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The vaccine-site microenvironment induced by injection of incomplete Freund's adjuvant, with or without melanoma peptides

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Abstract

Cancer vaccines have not been optimized. They depend on adjuvants to create an immunogenic microenvironment for antigen presentation. However, remarkably little is understood about cellular and molecular changes induced by these adjuvants in the vaccine microenvironment. We hypothesized that vaccination induces dendritic cell activation in the dermal vaccination microenvironment but that regulatory processes may also limit the effectiveness of repeated vaccination. We evaluated biopsies from immunization sites in two clinical trials of melanoma patients. In one study (Mel38), patients received one injection with an adjuvant mixture alone, comprised of incomplete Freund's adjuvant (IFA) plus granulocyte-macrophage colony stimulating factor (GM-CSF). In a second study, patients received multiple vaccinations with melanoma peptide antigens plus IFA. Single injections with adjuvant alone induced dermal inflammatory infiltrates consisting of B cells, T cells, mature dendritic cells (DC) and vessels resembling high endothelial venules (HEV). These cellular aggregates usually lacked organization and were transient. In contrast, multiple repeated vaccinations with peptides in adjuvant induced more organized and persistent lymphoid aggregates containing separate B and T cell areas, mature DC, HEV-like vessels, and lymphoid chemokines. Within these structures, there are proliferating CD4⁺ and CD8⁺ T lymphocytes, as well as FoxP3⁺CD4⁺ lymphocytes, suggesting a complex interplay of lymphoid expansion and regulation within the dermal immunization microenvironment. Further study of the physiology of the vaccine site microenvironment promises

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to identify opportunities for enhancing cancer vaccine efficacy by modulating immune activation and regulation at the site of vaccination.

Keywords

cancer vaccines; immunotherapy; melanoma; histology; dendritic cells; T-lymphocytes; chemokines

Introduction

Immune therapies that modulate T cell immunity can induce dramatic and durable clinical responses of advanced melanoma. These have been induced by interleukin-2 (IL-2), adoptive T cell therapy, and CTLA-4 antibody therapy (1–4). Also, cancer vaccines have been effective at inducing anti-tumor T cell responses (5–10), but their clinical impact has largely been disappointing. This may be due to low magnitude or persistence of vaccine-induced T cell responses or to immune regulatory processes that limit T cell expansion and persistence. A logical strategy to improve melanoma vaccines is optimizing adjuvants. However, the mechanisms of adjuvants remain poorly understood. Their effect is local, at the vaccine site, but studies of their impact are often limited to measures of systemic effects. Probably the most widely used is incomplete Freund's adjuvant (IFA), which was developed to augment antibody responses to protein vaccines (11) but very little has been done to characterize its effects at the vaccine site, as may impact T cell responses to peptide vaccines.

We have reported that short peptides used in melanoma vaccines have very brief half-lives in human serum, which may even be less than a minute.(12) Degradation is mediated by exopeptidases and endopeptidases that are present both in human serum and human skin. (12) This rapid degradation presents challenges for vaccinating with short peptides. The standard paradigm for understanding cutaneous immunization is that antigen is processed and presented by activated dermal dendritic cells. These dendritic cells can uptake particulate antigen when immature, then can present the antigen to T cells in the nodes, when the DC are mature. For peptide antigens, however, there is no need for uptake and processing; the peptides can bind directly to Class I MHC molecules on the cell surface. We have had concerns that the brief half-life of peptides in the skin could cause most of the injected antigen to be degraded before it can access MHC molecules on the surface of mature dendritic cells. Despite this concern, we have found that repeated vaccination with peptides in IFA induces T cell responses in most patients.(13–16) Thus, we have postulated that repeat vaccination with peptides in adjuvant may lead to accumulation of dendritic cells in the vaccine microenvironment, which is clinically inflamed (Figure 1A).

Others have also used GM-CSF as a local vaccine adjuvant (17–19). Murine studies have suggested that the immunogenicity of peptide vaccines administered in IFA may be increased by inclusion of GM-CSF in the adjuvant emulsion (17); thus, some of our prior and recent vaccine trials have incorporated this adjuvant combination (13–15). Though its effects are debated, it may impact the function of antigen-presenting cells. However, little is known about effects in vaccine sites in humans. The present study was undertaken to determine the effects of IFA, with or without GM-CSF, in the human dermis, with particular attention to effects on dendritic cells and T cells, and their organization within the vaccine microenvironment of melanoma patients. We hypothesized that IFA causes accumulation of DC, especially mature DC, in the dermis. For this manuscript, we evaluated skin and subcutaneous tissue at various time points over 14 days after injection of adjuvant (Mel38

study), as well as evaluating skin after several vaccinations with peptides plus incomplete Freund's adjuvant (Mel44 study).

Methods

Patients

Two patient groups were evaluated, both with resected melanoma, differing in the interventions and extent of biopsy. They are referred to as Group 1 and Group 2 patients.

Group 1 Patients: dermal changes after one injection of incomplete Freund's adjuvant and GM-CSF (UVA-Mel38)—Twenty-one patients were enrolled into a non-therapeutic clinical trial (UVA-Mel 38) that was designed for clinical AJCC stage I or II melanoma patients in order to evaluate the local effects of vaccination with GM-CSF and incomplete Freund's adjuvant (IFA, Montanide ISA-51, Seppic, Inc, Paris, France) in the skin. The IFA used in group 1 patients was the formulation containing oleic acid derived from beef tallow. (18) GM-CSF (made by Berlex/now Genzyme) and Montanide ISA-51 were purchased by the University of Virginia. For these group 1 patients, the “adjuvant” injected refers to the combination of 0.5 ml IFA, 110 mcg GM-CSF, and 0.5 ml saline solution. Participants were those who needed wide local excision of their primary melanoma as part of their standard clinical management, with or without sentinel node biopsy. After obtaining informed consent (HIC #8380, FDA IND #7593), patients were injected once with adjuvant into the dermis within the planned wide excision specimen. The surgical excision took place at 1, 2, 5, 7, 9, or 14 days after the injection, so that the inflammatory changes at the injection site could be assessed at multiple time points (Figure 1B). Three patients were accrued to each time point and, also, 3 patients were accrued to day 0 (no injection), for a total of 21 patients. Portions of skin were harvested from the wide excision specimen, including a 1 cm² area from the injection site and another separate control skin specimen. The tissue was divided so that one portion was formalin-fixed and paraffin embedded and the other portion was fresh frozen (Figure 1B). There was also a subsequent stage to that study, in which patients were randomized to injection with (i) saline, (ii) GM-CSF alone, (iii) a water-in-oil emulsion of IFA alone, or (iv) the emulsion of IFA plus GM-CSF. This second stage was closed after enrolling 3 patients; limited data are available.

Group 2 Patients: dermal changes after multiple vaccinations with incomplete Freund's adjuvant and peptides (UVA-Mel44)—Patients with resected stage IIB, III or IV melanoma were enrolled into a clinical trial of a multi-peptide melanoma vaccine designed to evaluate the effects of administering cyclophosphamide and/or melanoma-derived helper peptides on the immunogenicity of a class I MHC-restricted peptide-based vaccine in participants. (Figure 1C). Overall immunologic and clinical outcomes for that trial are reported separately (19). Patients received a total of 10 vaccines throughout the year and were vaccinated with melanoma peptide mixtures MELITAC 12.1 (12 melanoma peptides restricted by Class I MHC molecules, plus 1 tetanus helper peptide (15)) or MELITAC 12.6 (12 melanoma peptides restricted by Class I MHC molecules, plus 6 melanoma helper peptides (20;21)), in an emulsion with Montanide ISA-51 (incomplete Freund's adjuvant, IFA, without GM-CSF). The IFA used in group 2 patients was the formulation containing oleic acid derived from a vegetable source (olives, IFA-V) (18) for 16 of the 18 patients; for the other two patients, some vaccines used IFA-V, and some used IFA-A. Therefore, for group 2 patients, the term “adjuvant” refers to IFA, without GM-CSF. Each vaccine was a 2 ml emulsion consisting of peptides in aqueous solution plus 1 ml Montanide ISA-51 adjuvant, with half administered intradermally and half subcutaneously. We offered 3–4 mm punch biopsies of vaccination injection sites to group 2 patients, with informed consent (HIC 10598), as an optional intervention. Punch biopsies were performed either “early” (two

weeks after 3 vaccines, n=12, Group 2A), or “late” (after 6 or more vaccines, n=6, Group 2B, weeks 8 (2), 13 (2), 40, or 62).

Antibodies for characterization of cellular and molecular features of inflammatory reactions using immunohistochemistry

To characterize injection site lymphoid aggregates, we stained skin samples from patients in groups 1 and 2 with antibodies against the following markers: *In fresh frozen tissue*: CD1a (Dako Cytomation, Carpinteria, CA, M3571, mouse, 1:50), CD83 (Novocastra, Norwell, MA, NCL-CD83, mouse, 1:10) and DC-LAMP (Beckman-Coulter, Fullerton, CA, CD-208, mouse, 1:2.5). *In formalin fixed, paraffin embedded tissue*: CD8 (M7103, mouse, 1:50) and CD20 (MO755, mouse, 1:200), both from Dako Cytomation, Carpinteria, CA. In addition, CD3 (VP-RM01, rabbit, 1:75), CD4 (VP-C318, mouse, and Vector, 4b12, mouse 1:40), and Ki67 (VP-K452, mouse, 1:50), all from Vector, Burlingame, CA. CCL19 (MAB361, mouse, 1:80), CXCL12 (MAB350, mouse, 1:300), CXCL13 (AF801, goat, 1:50), and CCL21 (AF366, goat, 1:60) from R&D systems, Minneapolis, MN, and PNAd (BD Biosciences Pharmingen, San Jose, CA, 553863, rat, 1:75) and FoxP3 (eBiosciences, San Diego, CA, 14-4776, rat, 1:160). For optimization of antibodies in IHC, we used normal human lymph node tissue from non-melanoma patients (LN) as positive controls.

Immunohistochemistry procedures

All incubation steps were performed in a humidity chamber at room temperature. Between each step, sections were washed for 5 minutes in PBS. Following heat mediated antigen retrieval (formalin fixed paraffin embedded samples, only) with 10 mM sodium citrate, pH 6.0 in Pascal pressurized heating chamber (DakoCytomation) and rehydration with PBS (5 min), endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxidase. Next, nonspecific binding sites for secondary antibodies were saturated by incubation with normal serum (10% in PBS, Vectastain ABC kit) for 15 min. Endogenous biotin activity was blocked by incubation with avidin (15 min, Vector) and biotin (15 min, Vector). Sections were incubated with primary antibodies for the recommended incubation periods and biotinylated secondary antibodies (for 10 min, Vectastain ABC kit). Afterward, sections were incubated with ABC reagent (for 5 min, Vectastain ABC kit), followed by substrate solution consisting of Vector Blue, Vector Red or DAB or DAB-Ni (2–20 minutes, under microscope control, Vector). In cases of multiple antigen labeling, the series of steps was repeated again, for each additional label, beginning at the blocking of nonspecific sites for secondary antibodies. Sections were counterstained, in some cases, with hematoxylin (Vector). All sections were then dehydrated and coverslipped, using VectaMount permanent mounting media. For selected multiple-staining studies, a modified SIMPLE technique (22) was used. Slides were dewaxed with xylene, rehydrated with EtOH and water, washed, counterstained with hematoxylin, rinsed, then dipped in lithium carbonate bluing solution. This negative control is covered with 70% glycerol and coverslip, then imaged with the Aperio Scanscope. The coverslip is removed with PBS, then antigen retrieval is performed if indicated, followed by blocking endogenous peroxidase with sodium hydroxide and blocked with normal horse serum (ImmPRESS kit). Primary antibody is added, then washed, and then secondary antibody is added and washed. Color is added with AEC staining. Coverslipping again on 70% glycerol allows imaging of the stained slides. Then the AEC is removed with alcohol and water. The antibody is washed with acidified KMnO₄, then PBS, serum blocked, then restaining is possible sequentially with up to 5 total markers on the same slide.

Evaluation of stained tissue sections and cell counting methods

H&E and immunohistochemically-stained tissue sections were reviewed by two dermatopathologists (J.W.P. and J.T.S.). Cell counts were obtained in epidermis, papillary

dermis, reticular dermis, and subcutis, for DC-LAMP+ dendritic cells (as a measure of mature dendritic cells), CD1a+ cells (as a measure of immature dendritic cells/Langerhans cells), and CD8+ T-cells. For these analyses, cells were counted by a dermatopathologist in three representative high-powered fields of the cellular infiltrates, and the mean of these values was multiplied by the percent of the tissue cross-section containing the inflammatory infiltrates. The result was an estimate of the number of stained cells per high-powered field (hpf) overall in that skin layer (4.5 hpf = 1 mm²). The data are reported as cells per hpf. Analyses of the punch biopsies of trial #2 (Mel44) included enumeration of CD4+ cells, CD8+ cells, Ki67+ cells, and FoxP3+ cells, as well as enumeration, by double staining, of Ki67+CD4+ cells, Ki67+CD8+ cells, and FoxP3+CD4+ cells by a pathologist (LB) blinded to the specimen details, counting 5 high powered fields of stained cell clusters throughout the biopsy specimens.

Western blot analysis for CCL19—Several specimens were evaluated for CCL19, with each skin specimen placed in an equal volume of RIPA buffer (with protease inhibitors) in a tube, then submerged in liquid nitrogen. A pellet pestle motor was used in cycles of 30 seconds on followed by 30 seconds off, to grind tissue to liquefaction. After centrifugation, supernatant was transferred to a new tube. Protein yields were determined by BCA analysis, and proteins were resuspended in SDS-containing sample buffer, heated 5 min at 100 °C, and resolved by SDS-PAGE and transferred to Immobilon-P (Millipore). After blocking in 5% BSA in 50 mM Tris-Cl (pH 7.5), 0.9% NaCl, 0.05% Tween 20, and 0.01% antifoam A, the membranes were probed with antibody to CCL19. Proteins were detected with Pierce SuperSignal West Pico Chemiluminescent substrate (#34080) as recommended by the manufacturer and used to expose to Kodak BioMax film.

Results

Dermal aggregates containing lymphocytes and mature DC develop in the dermis after a single injection with IFA and GM-CSF (group 1, Mel38)—We first assessed the effects of IFA+GM-CSF on dermal accumulation of immature dendritic cells (Langerhans cells (LC), CD1a) and mature dendritic cells (DC-LAMP/CD83), and to assess whether this is associated with depletion of LC from the epidermis, as has been reported with other inflammatory stimuli. We biopsied skin from 3 patients at each of seven intervals (0, 1, 2, 5, 7, 9, 14 days) after one injection of IFA plus GM-CSF (UVA-Mel 38 study, Figure 1B) and all biopsy specimens were evaluated on H&E stained sections and with immunohistochemical stains for infiltrating CD8+ T cells (CD8) and dendritic cells (CD1a, DC-LAMP). Additional stains for B cells (CD20), T cells (CD3) and dendritic cells (CD83) were performed on representative biopsy specimens from 7, 5, and 6 patients, respectively. Morphologically, mononuclear cell aggregates were identified in the dermis within 24–48h and persisted over the first 7–9 days (Figure 2A–B). These aggregates were usually loosely organized (Figure 2B), but included more organized cell clusters surrounding dermal vessels (Figure 2A, C). T and B lymphocytes were prominent in the lymphoid aggregates, without separate B and T cell areas (Figure 3A). However, a prominent organized B cell area was evident in the dermis in one case (Figure 3B). Immune cells usually were most numerous between days 2 and 7, dissipating substantially by day 14, except that one outlier patient still had large numbers of CD8+ T cells even at day 14 (Figure 2G).

Dermal dendritic cells in cellular aggregates are primarily mature—All the tissue sections were stained for DC-LAMP, a specific marker for mature dendritic cells, and for CD1a, a marker for Langerhans cells, which are classically immature DC. Sections from six of these patients were also stained for CD83, another marker of mature DC. Most of the DC in the dermis were CD83⁺ and DC-LAMP⁺ (consistent with mature DC), and clustered with

lymphocytes in lymphoid aggregates (Figure 2C,D). These clusters specifically included both CD8⁺ T cells (Figure 2F) and CD4⁺ T cells (not shown). Unlike mature DC, the CD1a⁺ DC were located primarily in the epidermis and adnexal structures, consistent with Langerhans cells, and were not prevalent in the lymphoid aggregates (Figure 2E). Quantitatively, DC-LAMP⁺ mature DC were 2- to 4-fold more prevalent than (immature) CD1a⁺ DC in the dermis (Figure 2G). Even when there was a large accumulation of mature DC in the dermis, Langerhans cell populations in the epidermis persisted at fairly constant levels, from day 0 to 14, except at day 5, when all dendritic cell numbers were lower for those specimens (Figure 2G).

Dermal aggregates of lymphocytes and mature DC contain PNA⁺ microvessels resembling HEV

—The dermal aggregates of lymphocytes and mature DC encircled small vessels. To characterize them, four specimens from day 7–9 were evaluated for endothelial peripheral node addressin (PNA⁺). Endothelial PNA⁺ expression is characteristic of high-endothelial venules (HEV) in normal lymph nodes, and is the ligand for CD62L (L-selectin) on naïve lymphocytes. In this study, PNA⁺ microvessels were identified in the lymphoid aggregates in the dermis of 2 of these 4 patients (Figure 3B, 3C and data not shown). These PNA⁺ vessels are associated with the dermal aggregates of lymphocytes and DC described above (Figure 3B–C). CD83⁺ cells were interspersed with the lymphocytes surrounding these PNA⁺ microvessels (Figure 3C). When there were distinct B and T cell areas, the PNA⁺ vessels were in the T cell areas (Figure 3B), as is typical for normal lymph nodes.

Chemokine CCL19 induced after IFA emulsion injection—CCL19 is a chemokine that recruits CCR7⁺ dendritic cells and lymphocytes to secondary lymphoid organs and that has been implicated in mediation of lymphoid neogenesis outside of established lymphoid tissues.(23) Skin samples from 2 patients were evaluated by Western blot for CCL19: one biopsied 7 days after injection with IFA+GM-CSF (stage 1 of Mel38 study), and one biopsied 7 days after injection with GM-CSF alone (stage 2 of Mel38 study). There was markedly more CCL19 protein in skin injected with IFA + GMCSF than in control skin from the same patient (Figure 4). In skin from a patient injected only with GM-CSF, there was no clinical evidence of inflammation at that site (not shown), and Western blot analysis showed minimal CCL19 (Figure 4).

Effects of repeat vaccination with adjuvant and melanoma peptides, without GM-CSF (group 2, Mel44)—Having observed accumulation of mature DC and lymphocytes clustered around microvessels, and finding that these were very transient structures with adjuvant alone, we hypothesized that repeated injection with immunogenic peptides in adjuvant would lead to similar but more persistent lymphoid aggregates with mature DC. In evaluation of skin after repeated vaccination, we were also interested in preliminary evidence for regulatory T cell accumulation in the vaccine microenvironment.

Repeated vaccination with melanoma peptides in IFA induces organized dermal lymphoid aggregates at week 3—Patients on the Mel44 melanoma vaccine trial were immunized with 12 melanoma peptides and one or more helper peptides, plus IFA, without GM-CSF, on weeks 0, 1, 2, 4, 5, 6, 12, 26, 39, and 52. (Figure 1C) To determine effects of these repeat vaccinations with IFA plus peptides, a subset of patients underwent 3–4mm punch biopsies of skin at the vaccine site either 2 weeks after 3 vaccines, or after at least 6 vaccines (Figure 1C) on a companion protocol. Tissue specimens were evaluated for cellular infiltrates and for selected chemokines. Example immunohistochemical findings are shown in Figure 5 for biopsies 2 weeks after receiving 3 vaccines. There are lymphoid aggregates that are more organized than was typical in the

Mel38 study. These aggregates contain B cells and T cells with some tendency toward segregation into T and B cell areas and with a predominance of T cells (Figure 5A).

PNAd expression is present in vaccine sites—PNAd-stained vessels were prominent within these organized lymphoid aggregates in most specimens (12 of 18, 67%), and were surrounded by lymphocytes and associated with CD83⁺ dendritic cells (Figure 5b, after 3 vaccines, and data not shown). PNAd was expressed on dermal microvessels on 8 of 12 biopsies of dermis 2 weeks after 3 vaccines, and also in 4 of 6 specimens collected after 6 or more vaccines, at weeks 8–62. In each specimen, not all vessels stained with PNAd, but almost all vessels surrounded by substantial lymphoid aggregates were PNAd⁺. Normal skin controls were evaluated from 5 patients – dermal vessels were PNAd negative in 3 of them, and in the two where PNAd⁺ vessels were found, PNAd staining was faint, and there were perivascular lymphoid aggregates around those vessels.

Lymphocytes within dermal aggregates, induced by repeated vaccination with IFA and melanoma peptides, are proliferating—Lymphocytes in these aggregates are actively proliferating, as shown by nuclear Ki67 staining (Figure 5C). Double-staining with CD4 or CD8 antibodies identifies CD4⁺Ki67⁺ cells and CD8⁺Ki67⁺ cells, providing evidence that both CD4⁺ T cells and CD8⁺ T cells are proliferating in the vaccine site microenvironment (Figure 5E,F). The frequency of these double-stained cells is provided in Table 1 by study group, and by the interval from study initiation to biopsy. As an overall mean, 6% of CD8⁺ T cells and 3% of CD4⁺ T cells in the VSME were Ki67⁺. These may be slightly greater for biopsies at 4 weeks than later.

FoxP3⁺CD4⁺ T cells within dermal aggregates induced by repeated vaccination with IFA and melanoma peptides—Prominent FoxP3 nuclear staining is also evident in a subset of lymphocytes in these aggregates (Figure 5D). Additional sections double-stained for CD4 and FoxP3 show the FoxP3⁺ cells are CD4⁺ (Figure 5G), and double staining for CD8 and FoxP3 shows all FoxP3⁺ cells are CD8 negative (Figure 5H). Thus, the FoxP3⁺ cells appear to be CD4⁺CD8^{neg}FoxP3⁺. All 18 specimens were evaluated by the SIMPLE technique, and stained and double-stained cells were enumerated: Overall, 95% (range 83–100%) of the FoxP3⁺ cells were CD4⁺, and 12% (range 4–25%) of the CD4⁺ T cells were FoxP3⁺. Only 2% (mean; range 0–8.5%) of the FoxP3⁺ cells were Ki67⁺, compared to 6% of all CD8⁺ cells and 3% of all CD4⁺ cells. Proliferating FoxP3⁺ cells were identified only in biopsies after 3 vaccines (week 4), and were higher in patients whose vaccines included 6MHP (arms C & D, 5%) than in patients whose vaccines included the tetanus peptide (arms A and B, 1%). (Table 1)

Lymphoid chemokines are expressed within dermal lymphoid aggregates induced by repeated vaccination with IFA and melanoma peptides—Chemokines CXCL12, CXCL13, and CCL21 were detected in the VSME for 18 (100%), 13 (72%), and 17 (94%) of the 18 VSME biopsy specimens, respectively (Figure 6, Table 2). CXCL12 was expressed by multiple cell types in the VSME (Figure 6A–C): most of the lymphocytes in dermal LNLA expressed CXCL12 at high level (Figure 6A,B), and dermal blood vessels and epidermal dendritic cells (Langerhans cells) also expressed it (Figure 6A,B). Also, immune cells aggregated in the deep dermis and subcutaneous tissue often expressed CXCL12 (Figure 6C). All five normal skin biopsies also contained CXCL12⁺ dermal blood vessels, perivascular lymphocytes, and epidermal dendritic cells, though the intensity of staining was weaker than in the vaccine site biopsies.

Thirteen of the 18 biopsies (72%) contained CXCL13-positive cells in the LNLA, most in less than 10 cells in the whole tissue section but with prominent staining in those few cells; 3 specimens had more than 20 CXCL13⁺ cells in the tissue section, one each for arms B, C,

and D. One of these is shown in Figure 6D. CXCL13 was not expressed by other cells in these tissues. CXCL13+ cells also were not detected in the 5 normal skin biopsies.

CCL19 was detected in early experiments (Figure 6E) in lymphocytes and in endothelium of the HEV-like vessels, and by some cells in the perivascular aggregates; however, CCL19 was not evaluable for all specimens due to technical problems with this antibody.

CCL21 was detected on dermal immune cells in 17 specimens (94%, Figure 6F) and by dermal vessels in all 18 (100%, not shown). Mean values per hpf, among these 18 specimens, were approximately 10 superficial dermal immune cells, 32 deep dermal immune cells, and 2.7 dermal blood vessels staining for CCL21. In contrast, in five normal skin biopsies, there was weak staining of dermal vessels (1–2 per hpf), and CCL21+ lymphocytes were identified in only 1 (20%), at less than 5 per hpf.

Lymphoid aggregates persist in the dermis for many months, sometimes with chronic granulomatous changes—Biopsies from both patients on the Mel44 melanoma vaccine trial that were obtained at week 13 had extensive granulomas (Figure 7A), with the granulomas containing central vacuoles that presumably contained residual emulsion. These granulomas are highly cellular, with a predominant T cell infiltrate; B cells are essentially absent (Figure 7B, left side of figure). However, lymphocytic aggregates between and among the granulomas contained B and T cell areas (Figure 7B right side of figure; Figure 7C). Both patients also had extensive plasma cell infiltrates within their biopsy specimens, identified by their classic histologic appearance, especially in the deep dermis (not shown). CXCL12 was expressed by many cells in the granulomas in both patients, whereas CXCL13 was absent from both, and CCL21 was detected in granulomas in only one of the two patients (Figure 7D,E,F).

Discussion

Strategies are needed to optimize the frequency, magnitude, and persistence of anti-tumor T cell responses induced by cancer vaccines. Numerous approaches are being explored to optimize vaccine immunogenicity, including use of TLR agonists, cytokines, and antibodies to modulate costimulation. Integration of these agents into vaccine preparations is complicated by the myriad combinations possible and the additional variables of dose and timing, which are likely to be critical, and possibly patient-dependent. Tumor immunologists need to understand the cellular and molecular effects of adjuvants, over time, in the vaccine sites microenvironment (VSME), to guide rational use and combination of adjuvants. The present manuscript reports early efforts at evaluation of the effects of a standard incomplete Freund's adjuvant in the skin of humans with melanoma, over time, after one or multiple injections.

Murine studies suggest that dermal inflammation induces migration of Langerhans cells (immature DC) from the epidermis into the dermis, en route to the draining nodes, concurrent with their maturation; so that increased numbers of mature DC in the dermis were associated with depletion of Langerhans cells from the epidermis for a few days. We found that dermal injection of IFA+GM-CSF induced inflammation that peaked within a week and was associated with increased numbers of dermal mature and immature DC days 2–9 (Figures 3a,b). Mature DC (DC-LAMP⁺) outnumbered immature (CD1a⁺) DC 2:1 to 3:1 in the dermis during this time interval (compare Figures 3a,b), and DC in the perivascular aggregates were invariably mature. DC numbers were lower on day 5 than on days 2 and 7. This likely represents individual patient variation: three patients were enrolled at each time point (Mel38), but this was adequate to enable detection of large differences over time. However, epidermal LC (CD1a) were not identified in day 5 skin samples; so we

cannot rule out the possibility that there may be a transient depletion of epidermal Langerhans cells between days 2 and 7, which self-corrects, consistent with prior murine data.

One injection of IFA+GM-CSF also induced perivascular dermal accumulation of T cells and B cells. These aggregates were somewhat disorganized, peaked within 1 week after injection and typically dissipated by day 14. They rarely had formally organized T and B cell compartments, with the exception shown in Figure 3B. However, administering multiple vaccinations with melanoma peptides and adjuvant (Group 2, Mel44) induced formation of more organized lymphoid aggregates. Interestingly, these lymphoid aggregates have some features typically found in secondary lymphoid organs: (i) Organizing B and T cell areas, (ii) Proliferating lymphocytes, (iii) Mature dendritic cells, (iv) chemokines CXCL12, CXCL13, CCL19, and CCL21. These findings suggest interaction among multiple immune cell populations, with chemokines and organizational features typical of lymphoid organs, raising the possibility that the VSME induced after multiple vaccines may have some features of lymphoid neogenesis. Prior studies have identified lymphoid neogenesis in visceral locations, but the tissues in which it has been described do not include skin (23), and it has been suggested that lymphoid neogenesis does not occur in the skin (24). However, it is striking that PNAd was expressed by vascular endothelium in some biopsies after one injection with adjuvant (Group 1), and in most biopsies after multiple vaccine injections (Group 2). PNAd is characteristic of high-endothelial venules in lymph nodes and functions as ligand for L-selectin (CD62L) on naïve T cells. PNAd in the VSME could enable circulating naïve T cells to egress dermal vessels to encounter mature DC presenting vaccine peptides in the VSME. The presence of proliferating T cells in the VSME (Figure 6A, 6B) further raises a question of whether T cell expansion may occur in the VSME. If true, these findings would differ from the classic paradigm, where interaction of T cells with antigen-presenting dendritic cells is limited to their interaction within draining lymph nodes.

Normal skin may contain small perivascular immune cell aggregates in the superficial dermis, which resemble but are less dramatic than those observed in vaccine sites. These may reflect subclinical dermatitis of any of multiple possible etiologies. We found low-level PNAd expression in 2 of 5 normal skin specimens, in superficial dermal vessels surrounded by such small perivascular immune cell aggregates. Thus, the changes induced by vaccination with IFA and the inflammatory changes encountered by normal environmental exposures may differ more in quantity than in quality.

We observed increases in the expression of lymphoid chemokines CXCL12 (SDF-1), CXCL13 (B cell attracting chemokine-1), CCL19 (MIP-3 β), and CCL21 (SLC, 6Ckine) in the VSME compared to normal skin. These chemokines have been implicated in induction of lymphoid neogenesis (ref) and function to attract lymphocytes expressing CXCR4, CXCR5 or CCR7. The chemokines CCL19 and CCL21 are ligands for CCR7 and are implicated in recruitment of DC and lymphocytes into tertiary lymphoid organs. CXCL12 (ligand for CXCR4, also called SDF-1) is present in lymph nodes and regulates basal migration of some lymphocytes and monocytes (25). Interestingly, increased CXCL12 is observed in inflammation (26;27) and has been reported at increased levels in synovial fluid in rheumatoid arthritis (25;28). B cell recruitment also has been described as a critical component of TLO formation. B cells express CXCR5 and are recruited to the follicular zone by CXCR13 (29). Interestingly, CXCL13 is expressed only by cells in the LNLA of the VSME, though the cell type producing it is not firmly established. CXCL12 is expressed by many cell types in normal skin, but there are more cells expressing it in the VSME, both superficially and deep. Modulation of these and other chemokines may be useful for controlling the types of cells recruited to the VSME.

Patients biopsied “late” in group 2 had granulomatous changes within the dermis, and associated plasma cells. Granulomas can occur in the presence of persistent antigen. The granulomas appear to have formed around the adjuvant, and they have a lymphocytic cuff consisting primarily of CD8⁺ T cells. CXCL12 was detected in large numbers of cells in the granulomas. CXCL12 is the ligand for CXCR4, which is expressed on lymphocytes and some tumor cells. Granulomas were not detected in patients whose biopsies were performed at even later time points, when there were 3 month intervals after vaccines.

There is debate about the value of GM-CSF as a vaccine adjuvant, with recent data suggesting it may induce myeloid suppressor cells (30) and our own data that inclusion of GM-CSF in the IFA emulsion significantly decreases circulating CD4⁺ and CD8⁺ T cell responses to a multi-peptide vaccine (16). Evaluation of Group 2 (Mel144) biopsies reveals that lymphoid aggregates and mature DC are both prevalent and persistent in the VSME without inclusion of GM-CSF in the adjuvant. Thus, GM-CSF is not required for the changes observed.

To initiate investigations of immune regulation in the VSME, we evaluated for the presence of regulatory T cells. CD4⁺FoxP3⁺ lymphocytes were identified within dermal immune cell aggregates, by immunohistochemistry (Figure 6). Transient FoxP3 expression has been described in early T cell activation (31;32); so the observed FoxP3⁺ cells could be either early activated T cells or regulatory T cells. More recent work suggests, however, that detection of CD4⁺FoxP3⁺ cells by immunohistochemistry does, in fact, correspond to regulatory T cell function in patients with melanoma undergoing immune therapy.(33) Proliferation of T regs has been reported as a component of Treg homeostasis (34). In the present study, only 2% of the FoxP3⁺ cells in the VSME are proliferating; at week 4, this percentage is higher for patients in the Mel144 trial whose vaccines include melanoma helper peptides (19) than those whose vaccines included a tetanus helper peptide (5% vs 1%). If this difference persists in a larger sample, it could reflect differences in the effect of helper peptides on Treg proliferation. Regardless, regulatory T cells may be a potential therapeutic target for optimizing vaccine adjuvants.

In summary, the present report identifies cellular architecture and temporal evolution of the VSME induced by an incomplete Freund's adjuvant. Mature dendritic cells associate with proliferating T cells in perivascular dermal aggregates, with some features suggestive of lymphoid neogenesis at sites of repeated immunization. Future work will characterize the function of effector and regulatory cells in the VSME, how they may be modulated by other adjuvants, and whether such modulation at the VSME may improve T cell responses to vaccination.

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Figure 1. Vaccination sites and their biopsies

(A) A typical vaccination site on the upper arm of a patient on a clinical trial of a multi-peptide vaccine administered weekly in Montanide ISA-51 adjuvant, (B) protocol schema for Mel38 trial (Group 1 patients). Patients were injected intradermally with an emulsion containing IFA and GM-CSF, prior to wide excision of skin, and a 1 cm² section of skin at that injection site, marked with an X, was excised for histologic studies. This included full-thickness skin and subcutis, (C) protocol schema for Mel44 trial, plus optional biopsies of vaccine sites (Group 2 patients). Punch biopsies were obtained of vaccine sites, containing full-thickness skin (epidermis and dermis).

Figure 2. Histologic & immunohistologic findings on Mel38 trial biopsies

(A) H&E staining of epidermis and dermis, 40 $\times$, on frozen tissue section. Extensive mononuclear cell infiltration in dermis is observed. A prominent perivascular aggregate of mononuclear cells is shown in greater detail in the inset (day 9); (B) H&E staining of deep dermis, 100 $\times$, on frozen tissue section. Extensive mononuclear cell infiltration in dermis (day 9); (C) Immunohistochemical stain for CD83 in reticular dermis at higher power, on frozen tissue. CD83 $^{+}$ dendritic cells are prominently highlighted and are present in large numbers both diffusely and in aggregates. Faintly blue-stained lymphocytes are aggregated in close association with these mature dendritic cells. Dendritic morphology is evident in the inset (day 9); (D) Immunohistochemical stain for DC-LAMP in reticular dermis on frozen tissue. Numerous DC-LAMP $^{+}$ dendritic cells are observed, also in close association with faintly staining lymphocytes (day 9); (E) Immunohistochemical stain for CD1a in reticular dermis (**left**) and in superficial dermis (**inset right**). The highly cellular mononuclear infiltrate in the deep dermis contains CD1a $^{+}$ dendritic cells, but they are limited in number in these areas. Many of the CD1a $^{+}$ DC identified and counted in the papillary dermis are in (or associated with) adnexal structures, consistent with Langerhans cells (day 7); (F) Immunohistochemical stain for CD8 in dermis, on formalin-fixed paraffin-embedded tissue (day 9); (G) Cell counts, per hpf, of dendritic cells and CD8 T cells. Numbers of mature dendritic cells (DC-LAMP), immature dendritic cells (CD1a), and CD8 T cells, in epidermis (EP), papillary dermis (PD), reticular dermis (RD), and subcutaneous tissue (SQ) identified by immunohistochemistry were calculated based on counts averaged over 3 hpf in each skin layer, for skin biopsies in Group 1 (Mel38) after single injections with Montanide ISA-51 and GM-CSF. The values represent mean values for 3 patient samples at each time point, except that data for CD8 counts at day 14, we excluded one outlier (17, 105, 66, 84 CD8 cells per hpf for EP, PD, RD, SQ respectively [sum 272], compared to sums of 1.0 and 4.2 for the other two patients in this group). Standard deviations for the sums are shown for each bar.

Figure 3. Immunohistologic localization of T cells, B cells, dendritic cells, and PNAd+ vessels in skin biopsy sites, Mel38

(A) interspersed B cells (CD20, Vector Red, red) and T cells (CD3, DAB-ni, black), (B) organized cluster of B cells (CD20, DAB-ni, black) and nearby PNAd-expressed vessels (red) with intervening cluster of small mononuclear cells (CD3 by separate staining, not shown). The brown pigment in the upper left is melanin or hemosiderin but not immunostain. (C) lymph node-like aggregate with PNAd+ vessel (red) surrounded by lymphocytes (hematoxylin stain only) and CD83+ dendritic cells.

Figure 4. CCL19 protein in dermal specimen after adjuvant injection

Two patient samples from the 2nd stage of the Mel38 study have been evaluated by Western blot. Lane 1 = skin injected with GM-CSF only; lane 2 = negative control skin from that patient; lane 3 = skin injected with Montanide ISA-51 and GM-CSF; lane 4 = negative control skin for that patient. GAPDH was run as a loading control.

Figure 5. Cellular changes after repeated vaccination with multi-peptide vaccine in IFA, without GM-CSF (Mel44)

A patient on the Mel44 vaccine trial underwent vaccine site biopsy two weeks after the third vaccine; the formalin-fixed paraffin-embedded biopsy was evaluated by immunohistochemistry, for with the stated chromogens: (A) CD3 (DAB-nickel, black) and CD20 (NovaRED), (B) PNA (Vector-Red) and CD83, (C) Ki67 (DAB, brown), (D) FoxP3 (NovaRED); hematoxylin counter-stain is used. Dermal cellular aggregates also were double-stained, without counterstain, for (E) CD4 and Ki67, (F) CD8 and Ki67, (G) CD4 and Fox P3, or (H) CD8 and Foxp3. CD4 and CD8 are stained orange-red (Vector NovaRED) at the cell surface; Ki67 and FoxP3 are nuclear-stained blue-gray (Vector SG). In (E), red arrows indicate CD4⁺Ki67⁺ cells, and blue dotted arrows indicate CD4-negative Ki67⁺ cells. In (F), blue arrows indicate CD8⁺Ki67⁺ cells. In (G), two separate areas are shown. There is significant diffuse background staining with the CD4 antibody which is common with this antibody; CD4⁺ cells staining strongly at the cell surface are evident. Double staining CD4⁺FoxP3⁺ cells are strongly staining for CD4. In (H), Foxp3⁺ cells are CD8 negative. These results are representative of 8 patients studied in this way.

Figure 6. Chemokine expression in the VSME

Patient biopsies were evaluated for CXCL12, CXCL13, CCL19, and CCL21 by immunohistochemistry with AEC or DAB chromogen. (A) endothelium and perivascular lymphocytes expressing CXCL12 (week 4), (B) epidermal DC and superficial dermal LNLA strongly staining for CXCL12 in the endothelial cells and immune cells (week 4), (C) deep dermal lymphoid aggregate with CXCL12⁺ cells (week 13), (D) CXCL13⁺ cells in superficial lymphoid aggregate (week 4), (E) CCL19 staining of endothelium and cells of the lymphoid aggregate (week 4), (F) CCL21 staining of individual cells in immune cell aggregate deep in vaccine site (Week 13).

Figure 7. Patient with granulomas on biopsy at week 13

(A) H&E of deep granulomatous reaction with vacuoles at sites of emulsion. (B) IHC for CD3 (T cells, brown) and CD20 (B cells, red). Granulomas contain T cells, but B cells are absent; however, adjacent to, and between granulomas, there are infiltrates with both T cells and B cells. (C) IHC for CD20 (B cells, black) and PNAd (red) shows some organization of B cells, and PNAd+ vessel in T cell rich area. (D) CCL21 is largely absent from the granulomas, as is CXCL13 (E), whereas cells in the granuloma express CXCL12.

Table 1
Frequency of CD4 and CD8 cells expressing Ki67 or FoxP3 in the vaccine site microenvironment after injection of peptides in iFA

Patient subsets		Mean number of stained cells per HPF						Ratio of CD8:CD4 cells	% FoxP3+ cells expr CD4	% T cells expressing Ki67 or FoxP3			Ratios of cell types				Ratio Ki67+ CD4+: FoxP3+ CD4+
Interval from 1 st vaccine to biopsy	N	CD4	Ki67	CD4 Ki67	CD8	CD8 Ki67	FoxP3	CD4 FoxP3		CD4 expr FoxP3	CD8 expr Ki67	CD4 expr Ki67	CD4: FoxP3 ratio	CD8: FoxP3 ratio	CD4: Ki67 ratio	CD8: Ki67 ratio	
Week 4	12	93	11	3	20	1	12	11	94%	3%	12%	7%	4%	2.0	9.6	2.2	0.31
Week 8	2	95	11	1	45	6	6	5	97%	0%	6%	7%	0%	8.3	11.8	3.8	0.04
Week 13	2	69	9	1	65	1	9	9	94%	0%	15%	2%	2%	9.8	8.3	7.0	0.15
Week > 13	2	38	4	0	12	1	4	4	100%	0%	10%	4%	0%	1.4	8.8	3.1	0.00
Sum weeks 8-62	6	67	8	1	46	2	6	6	97%	0%	11%	4%	1%	6.5	9.3	5.2	0.06
All	18	84	10	2	28	2	10	9	95%	2%	12%	6%	3%	3.5	9.5	3.0	0.23

Study arm(s), for those biopsied week 4:

A (12MP/tet)	3	80	10	2	25	2	13	12	93%	1%	15%	6%	2%	2.0	9.9	2.8	0.17
B (Cy/12MP/tet)	2	66	6	1	11	1	7	6	97%	1%	9%	6%	2%	2.3	10.8	2.2	0.29
C (12MP/6MHP)	3	119	16	5	16	2	10	10	94%	5%	10%	13%	6%	1.6	8.9	1.1	0.57
D (Cy/12MP/6MHP)	4	96	8	3	24	1	15	14	93%	4%	14%	3%	3%	2.2	9.2	2.8	0.23
A+B (12MP/tet)	5	74	8	2	19	1	10	10	95%	1%	13%	6%	2%	2.1	10.2	2.6	0.22
C+D (12MP/6MHP)	7	106	12	4	20	1	13	12	94%	5%	12%	7%	4%	2.0	9.1	2.0	0.37

Table 2

PNAd and Chemokines CXCL12, CXCL13, and CCL21 in the vaccine site microenvironment after injection of peptides in IFA

Patient subsets: Interval from 1 st vaccine to biopsy	N	# PNAd+	% PNAd+	Mean# PNAd+ vessels / HPF	# with CXCL13 + cells	% CXCL13+	Intensity of CXCL12+ dermal immune cells (0-3) mean	Intensity of CXCL12+ dermal vessels (0-3)	Intensity of CXCL12+ epidermal DC (0-3)	CCL21+ dermal vessels/hpf	CCL21+ dermal immune cells/hpf (median)	# with CCL21+ immune cells
Week 4	12	8	67%	2.3	8	67%	3	3	3	1	56	11
Week 8	2	1	50%	1	2	100%	1.5	2	2	4	16	2
Week 13	2	2	100%	5	2	100%	2	3	1.5	2	34	2
Week > 13	2	1	50%	0.5	1	50%	2	3	2	3.5	67	2
Sum weeks 8-62	6	4	67%	2.3	5	83%	2	3	3	3.2	35	6
All	18	12	67%	2.2	13	72%	3	3	1.5	1.7	53	17
Study arm(s), for those biopsied week 4 (means):												
A (12MP/tet)	3	2	67%	3.3	2	67%	3	3	3	0.7	102	3
B (Cy/12MP/tet)	2	1	50%	2	1	50%	2	1.5	2.5	1.5	5	1
C (12MP/6MHP)	3	1	33%	1	2	67%	3	3	3	1	52	3
D (Cy/12MP/6MHP)	4	4	100%	2.8	3	75%	3	3	2.5	1	65	4
A+B (12MP/tet)	5	3	60%	2.8	3	60%	3	3	3	1	10	5
C+D (12MP/6MHP)	7	5	71%	2	5	71%	3	3	3	1	60	7