.4G2; BD Pharmingen, San Diego, CA) and 10 μg of rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Cells were stained with a mixture of PE-conjugated anti-Ia (clone 2G9; BD Pharmingen) and PE-conjugated anti-CD86 (clone GL1; BD Pharmingen) or PE- and FITC-rat IgG2a isotype control per manufacturer’s instructions. In some experiments, cells were additionally stained with combinations of FITC-anti CD3 (clone 17A2), PE or allophycocyanin-anti-CD11c (clone HL3), FITC-anti-CD11b (clone M1/70), FITC or PE-anti-CD40 (clone 3/23) which were all purchased from BD Pharmingen. PE-anti-Dec205 (clone NLDC-145) was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). FITC-anti-γδTCR (clone GL3) was a generous gift from Dr. H. Xu (Department of Dermatology, University of Alabama, Birmingham, AL). Viable cells were by gated by forward angle and side scatter characteristics and analyzed using a FACSscan flow cytometer and CellQuest Software (BD Immunocytometry Systems, San Jose, CA).

Migratory LC from gene gun-transfected skin

The dorsal and ventral sides of mouse ears were each subjected to two gene gun shots, delivering a total of 1 μg of DNA per side and harvested 1 h after bombardment. Harvested ear specimens were pulled apart with forceps and split into dorsal and ventral halves placed on droplets of sterile PBS dermal side down. Using the rounded end of curved forceps, collage was gently scraped from the bottom of the ventral half to enhance the passage of migrating LC through the epidermal and dermal layers into surrounding medium. Prepared ear halves were floated dermal side down and cultured on complete RPMI 1640 medium (containing 10% FBS) for 4 h. Following the manufacturer’s instructions, XS106 cells were transfected by gene gun as previously described (12). The level of gene expression achieved by gene gun delivery of pCMV-Luc DNA was determined by ELISA and cytotoxicity assays. As a positive control, we inoculated mice by standard GI. To serve as a parallel negative control group, some of the GI-treated mice were subjected to ear removal 1 h after gene gun treatment (van Gogh experiments).

ELISA was performed on sera obtained at 3 wk, as described previously (12). Serial dilutions of human AAT-specific mouse mAb (Calbiochem, La Jolla, CA) provided a standard curve for determining the reported microgram per milliliter values.

To test for the generation of AAT-specific CTLs, mice were boosted at 3 wk with pCMV-Luc by standard GI and their spleens were harvested after 8 days. Spleen cells from individual mice were cultured for 5 days in the presence of 30 μg/ml heat-denatured AAT protein and 5% rat Con A-stimulated spleen cell supernatant as a source of IL-2 (T-Stim; Collaborative Biomedical Products, Bedford, MA). Viable cells were harvested from these cultures by centrifugation, washed twice, and examined for their cytotoxic activity. For these assays, we used NS46 fibroblasts, derived from A/J mice, pulsed with or without heat-denatured AAT protein at 1 mg/ml (18) and labeled 1 h in 51Cr solution. Washed target cells, at 5 × 10³ per well, were incubated for 4 h at 37°C with effector cells in triplicate. Maximum and spontaneous releases were determined from wells containing 2% Triton X-100 or medium alone, respectively. Specific lysis was calculated as [(experimental 51Cr-release - spontaneous 51Cr-release)/(maximum 51Cr-release - spontaneous 51Cr-release)] × 100%.

Inducible genetic vector system

A mifepristone (RU486)-inducible plasmid system (GeneSwich Systems) was a generous gift from J. L. Nordstrom (Valentis, The Woodlands, TX). The parent two-plasmid system originally described by Wang et al. (19), was modified by Valentis and ourselves for better control of gene expression in transferred LC. The effecter plasmid, GS1158 (referred to in this report as pCMV-Gal4), expresses a chimeric RU486-regulated transactivator through a CMV promoter. The regulated expression plasmids, p4U-AAT or p4U-Luc, express AAT or Luc, respectively, in the presence of RU486. The chimeric transcription activator is a three-domain fusion protein consisting of an N-terminal DNA binding domain of yeast Gal4, a RU486 ligand-binding domain from the human progesterone receptor, and a transcription activation domain from human Rel (p65) NF-κB (20). This chimeric molecule binds to the yeast-specific Gal4 upstream activation sequence elements on the regulated plasmid and in the presence of RU486 activates expression of AAT or Luc gene expression. Gene gun inoculation of 2.0 μg of DNA, containing both plasmids pCMV-Gal4 and p4U-AAT or p4U-Luc at a ratio of 1:10, was administered to dorsal and ventral sides of the ear. Migratory LC were harvested as described previously. For maximal gene induction in vivo, RU486 was given i.p in 100 μl of saline (dilution vehicle) at 0.5 mg/kg body weight. Mock-treated animals received vehicle alone. For in vivo induction, recipient mice received three doses of RU486 per week over 3 wk, with the first dose given 1 h before LC transfer.

Statistical analysis

A one-tailed Student’s t test was applied and the p values are indicated in the text and figure legends.
Results

Impact of gene gun treatment on LC migration

To determine whether gene gun treatment would enhance the migration of LC, we studied the effect of biolistic gene gun bombardment on LC migration. Gene gun-treated or untreated ear skin samples were cultured over 2 days, and the numbers of LC that remained in the epidermis were counted after immunofluorescence staining with anti-Ia mAb. We observed a dramatic reduction of LC density as a result of gene gun treatment (Fig. 1A). The few remaining LC were larger in size than those in untreated epidermal sheets and stained with higher intensity with anti-Ia Abs, indicating an activated phenotype. In the absence of bombardment we observed 30% reduction in LC surface density during culture, which is in agreement with reports by others (17) (Fig. 1B). Surprisingly, LC densities dropped by 86–92% in skin treated by gene gun, either our standard gene gun (Rumsey-Loomis) or the Helios, as compared with the levels found in fresh skin. This reduction in LC density from gene gun-treated ears was also seen when animals were sacrificed after 24 h and then examined (data not shown), indicating that the in vitro culture closely mimics in vivo conditions. These results indicate that the introduction of gold into skin tissue plays an important role in eliciting the local LC migration effect. These data are consistent with two possible interpretations, either LC at the GI site migrated out of the epidermis or they died due to gene gun-mediated events. To distinguish between these two possibilities, we enumerated the number of Ia-positive cells that migrated out of skin into the culture medium. We observed that concomitant with gene gun-mediated LC disappearance from the epidermis, 3-fold higher numbers of Ia-positive migratory cells were recovered from the medium as compared with untreated skin cultures (Fig. 2A).

The migratory cell population was analyzed by flow cytometry for leukocyte and DC markers, as indicated in Table I. Both small and large cell subsets were identified by light scatter properties. The small subset of cells comprised 45% of the migratory population and was double-positive for both CD3, as shown in Table I, and γδ TCR, but was negative for B220 and Ia molecules (data not shown). The remainder of cells were large and coexpressed CD11c, CD11b, Dec205, CD40, and Ia molecules. In addition, 14–27% of total cells were large CD11b-positive but Ia-negative cells, suggesting that they represent a subset of inflammatory granulocytes. The maturation state of migratory LC was examined by measuring the expression levels of DC-specific activation markers CD86 and CD40. Interestingly, while migratory LC from control skin cultures exhibited uniformly high levels of Ia and CD86 on their surface, a large proportion of the migratory LC from gene gun-treated skin expressed low levels of CD86 (Fig. 2B) but higher levels of DC-specific markers and activation marker CD40 (Table I).

FIGURE 1. Impact of gene gun treatment on epidermal LC density. A. Photomicrographs of Ia-positive cells in untreated and gene gun-treated epidermal sheets obtained from skin organ cultures after 2-day in vitro culture. Ia-positive cells are visualized by FITC-conjugated anti-Ia mAb (×200 magnification). B. Enumeration of LC remaining in epidermis after 2 days of in vitro skin organ culture. Three nonoverlapping fields per sheet obtained from three animals per group were evaluated. The number of LC per group of either freshly obtained epidermal sheets or epidermal sheets obtained from 2-day cultures of mock or gene gun-treated skin is shown. Mock treatment consisted of subjecting ears to gene gun treatment without gold before culture. Mean and SEM values are shown. *, p < 0.001; **, p < 0.0001.

FIGURE 2. Enhanced LC migration from gene gun-treated skin. A. Untreated, gene gun (Rumsey-Loomis) or Helios (Bio-Rad)-treated ear specimens were cultured for 2 days. Cells in the culture medium were harvested, counted, and stained with FITC-anti-Ia mAb. The percent of Ia+ cells, obtained by flow cytometry, was applied to the total cell count to determine the number of Ia+ cells per culture. Triplicate cultures were counted and analyzed by flow cytometry for each group. The mean and corresponding SD is shown. *, p < 0.05; **, p < 0.02. B. Two-color flow cytometric analysis. Cells were double-stained for Ia (FITC-anti-IA<sup>β</sup>/IE<sup>β</sup>) and CD86 (PE-anti-CD86) or with PE- and F-Rat IgG2a isotype control. The data shown are representative of 13 independent experiments.
Collectively these data indicate that LC disappearance from the epidermis is not due to significant levels of cell death, but is due to their activation and enhanced migration out from the epidermis. Further, the identification of a subset of Ia-positive cells that are low in CD86 suggest that the ability to migrate from the epidermis can precede a fully activated phenotype, or they may represent a distinct subset of cells that are also activated by gene gun treatment derived from the dermis such as dermal DC or macrophages.

Transgene expression in migratory cells

To determine whether any migratory cells were transduced by gene gun, ear skin specimens inoculated with a GFP reporter plasmid were cultured over 2 days and migratory cells were examined for GFP expression. A faint green fluorescent signal was detected in cells that had migrated from skin inoculated with the GFP reporter plasmid but not in cells from control skin inoculated with the Luc-encoding plasmid (Fig. 3A). The GFP-positive cells were large and demonstrated dendritic or veiled LC morphology. Further, the GFP-positive cells contained a gold bead in the cytoplasm consistent with the hypothesis that these cells were directly transfected.

To formally exclude the possibility that GFP-positive cells simply incorporated the transgene product derived from other nonmigratory cells, expression of transgene mRNA in migratory cells was examined by RT-PCR. The primers used for AAT sequence amplification were designed to amplify only plasmid-derived, processed mRNA. In addition, we tested without RT treatment, and the AAT-specific PCR product was not detected, indicating that DNA did not contaminate the RNA preparations or, if present, could not serve as a template (Fig. 3B). The same observation was made for RNA preparations from a gene gun-treated LC line, XS106. With RT treatment, a 590-bp AAT-specific PCR product was detected in both LC and control XS106 cell populations. Collectively, these results demonstrate that at least some of the migratory cells express the transgene. In our cultures, small cells, which were identified as T cells by flow cytometry (Table I), were not observed to express GFP, but we cannot exclude that such Ia-negative cells might also be transduced by gene gun and contribute to the PCR signal detected. The role of these migratory Ia-negative cells in genetic immunization was investigated in the adoptive transfer experiments presented below.

Adoptive transfer of migratory LC leads to AAT-specific humoral responses

The gene gun (Rumsey-Loomis model) covers an area of ~1 cm² of skin. Because the average density of LC in ear epidermis of A/J mice is 10³ cells/mm² (see Fig. 1B), the theoretical number of LC affected by each gene gun treatment would be 10⁷. Based on this theoretical calculation we chose an inoculation dose of 2 x 10⁵ cells (equivalent to the number of LC affected by two gene gun

Table 1. Phenotype of migratory cells from gene gun-treated vs control skin cultures

<table>
<thead>
<tr>
<th>Marker</th>
<th>Gene Gun</th>
<th>Control</th>
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<tbody>
<tr>
<td>I-A</td>
<td>49.9</td>
<td>51.5</td>
</tr>
<tr>
<td>CD3</td>
<td>45.2</td>
<td>44.4</td>
</tr>
<tr>
<td>CD11c</td>
<td>40.6</td>
<td>43.5</td>
</tr>
<tr>
<td>CD11b</td>
<td>63.8</td>
<td>49.0</td>
</tr>
<tr>
<td>CD40</td>
<td>50.4</td>
<td>55.2</td>
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<tr>
<td>Dec 205</td>
<td>34.2</td>
<td>ND</td>
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</table>

* BALB/c mice were treated by gene gun or mock-treated as described in Materials and Methods. Migratory cells from skin cultures were stained with various combinations of the Abs listed above. The data are representative of two separate experiments.

FIGURE 3. Transgene expression in migratory LC. A, Migratory cells from 2-day skin organ cultures from GFP or Luc gene gun-treated skin was examined at x450 magnification by both phase contrast (light background) and epifluorescence microscopy (dark background). Filled arrows indicate large veiled and DCs. Migratory gold heads, open arrows indicate GFP-positive cells and chevron arrowheads show small cells in culture. B, Migratory cells express transgene mRNA. Total RNA was extracted from migratory LC cultured from skin transfected by gene gun with pCMV-i-AAT DNA or from a XS106 DC line transfected by gene gun, in parallel. RT-PCR for β-actin and AAT expression were performed with (+) or without RT (−) as indicated.
Adoptive transfer of migratory LC generates CTL.

Migratory LC from gene gun-treated skin and gene gun-transfected XS106 cells were examined for their ability to initiate CTL responses. Transfection was achieved in both populations, as determined by RT-PCR signal analysis of the AAT gene (Fig. 3B). As described above, a single dose (2 × 10^5) of migratory LC or XS106 cells was transferred into naive mice by s.c. injection into the ear. Positive control mice received GI treatment with the AAT gene (GI-AAT). A group of mice were subjected to removal of the inoculated ear 1 h after gene gun treatment (VG experiments), serving as a parallel control (VG-AAT). After 3 wk, all mice were boosted with GI, their splenocytes harvested after 8 days, restimulated in vitro for 5 days, and assessed for CTL activities against AAT protein-pulsed or unpulsed target cells by standard 51Cr-release assays. Fig. 5 shows that the level of CTL activity generated by LC (LC-AAT) was comparable to that obtained with transfected XS106 cells (XS106-AAT) and to that after standard GI (GI-AAT). This activity was Ag-specific because cytotoxicity was

FIGURE 4. Ia⁺ migratory cells generate AAT-specific humoral responses after transfer into naive mice. A, Total migratory cells obtained from cultures of ear skin inoculated with the pCMVi-AAT plasmid were collected and transferred to naive mice (LC-AAT). In parallel, transfected XS106 cells were also transferred (XS-AAT). Positive control GI-treated mice were immunized with pCMVi-AAT (GI-AAT) or, serving as a parallel negative control, with pCMVi-Luc (GI-Luc). Humoral responses from individual mice, three per group, were measured 3 wk after immunization in an AAT-specific ELISA. The mean and SEM is shown for each group and is representative of results obtained from three similar experiments. *, p < 0.025; **, p < 0.001. B, Ia-positive transfected migratory cells mediate the AAT-specific humoral response. Cells obtained from skin cultures were subject to MACS enrichment or depletion based on Ia expression. Three mice per group received 1 × 10^6 total Ia-enriched (75% Ia⁺), depleted (6% Ia⁻), or unfractionated (42% Ia⁺) cells and humoral responses measured at the time point indicated by AAT-specific ELISA. Mean and SEM are shown. Results are representative of two separate experiments. *, p < 0.025; **, p < 0.01; ***, p < 0.001.

FIGURE 5. CTL activity is generated in mice receiving transfected migratory cells. Transfected migratory LC were collected from 2-day cultures of gene gun-treated ear skin (LC). XS106 cells were transfected by gene gun in parallel and also cultured over 2 days (XS). GI-treated mice were either left alone (GI) or were subjected to removal of the inoculated ear 1 h after gene gun treatment (VG). A single dose (2 × 10^5) of transfected LC or XS106 cells was transferred into naive mice. All mice were boosted with GI after 3 wk, their splenocytes were harvested 8 days later, restimulated in vitro for 5 days, and assessed for CTL activity against AAT-pulsed or unpulsed target cells by standard 51Cr-release assays. Mean and SEM values are shown for cytotoxicity at an E:T ratio of 80:1. *, p < 0.01; **, p < 0.001.
Inducible LC gene expression correlates with immune responses. Mice were shot with inducible AAT plasmids, ears were cut after 1 h, and cultured for 2 days. As indicated, skin explants were cultured either in the presence or absence of 100 ng/ml RU486. Continuous animals received cells cultured in the absence of RU486 during culture and after transfer to recipient animals. Three migratory LC groups were derived from the following gene expression conditions: 1) “Continuous” expression, in which donor GI-treated animals, during culture of the donor specimen, and after transfer of migratory LC in recipient animals. These conditions attempt to mimic those of standard GI, permitting both direct (by transfection) and indirect loading of Ag (produced by surrounding transfected skin cells) during LC migration. 2) “Induced” expression, in which donor ear specimens are derived from untreated animals, LC migrate into culture medium in the absence of RU486 and induced only after transfer to recipient mice treated with RU486. Without RU486 in culture, gene expression remains undetectable indicating that Ag is not produced under these conditions (Fig. 6). Therefore during migration, LC cannot be loaded with Ag. Only after transfer into mice treated with RU486 is gene expression observed against nonpulsed target cells. These data provide evidence that LC which initially migrate from the immunization site give rise to the majority of the CTL response generated by gene gun-mediated GI.

Inducible transgene expression in skin organ cultures

The migratory LC collected from 2-day skin organ cultures may be loaded with Ag by either direct transfection or indirect uptake of Ag derived from neighboring transfected cells. To distinguish these two possibilities, we used an inducible GI vector system.

The two plasmid system consists of an activator and reporter plasmid. The activator plasmid encodes a chimeric transactivator that, upon binding the inducing drug RU486, activates transcription on a second reporter plasmid encoding either Luc or AAT. To test the effectiveness of this system in mouse skin organ culture, mouse ears were inoculated with the inducible Luc vector by gene gun and cultured in various doses of RU486. As shown in Fig. 6, we found that Luc gene expression occurred in an RU486 dose-dependent manner. Importantly, basal activity was undetectable in the absence of RU486. Another group of mice was pretreated with the drug RU486 an hour before gene gun inoculation in an attempt to maximize the induced transcription levels. We found that pretreatment of drug was sufficient to induce detectable expression of the Luc gene in the absence of further drug exposure during culture. This expression was further augmented when pretreated ear specimens were cultured in the presence of RU486, again, in a dose-dependent manner. Luc gene induction peaked at 100 ng/ml RU486 resulting in a 200-fold induction in this skin organ culture system and this concentration was used in combination with in vivo pretreatment in subsequent experiments. The peak level of Luc activity induced in vitro approached levels achieved by in vivo induction (as shown by a (+) symbol in Fig. 7) illustrating the effectiveness of the in vitro system.

FIGURE 7. Inducible LC gene expression correlates with immune responses. Mice were shot with inducible AAT plasmids, ears were cut after 1 h, and cultured for 2 days. As indicated, skin explants were cultured either in the presence or absence of 100 ng/ml RU486. Continuous animals received cells cultured during culture and were treated in vivo with RU486 (Continuous). Induced animals received cells cultured in the absence of RU486 then treated in vivo with RU486 (Induced). Uninduced animals received cells cultured without drug and treated in vivo with vehicle only (Uninduced). As a specificity control, LC were cultured from skin shot with pCMV-Luc (Luc). Three weeks after transfer, serum from individual mice were titered in the AAT-specific ELISA. NMS, normal mouse serum. The mean and SEM values from three mice per group are shown from one of three similar experiments. *, p < 0.001.
expression induced. Thus, the Ag source in the Induced group can only be derived from transfected LC. 3) Uninduced LC are never exposed to RU486 induction in the donor, in culture, or in vivo upon adoptive transfer. Thus, immune response levels in the Uninduced group reflect background transcription of the transgene.

By comparing the magnitude of the immune response generated from Continuous vs Induced conditions, we assessed the contribution of indirect Ag loading in LC vs direct loading by transfected genes. As shown in Fig. 7, both Continuous and Induced groups generated significantly higher levels of anti-AAT Abs than the Uninduced group ($p < 0.001$). More importantly, we observed no significant difference in their responses when paired with each other, i.e., Continuous vs Induced groups. Furthermore, the Uninduced group did not generate significant levels of AAT-specific Ab when compared with the negative control Luc group, indicating that AAT-specific Abs could only be generated upon RU486 induction. Because no significant difference in the immune response was observed between Continuous and Induced groups, the results indicate that the majority of the immune response generated by inducible GI vectors was due to directly transfected LC and that uptake of exogenous Ag, under these conditions, played only a marginal role.

**Discussion**

These studies represent the first demonstration of inducible control of gene expression in transfected LC. Using this strategy, we were able to temporally control transgene expression, and therefore the timing of LC Ag loading. In adoptive transfer experiments, we were able to distinguish between the contribution of exogenous and endogenous Ag loading in LC, and demonstrate that direct transfection in LC plays a significant role in GI.

Previous studies by others have demonstrated small but significant numbers of transgene-expressing LC become detectable in the draining lymph node after gene gun treatment (11, 21). The functional contribution of these directly transfected LC on the immune response has been addressed in various ways. For example, some investigators used nonsecreted forms of Ags in GI so that the gene product is less likely to be transferred to neighboring LC (15, 21–23). In other studies, the role of cross-priming in GI was addressed by studying CTL priming in which expression of two genes, influenza nucleoprotein Ag and CD86, were required. They showed that bombardment with gold particles coated with both genes, and not a mixture of particles coated with individual genes, was necessary for developing gene gun-mediated responses, indicating that presentation of both gene products in the same cell could only be achieved by direct transfection (16). Together with our results the findings are all consistent with the hypothesis that uptake of exogenous Ag, in secreted or cell-associated form, is less efficient than direct transfection for LC Ag loading at the immunization site.

To address the role of migratory cells in mediating GI, Kleinman et al. (24) studied the capacity of gene inoculated skin to transfer immunity to naive recipients. When naive mice were grafted with immunized skin, harvested from the donor as early as 1 day after GI, they failed to fully develop primary and memory immune responses. These results support our conclusion that migratory LC from the immunization site are responsible for initiating Ag-specific responses. Furthermore, Boulo et al. (25), using a different GI technique of s.c. injection of DNA in saline, also showed that migratory LC expressed transgene RNA and conferred immunity in adoptive transfer experiments. We have extended their observations by examining the direct impact of gene gun treatment on LC migration and by testing the contribution of transfected LC, as well as the role of exogenous uptake for Ag loading to the immune response mediated by the first wave of migratory LC. We propose that cross-priming may play a more pronounced role in loading Ag into LC that repopulate the GI site. This interpretation is consistent with findings from Cho et al. (26), who addressed the role of cross-priming without direct transfection of LC, by demonstrating the ability to activate adoptively transferred Ag-specific CD8 T cells 7 and 21 days after GI, well after the first wave of LC migrated from the GI site. Although their data provide strong evidence that cross-priming plays a predominant role in generating humoral and cellular responses when using a keratinocyte promotor-driven GI vector, they did not directly examine the transfection of migratory cells from the GI site. Using DC-specific promoters to drive Ag expression, they were unable to demonstrate strong humoral responses and demonstrated a reduced capacity to activate CTL. Their data may differ from ours due to differences in the promoters used, as they demonstrated weak levels of Ag expression obtained with the keratinocyte and DC-specific promoters tested, and their use of a nonsecreted model Ag may have been less effective in promoting humoral responses as compared with the secreted AAT Ag used in our studies. Together, the previous findings by others and our results enforce the concept that presentation of Ag by directly transfected LC can be an important contributor in mediating GI.

Our results indicate that biolistic bombardment of skin by gene gun induces a synchronous, rapid, and enhanced emigration of LC from epidermis in skin organ cultures. The disappearance of LC after bombardment was also observed in vivo and was not dependent on the presence of DNA (Ref. 27 and data not shown). The migration of untransfected and transfected LC from the site of inoculation is consistent with other reports showing that in addition to identifying rare transgene-expressing cells in draining lymph nodes, significant increases in total DC numbers were also observed (25). The mechanism by which LC are activated in the epidermis by gene gun requires further investigation. It is possible that microdamage of epidermal cells initiates stress signals, perhaps through apoptotic/necrotic mechanisms (28) and/or the elaboration of proinflammatory cytokines (29). We examined a number of DC-specific and leukocyte-specific markers to characterize the cells that migrate in response to gene gun. We found both LC and γδ T cells migrate, and that in general, LC markers were expressed at higher levels in gene gun-treated samples as compared with controls, indicating that LC were indeed activated. Unexpectedly, not all of the migratory LC from gene gun-treated skin exhibited typical phenotypic features of mature DC. One may interpret this to suggest that the signals generated by gene gun were sufficient to trigger migration but not full maturation of all LC. Immature DC have high levels of intracellular “empty” MHC class II molecules ready for loading processed Ag while mature DC traffic all their MHC II to the plasma membrane, and, therefore, have no MHC molecules available for intracellular Ag loading (30). Thus, it may be advantageous that gene gun produces some DC that are not fully mature because GI-transfected migratory DC may have time to express, process, and load Ag-derived peptides onto MHC II before they fully mature in the lymph node.

In this report, we demonstrate the novel use of inducible GI vectors to address questions regarding the Ag-loading mechanisms involved in mediating GI from the first wave of LC that leave the GI site. Using this tool we showed that directly transfected LC have a predominant role in loading the first wave of LC that migrate from the GI site. Thus, future strategies for targeting gene expression exclusively to LC may offer more effective activation of desired immune responses. Collectively, our results with inducible vectors have important implications for the future development of engineered vaccines. Incorporating elements of inducible control in the design of vectors for GI or engineered DC-based
vaccines may permit temporally precise and perhaps more predictable regulation of immunologic outcome in vivo.

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References