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A Combination of Local Inflammation and Central Memory T Cells Potentiates Immunotherapy in the Skin

Salvatore Fiorenza,* Tony J. Kenna,* Iain Comerford,† Shaun McColl,† Raymond J. Steptoe,* Graham R. Leggatt,* and Ian H. Frazer*

Adoptive T cell therapy uses the specificity of the adaptive immune system to target cancer and virally infected cells. Yet the mechanism and means by which to enhance T cell function are incompletely described, especially in the skin. In this study, we use a murine model of immunotherapy to optimize cell-mediated immunity in the skin. We show that in vitro–derived central but not effector memory-like T cells bring about rapid regression of skin-expressing cognate Ag as a transgene in keratinocytes. Local inflammation induced by the TLR7 receptor agonist imiquimod subtly yet reproducibly decreases time to skin graft rejection elicited by central but not effector memory T cells in an immunodeficient mouse model. Local CCL4, a chemokine liberated by TLR7 agonism, similarly enhances central memory T cell function. In this model, IL-2 facilitates the development in vivo of effector function from central memory but not effector memory T cells. In a model of T cell tolerogenesis, we further show that adoptively transferred central but not effector memory T cells can give rise to successful cutaneous immunity, which is dependent on a local inflammatory cue in the target tissue at the time of adoptive T cell transfer. Thus, adoptive T cell therapy efficacy can be enhanced if CD8+ T cells with a central memory T cell phenotype are transferred, and IL-2 is present with contemporaneous local inflammation. The Journal of Immunology, 2012, 189: 000–000.

Epithelial cancers and chronic viral infections at epithelial surfaces represent a significant cause of morbidity and mortality worldwide (1, 2), for which adoptive transfer of ex vivo–activated Ag-specific T cells is mooted as potential therapy. Adoptive T cell transfer, which uses the specificity of the TCR, conveys an advantage over immunization, because individual Ag-specific CD8+ T cells of high affinity can be expanded ex vivo to produce large numbers of cytotoxic effector precursors. Adoptive cell therapy holds great promise for the treatment of epithelial cancers and chronic viral infections (3). However, the reported success rates for adoptive cell therapy in humans are highly variable, ranging between 20 and 70% (4–6). The variable response observed to adoptive immunotherapy suggests a need to better understand the requirements for optimization of in vivo outcomes after adoptive transfer of T cells.

Once activated, naive CD8+ T cells differentiate into short-lived CD44hiCD62Llo effector and effector memory T cells (T Eff/T EM), or lymphoid organ-residing CD44hiCD62Lhi central memory T cells (T CM) (3). Previous work investigating the means by which to optimize T cell therapy has shown that, although T Eff/T EM demonstrate the capacity for rapid target lysis, the in vivo durability of T CM make this cell type best suited for use in adoptive cell therapy (7, 8). However, the mechanisms underlying enhanced outcomes with T CM and the means by which to further augment the function of T CM are poorly understood. Furthermore, the relative potential of T EM and T CM in adoptive cell therapy targeted to the skin has not been described.

CD8+ T cell–mediated immunity occurs in a tissue-specific manner. CD8+ T cell activation via s.c. immunization, for example, induces activated T cells that home to skin (9). Thus, optimization of immunotherapy for cutaneous viral infection and malignancy requires an understanding of the immunobiology of adoptively transferred T cells in skin-specific immunotherapy. We have used a system in which murine skin expressing a model Ag is grafted onto a naive host to study the requirements for effective skin-targeted immunotherapy (10–13). In this system, a model Ag, such as OVA or the viral oncogene E7, is expressed as a transgene in keratinocytes under the control of basal keratinocyte-specific promoters for the genes keratin 5 or keratin 14. In the case of OVA, this system models the immune consequences of Ag expression in epithelial cells without the confounding effects of tumor-related immune modulation. Rejection of the transgenic graft is easily visualized and is a measure of local immune effector function (12). This system allows investigation of induction of immune responses by Ag expressed in skin and effector functions elicited within this site by endogenous or adoptively transferred naive T cells (10–13). In this study, we investigate the capacity of different populations of ex vivo–activated memory T cells to elicit immune effector functions in the skin and the requirements for an optimal response.

Materials and Methods

Mice

C57BL/6 and Rag1−/− mice were sourced from the Animal Resources Centre (WA, Australia). TCR transgenic mice specific for H-2Kb–bound SIINFEKL peptide (OT-I mice) were originally sourced from W. R. Heath (University of Melbourne, Parkville, VIC, Australia). K5mOVA transgenic C57BL/6 mice, in which OVA, is expressed under control of the keratin 5

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The online version of this article contains supplemental material.

Abbreviations used in this article: T CM, central memory T cell; T Eff, effector T cell; T EM, effector memory T cell; T Reg, regulatory T cell.

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promoter, were provided by H. Azukizawa (Osaka University, Osaka, Japan) (14). CD11c-OVA mice, in which OVA expression is under the CD11c promoter, were bred at the Biological Resources Facility (Brisbane, QLD, Australia). All mice were housed under specific pathogen-free conditions, used at 6–10 wk of age, and sex-matched for all experiments. All animal procedures were approved by the University of Queensland Animal Ethics Committee.

Flow cytometry and intracellular Ag staining

mAbs to murine CD8, CD3, CD44, CD62L, IFN-γ, T-bet, IL-2, and associated Ig controls were purchased from BD Biosciences (San Jose, CA) and eBioscience (San Diego, CA) and used as per manufacturers’ protocol. For intracellular cytokine staining, cells were stimulated with 25 ng/ml PMA (Sigma-Aldrich, Castle Hill, NSW, Australia) and 1 μg/ml ionomycin (Sigma-Aldrich) and monensin (BioLegend, San Diego, CA). Permeabilization, fixation, and staining were performed as per the manufacturer’s instructions (Mouse Intracellular Cytokine Staining Kit; BD Biosciences). Staining of the transcription factor T-bet was performed with fixation and permeabilization reagents from the Foxp3 staining kit as per the manufacturer’s instructions (BD Biosciences). Data were acquired using the FACS Calibur flow cytometer (BD Biosciences) and analyzed using FlowJo version 8.7.3 (Tree Star, Ashland, OR).

Skin grafting

Ear skin was grafted onto the flanks as described previously (12, 15). Briefly, donor ear epidermis was placed onto graft beds of 1 cm² on the flanks of anesthetized mice and fixed with Elastoplast fabric strips (Beiersdorf, North Ryde, NSW, Australia). Bandages were removed 7 d later, and grafts were allowed to heal completely for 6 wk during which time all inflammatory cytokines return to resting levels (16, 17). Grafts were considered rejected when >80% of the graft area was visibly ulcerated and necrotic.

Imiquimod treatment

Skin grafts were treated with 30 mg 5% imiquimod cream (Aldara; iNova Pharmaceuticals, Thornleigh, NSW, Australia) under occlusive dressing for 10 consecutive days or until rejection occurred (15). Aqueous cream (Sorbolene; Redwin Skin Care, Moorabbin, VIC, Australia) was applied as a control.

Central and effector memory T cell culturing and adoptive transfer

An existing protocol was modified for in vitro differentiation of TCM and TEM from wild-type, Ag-naive OT-I mice at 6–8 wk of age (18). For differentiation of TEM, splenocytes from naive OT-I mice were cultured overnight with 100 ng/ml SIINFEKL peptide, washed, and then grown with 20 ng/ml rIL-2 (PeproTech, Rocky Hill, NJ). TCM were differentiated similarly using lymphocytes from naive OT-I mice and following peptide exposure, were cultured with 10 ng/ml rIL-7 and IL-15 (PeproTech). All cells were grown in RPMI 1640 medium (Gibco, Carlsbad, CA) supplemented with 10% FCS (Bovogen, Keilor East, VIC, Australia). Prior to tail vein adoptive transfer, nonviable cells were eliminated by centrifugation with Histopaque 1077 as per the manufacturer’s instructions (Sigma-Aldrich).

Lymphocyte purification from skin and liver

Skin grafts were excised and incubated in 1 mg/ml collagenase at 37°C for 3 h with mechanical dissociation. Cells were then strained and resuspended in buffer containing 5% FCS. Single-cell suspensions of liver cells were purified with a 32% Percoll gradient (GE Healthcare, Uppsala, Sweden) as per the manufacturer’s instructions, and RBCs were removed with the use of ACK (Ammonium-Chloride-Potassium) Lysing Buffer (Life Technologies, Grand Island, NY).

mAb production and treatment

Anti-CD8 (Ab 53-5.8) was produced from hybridoma cell lines using a sequential serum dilution technique as described previously (15). For grafting experiments, 100 μg 53-5.8 in PBS was injected i.p. on the day prior to skin grafting. On days 2 and 7, postgrafting animals were treated with an additional 150 μg 53-5.8. On day 9 postgrafting, CD8+ T cell depletion was assessed by analysis purification of leukocytes from a peripheral blood sample using CD3 and CD8α. Anti-IL-2 rat IgG mAbs from clones S4B6 and JES6-1A12 were sourced from BioXCell (West Lebanon, NH), a fermentation and purification service that produces endotoxin-negative in vivo–verified Abs. One hundred microliters of both S4B6 and JES6-1A12 were injected daily for 10 d from the day of cell transfer as described previously (19).

In vitro cytotoxicity assay

Cytotoxicity assay was performed as described previously (20). EL4 H-2Kb expressing thymoma cells were labeled with 100 μCi 35Cr with or without 1 μM SIINFEKL peptide. Labeled cells were then incubated for 2.5 or 5 h with TEM. TEM, media (spontaneous release control), or 5% Triton X-100 (maximum release control). Cells were then spun down, and supernatant was dried onto a Lumaplate-96 Microplate overnight (Packard, CT) and scintillation detected by TopCount NXT (PerkinElmer, Wellesley, MA). Percent lysis was calculated from means of triplicate rows as follows: [(sample – spontaneous)/(maximum – spontaneous)] × 100%.

Chemokine injection and chemokine receptor antagonists

All recombinant chemokines were sourced from PeproTech. The chemokine antagonist CCL5 (5–68) and inert control peptide CCL2 (AA5) were synthesized as described previously (21). Mice were treated with 100 μg CCL5 (5–68) or the inert control peptide CCL2 (AA5) via i.p. injection every other day following day 4 postgrafting. The in vivo activity of this antagonist has been validated as described previously (22).

Statistics

Skin graft survival curves were depicted on Kaplan–Meier curves and log-rank tests performed to assess statistical significance differences in survival. All data were analyzed by Prism Version 4 (GraphPad, San Diego, CA).

Results

Ag-specific CD8+ T cells are necessary and sufficient for K5mOVA skin graft rejection

Rejection of skin grafts expressing non–self-Ag from a keratin promoter has been used to study T cell immunotherapy (12, 15, 23). We studied the capacity of T cells to mediate rejection of skin grafts expressing OVA from the keratin 5 promoter (henceforth, K5mOVA mice) (14). Newly placed K5mOVA skin grafts were rejected spontaneously by naive, immunocompetent C57BL/6 mice but not by lymphodeplete Rag1−/− animals and not by wild-type animals depleted of cells expressing the CD8 chain, confirming that K5mOVA graft rejection is dependent on CD8+ T cell effector function (Fig. 1A). Transfer of TCR transgenic T cells specific for the H-2Kb-bound dominant OVA epitope, SIINFEKL (Rag-OT-I), but not of TCR transgenic T cells specific for the H-2Kb allo-epitope, SIYRYGL (Rag-2C), induces graft rejection in Rag1−/− animals bearing healed K5mOVA grafts, confirming that Ag-specific CD8+ T cells are sufficient for induction of graft rejection (Fig. 1B).

A local inflammatory cue enhances skin graft rejection by activated CD8+ T cells

Using a papillomavirus Ag, HPV16 E7 protein, expressed as a transgene in skin grafts, we have previously demonstrated that healed grafts are less effectively rejected by transferred Ag-specific T cells than newly placed grafts (12). To investigate whether local inflammation alters the efficiency of Ag-experienced CD8+ T cells in skin graft rejection, mice were primed to OVA by placement of a K5mOVA graft, which was rejected. Subsequently, these mice were depleted of CD8+ T cells, and given two additional K5mOVA grafts, which were not rejected. After T cell recovery, a third K5mOVA graft was given, and one of the two well-healed grafts was treated with imiquimod. The newly placed graft was rejected, confirming restoration of functional OVA-specific T cells. The imiquimod-treated healed graft was rejected more rapidly than the untreated graft (Fig. 1C), demonstrating that local inflammation contributes to immune effector function. To confirm that imiquimod alone does not induce rejection of K5mOVA skin, Rag1−/− mice bearing well-healed K5mOVA grafts were treated with imiquimod or control cream; neither resulted in grafts being rejected (Supplementary Fig. 1). To investigate the mechanism by which local inflammation could enhance CD8+ effector function, we first studied whether activated,
control cream. Weeks later, animals received a third contralateral graft, and one of the two original grafts was treated for 10 d with imiquimod, whereas the other received graft rejection in the absence of other lymphocytes. Adoptive transfer of 10⁶ SIINFEKL-specific Rag.OT-I splenocytes or SIYRYYGL-specific Rag.2C splenocytes. Grafts were then monitored for rejection.

To compare the capacity of TEM and TCM T cell subsets to induce skin graft rejection, and the extent to which this was enhanced by local inflammation, Rag1⁻/⁻ mice bearing two well-healed K5mOVA skin grafts received in vitro–differentiated TCM or TEM, and one of the two grafts was treated with topical imiquimod. Rejection of the K5mOVA skin grafts was significantly quicker in recipients of TEM than in recipients of equivalent numbers of TEM (Fig. 3). Furthermore, grafts treated with topical imiquimod on animals recipient of TEM showed accelerated rejection compared with untreated grafts, whereas this was not observed for animals recipient of TEM. The effect of rapid TCM-mediated graft rejection augmented by imiquimod was consistently observed across a range of transferred OT-I TCM phenotype (Supplemental Fig. 2).

Local TLR7 ligation augments cutaneous immunotherapy elicited by TCM but not TEM

We next aimed to determine which subset of adoptively transferred, Ag-specific CD8⁺ T cells could promote graft rejection in the absence of other lymphocytes. Rag1⁻/⁻ animals received two K5mOVA skin grafts and were allowed to heal for 6 wk. Following healing, grafted animals received an adoptive transfer of OT-I splenocytes and s.c. immunization with OVA/Quil A. When one of the two healed K5mOVA grafts was treated with topical imiquimod, rejection of the treated graft was significantly faster than rejection of the untreated graft across a range of OT-I cell precursor frequencies (Fig. 1D–F). The putative TEM population expressed high levels of T-bet, whereas the TCM and naive cell populations exhibited intermediate and low levels of T-bet, respectively (Fig. 2F). In vitro–differentiated TCM exhibited enhanced in vitro killing capacity as compared with TEM (Fig. 2I, 2I). In vitro–differentiated memory T cell populations, therefore, demonstrated the functional and phenotypic attributes held characteristic of TCM and TEM-like cells.

We next investigated whether TCM-mediated graft rejection was a consequence of prolonged OT-I T cells survival in vivo. In vitro–differentiated TCM and TEM were transferred into Rag1⁻/⁻ mice bearing well-healed K5mOVA skin grafts. Given that lymphodeplete environments can prolong survival of transferred T cells through the availability of homeostatic cytokines (24), TCM and TEM were also transferred into lymphodeplete Rag2.2C mice bearing well-healed K5mOVA skin grafts – Rag2.2C mice possess a complement of functional T cells solely specific for the SIYRYYGL alloepitope. Forty-five days following transfer, the spleen, lymph nodes, and the liver were harvested, processed into single-
cell suspensions, stained for CD8, Vα2, and CD62L, and analyzed by flow cytometry. Fig. 4A, 4C, and 4E show that TCM are present in larger numbers than TEM 45 d following transfer into either lymphodeplete Rag1^−/− or lymphoreplete Rag.2C mice. Consistent with memory T cell phenotype, transferred TCM and TEM differentiate into CD62L^hi (present in lymph nodes) and CD62L^lo (present in liver) T cells, indicating that these two cell types can trans-differentiate in vivo following transfer (Fig. 4B, 4D, 4F).

**Inhibition of IL-2 mitigates enhanced rejection by TCM**

Autocrine production of IL-2 has been shown to drive the secondary expansion and survival of TCM (25). Fig. 5A and 5B show that in vitro–differentiated TCM but not TEM produce large amounts of IL-2 upon stimulation with PMA/ionomycin. We thus hypothesized that IL-2 played a role in enhancing TCM-mediated graft rejection. Treatment with IL-2–inhibiting mAbs JES6-1 and S4B6 was associated with significantly delayed well-healed K5mOVA graft rejection in recipients of TCM but not TEM (Fig. 5C). Furthermore, inhibition of IL-2 binding to the high-affinity IL-2Rα, CD25, by JES6-1 alone also delayed TCM-mediated skin graft rejection to that same extent as combination of JES6-1 and S4B6, which blocks IL-2 completely (Fig. 5D). This confirms a role for autocrine IL-2 signaling through the trimeric high-affinity IL-2R complex in the acquisition of effector function by TCM but not TEM.

Lymphoreplete environments have been reported to inhibit adoptive cell therapy by decreasing the availability of γ-chain cytokines to adoptively transferred, effector-phenotype cells (24). Given that TCM are able to produce the γ-chain cytokine, IL-2, we proposed that TCM but not TEM would reject skin grafts in lymphoreplete environments. Fig. 5E shows that TCM but not TEM-rejected well-healed K5mOVA skin grafts in lymphoreplete Rag.2C animals.

**Intradermal CCL4 but not CCL2 nor CXCL2 is sufficient to recapitulate the effect of imiquimod on TCM-mediated graft rejection**

We next examined the mechanism by which imiquimod enhances TCM-mediated graft rejection. Imiquimod treatment of the flank of C57BL/6 mice induces a dense inflammatory infiltrate into the epidermis and dermis (Fig. 6A, 6B). To assess whether imiquimod increased trafficking of TCM-derived cells into inflamed skin, Rag1^−/− mice bearing two well-healed K5mOVA skin grafts were adoptively transferred with 10^5 CFSE-labeled TCM, and one of the two grafts was treated daily with imiquimod. Analysis of single-cell suspensions of skin grafts by flow cytometry on day 5 posttransfer showed imiquimod increased T cell traffic into imiquimod-inflamed skin. All cells analyzed were CFSE negative, indicating that these cells were divided progeny of transferred TCM, because Rag1^−/− mice have no endogenous T cells (Fig. 6C).

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**FIGURE 2.** Ex vivo–differentiated TCM and TEM exhibit extracellular markers, intracellular molecules, and killing capacity characteristic of TCM and TEM. (A and B) CD44 and CD62L staining of OT-I in vitro–derived TCM (black lines) and TEM (gray lines) cultured as per Materials and Methods. Isotype control shown in filled gray histograms. (C–E) Intracellular IFN-γ, TNF-α, and granzyme B expression in in vitro–differentiated TCM and TEM following stimulation with PMA/ionomycin. TCM shown as black lines, TEM shown as gray lines, and unstimulated cells shown as filled gray histograms. (F) Intracellular expression of the T-box transcription factor T-bet. TCM shown as black lines, TEM shown as gray lines, and naive cells shown as filled gray histograms. (G and H) Micrograph of OT-I TEM (G) and TCM (H). Photos represent one of two individual experiments using pooled lymphocytes. Scale bar, 20 μm. (I and J) In vitro cytotoxic killing assays of TCM (black lines) and TEM (gray lines) demonstrating chromium release following 2.5 h (I) and 5 h (J) of exposure to SIINFEKL-pulsed (solid lines) and nonpulsed targets (broken lines). **p < 0.01 by one-way ANOVA test with Bonferroni post hoc analysis. Target lysis calculated by ([sample − spontaneous]/[maximum − spontaneous]) × 100%.
We next hypothesized that local inflammation could enhance graft rejection in part via inflammatory chemokines. We have previously shown in a microarray screen that local imiquimod application upregulates the expression of the chemokines CCL2, CXCL2, and CCL4, all of which are known to induce T cell trafficking (15, 26, 27). To determine whether chemokines alone could enhance graft rejection, one of two grafts on Rag1<sup>−/−</sup> animals bearing well-healed K5mOVA grafts that received in vitro–differentiated TCM were injected s.c. with rCCL2, CXCL2, or CCL4, and the other with vehicle control. Only CCL4 accelerated graft rejection.

**FIGURE 3.** In vitro–differentiated central but not effector memory CD8<sup>+</sup> T cell rejection of skin grafts is enhanced by a local inflammatory stimulus. Rag1<sup>−/−</sup> mice bearing two well-healed K5mOVA grafts received either in vitro–differentiated TCM (black lines) or TEM (gray lines). Grafts were then treated with either imiquimod or control cream, and graft rejection was plotted on a Kaplan–Meier survival curve. (A) A total of 10<sup>4</sup> adoptively transferred TCM and TEM CD8<sup>+</sup> T cells. Data were analyzed by a log-rank test, n = 7 for each group, "p < 0.05. (B) A total of 10<sup>5</sup> adoptively transferred TCM and TEM CD8<sup>+</sup> T cells. Data were analyzed by a log-rank test, n = 7 for each group. **"p < 0.01. (C) A total of 10<sup>6</sup> adoptively transferred TCM and TEM CD8<sup>+</sup> T cells. Data were analyzed by a log-rank test, n = 8 for each group. **"p < 0.01.

**FIGURE 4.** T<sub>CM</sub> persist to greater extent than T<sub>EM</sub> in both Rag1<sup>−/−</sup> and Rag.2C mice. Rag1<sup>−/−</sup> (filled symbols) and Rag.2C (open symbols) mice bearing well-healed K5mOVA grafts were adoptively transferred with 10<sup>6</sup> TCM (circles, black lines on histogram) or TEM (squares, gray lines on histogram). Forty-five days following transfer, spleen (A, B), pooled inguinal, brachial, axillary, and mesenteric lymph nodes (C, D) and liver (E, F) were harvested and cells stained for CD8, V<sub>a</sub>2, and CD62L. (A, C, E) CD8<sup>+</sup>V<sub>a</sub>2<sup>+</sup> cells as percentage of total cells in each organ. n = 4/group. **"p < 0.01 when comparing Rag1<sup>−/−</sup> to Rag1<sup>−/−</sup> and Rag.2C to Rag.2C; analyzed by one-way ANOVA analysis with post hoc multiple comparison test. (B, D, F) Representative CD62L expression of CD8<sup>+</sup>V<sub>a</sub>2<sup>+</sup>CD44<sup>+</sup> in various organs analyzed by flow cytometry 45 d following transfer into Rag1<sup>−/−</sup> mice.
either 105 TCM or TEM treated for 10 d with i.p. injections of PBS or IL-2–expressing in vitro–differentiated TCM and TEM. Data from three individual experiments. Data were analyzed by a Student t test. Error bars represent SEM. ***p < 0.001. (C and D) Kaplan–Meier survival curve of well-healed K5mOVA skin grafted on Rag1−/− mice, which received an adoptive transfer of either 105 TCM or TEM treated for 10 d with i.p. injections of PBS or 100 μg of the IL-2–blocking mAbs Jes6-1 and S4B6 (C) or Jes6-1 alone or PBS (D). Animals were then monitored for graft rejection. n = 8 for TCM and n = 7 for TEM. **p < 0.01. Data were analyzed by a log-rank test. (E) Rag.2C mice bearing well-healed K5mOVA skin grafted were adoptively transferred with 105 TCM or TEM. Following transfer, grafts were monitored daily for graft rejection. n = 8/group. ***p < 0.001. Data were analyzed by a log-rank test.

If CCL4 was the only determinant of imiquimod-enhanced TCM-mediated graft rejection, then we predicted that blockade of the dominant CCL4 receptor CCR5 would reverse imiquimod-enhanced graft rejection. However, inhibition of CCR5, using a small peptide antagonist (CCL5[9–68]) failed to alter the effects of imiquimod on TCM-mediated graft rejection (Fig. 6H), even though this antagonist is able to inhibit CCL4-mediated migration of CD8+ T cells in vitro and T cell–dependent delayed-type hypersensitivity reactions in vivo (Fig. 6I, 6J).

Local TLR7 stimulation and adoptive transfer of T cells are sufficient to overcome tolerance of skin-expressed Ag

Therapy mediated by adoptively transferred Ag-specific T cells may be inhibited by tolerance (28–33). To investigate whether local inflammation might overcome tolerance induced in adoptively transferred T cells, we used CD11c.OV A mice, in which expression of OV A in dendritic cells from the CD11c promoter in dendritic cells induces tolerance in adoptively transferred OVA-specific memory T cells (34–36). K5mOVA skin grafts were not rejected by CD11c.OV A mice, consistent with the impaired response of this transgenic mouse strain to OV A (Fig. 7A) (34). CD11c.OV A mice bearing bilateral well-healed K5mOVA skin grafts received an adoptive transfer of either TCM or TEM, and one graft was treated daily with imiquimod. Animals that received OVA-specific TCM rejected only the grafts treated with imiquimod, whereas animals that received OVA-specific TEM did not reject grafts, whether treated with imiquimod.

OVA-specific T cells transferred to CD11c.OV A mice more than 4 wk previously are fully tolerated (34). To demonstrate whether such tolerance could be overcome by local proinflammatory signals, we grafted CD11c.OV A mice with K5mOVA skin. When grafts were healed, animals received OVA-specific TCM by adoptive transfer. After an additional 30-d grafts were treated with imiquimod, and no rejection was observed (Fig. 7B). Thus, once tolerance is established, local inflammation can no longer establish a successful adaptive immune response mediated by TCM.

Discussion

In this study, we demonstrate that cells differentiated in vitro to acquire TCM characteristics exhibit more rapid in vivo cutaneous immune effector function, manifest as rejection of transgenic skin, than those differentiated to TEM; Rapid TCM- but not TEM-mediated graft rejection is mitigated by the inhibition of IL-2. We further show that TCM-mediated graft rejection is enhanced by local inflammation induced by a TLR7 ligand or by the local provision of the chemokine CCL4. In contrast, graft rejection mediated by TEM is not significantly enhanced by TLR7 ligation. Local inflammation in skin allows TCM effector capacity in a tolerogenic environment that is otherwise induced by presentation of cognate Ag by APC.

The long-lived nature and rapid responses of memory T cells has led to the proposal that these cells are the most appropriate for T cell–based immunotherapies (37). The ability of local inflammation to enhance the development of fully competent T effector cells from memory T cell populations, as demonstrated in this study using parallel transgenic graft targets, has only recently been recognized, yet no studies have investigated the effect of local inflammation on TCM- and TEM-mediated immune responses (38–41). Local TLR7 ligation augments rejection of human growth hormone transgenic skin grafts from a graft-primed animal (15). Well-healed grafts expressing the E7 protein of human papillomavirus are, unlike newly placed and inflamed grafts, protected against rejection by E7-specific CTL passively transferred and...
primed in vivo (12). In this study, we show that time to effective TCM-mediated immune response in skin is decreased by local inflammation, whereas TEM-mediated immune responses are not affected. Traditionally, TEM, which express cytotoxic molecules, such as granzyme B, and demonstrate enhanced in vitro cytotoxicity, have been considered the ideal cell type for adoptive immunotherapy (42, 43). Yet, TCM have been reported to mediate disease in mouse model of skin graft versus host disease (44), and TCM mediate regression of transplanted melanoma in mice more effectively than TEM (45), which is considered a product of their prolonged in vivo persistence (46). Likewise, recently described human and murine T memory stem cells possess prolonged in vivo persistence that has been attributed to comprehensive regression of established tumors in mice and may hold future therapeutic promise in humans (47, 48).

We have previously demonstrated generation of enhanced effector function for skin graft rejection following systemic administration of the TLR4 agonist LPS in wild-type mice (49). In this study, we show that an inflammatory cue provided at the effector site decreases the time to CD8+ TCM-mediated attack in immunodeficient mice. Although the effect of imiquimod in enhancing TCM- but not TEM-mediated graft rejection is subtle, the effect is consistently reproducible across multiple different cell precursor frequencies and multiple conditions. Rejection of skin grafts in the tolerogenic CD11c.OVA environment is, furthermore, dependent on a local inflammatory cue. This may indicate that

FIGURE 6. Imiquimod enhances inflammatory infiltrate and traffic of adoptively transferred CD8+ T cells into inflamed grafts. The effects of imiquimod on TCM-mediated graft rejection are recapitulated by the chemotactic molecule CCL4. (A and B) H&E micrograph of flank skin of C57BL/6 mice treated for 5 d with imiquimod (A) or control cream (B). Scale bars, 50 μm. (C) Rag1−/− mice bearing two bilateral K5mOVA received an adoptive transfer of 10^5 CFSE-labeled skin grafts. At time of transfer, one of the two grafts was treated with imiquimod daily for 5 d, whereas the other received control cream. Grafts were then monitored by flow cytometry for CD3, CD8, and CFSE staining. n = 7/group. *p < 0.05 by a paired Student t test. (D–G) Rag1−/− bearing two well-healed K5mOVA skin grafts were adoptively transferred with TCM and treated with the CCR5-inhibiting peptide CCL5(9–68) or the nonfunctional mutated chemokine CCL2(4Ala), whereas one of the two grafts was treated with imiquimod. Grafts were then monitored for rejection. n = 8 for each experiment. **p < 0.01; log-rank test. (H) Two groups of Rag1−/− mice bearing two well-healed K5mOVA skin grafts were adoptively transferred with TCM and treated with the CCR5-inhibiting peptide CCL5(9–68) or the nonfunctional mutated chemokine CCL2(4Ala), whereas one of the two grafts was treated with imiquimod. Grafts were then monitored for rejection and plotted on a Kaplan–Meier survival curve. n = 8 for all groups. ***p < 0.001, log-rank test. (I) Transwell migration assay of purified CD8+ T cells stimulated with CD3 and CD28 mAbs, migrating in response to CCL4. Inhibition of T cell migration by CCL5(9–68) was evaluated by increasing concentration of CCL5(9–68) and assessing the percentage of migrated cells relative to wild-type CCL4. Nonfunctional CCL2(4Ala) peptide was used as control. n = 2. *p < 0.05 by one-way ANOVA. (J) Delayed-type hypersensitivity reaction was established by tail-base immunization of C57BL/6 mice with OVA in CFA. Seven days later, a recall was conducted in a footpad, and an increase in footpad thickness was assessed by calipers. To assess for in vivo activity of CCL5(9–68), 1 d prior to recall animals were treated with CCL5(9–68) or control peptide CCL2(4Ala). Data are presented as mean ± SEM. Data were analyzed by a Student t test. n = 12/group. *p < 0.05.
the primary role of inflammation in augmenting effector T cell responses is in influencing other adaptive immune cells. Local enhancement of effector function by local proinflammatory signaling through TLRs might also reflect increased recruitment of effectors (41), increased proliferation and maturation (50) of effector precursors, and/or induction of helper functions that override local inhibitors in skin (51). Local inhibitors include the anti-inflammatory cytokines IL-10 and TGF-β; NKT cells are also inhibitory in skin through an IFN-γ-dependent mechanism (10, 11, 52, 53).

Imiquimod can induce T cell migration to inflamed sites presumably through upregulation of chemokines and cellular adhesion molecules (15, 54–57). CCL4, CCL2, and CXCL2 expression is increased in imiquimod-inflamed skin (15), we show in this paper that only intradermal injections of CCL4 but not CCL2 and not CXCL2 are able to recapitulate the effects of imiquimod. However, inhibition of CCL4 through a small peptide antagonist that binds to the dominant CCL4 receptor CCR5 does not reverse imiquimod-augmented graft rejection. The lack of effect of the CCR5 antagonist indicates a potential redundancy in the chemokine receptors seen on TCM effector progeny and TEM.

This is of consequence given that we have shown there that inhibition of signaling through the high-affinity trimeric IL-2R complex has been shown (59). In this study, we show that CD8+ T cells differentiated in the presence of IL-15 produced IL-2, whereas TEM differentiated in the presence of IL-2 did not produce autocrine IL-2. Our data indicated that autocrine IL-2 production is a consequence of IL-15 stimulation and may play a pivotal role in the effector function of TEM and TCM. In current adoptive cell therapy strategies used in humans, IL-2 is administered along with effector–phenotype cells to prolong the persistence adoptively transferred cells (2). IL-2, however, has significant adverse effects including cardiomyopathy and pleural effusion (61). The use of TEM, which self-produce IL-2 and display enhanced in vivo persistence, may obviate the need for exogenous use of IL-2 in the clinic.

Failure of adoptive immunotherapeutic regimes may be accounted for by the tolerogenic effects of malignant disease (28, 29, 31–35, 62, 63). To address the effects of tolerance induction, we used the CD11c.OVA murine model. This mouse strain lacks a functional OVA-responsive repertoire and induces tolerance in adoptively transferred naive and memory CD8+ T cells (34–36). In this model, we show that adoptive transfer of Ag-specific T cells alone did not reject K5mOVA skin grafts. Rather, only skin grafts that were inflamed by imiquimod in animals that had received TEM were rejected. Imiquimod treatment could not overcome established tolerance as treating skin grafts with imiquimod after TEM had undergone tolerance induction failed to induce graft rejection. Other approaches to breaking or preventing tolerance have used CD40, IL-2, and CD4+ T cell-based regimens. Our method using contemporaneous proinflammatory signals and T cell transfer may
complement these (38, 64–66) and provides a means to enhance the efficacy of skin-directed adoptive immunotherapy in a potentially tolerogenic environment. Holcmann et al. (67) reported that skin inflammation alone does not break tolerance and cause skin disease when naive CD8+ T cells are adoptively transferred into animals that express tamofoxifen-inducible K5mOVA (68). This disparity may be accounted for by the observation that memory and naive CD8+ T cells display differing sensitivities to tolerance induction (34, 35, 62).

The mechanisms by which imiquimod breaks tolerance in the skin are currently under investigation. Imiquimod-inflamed skin tumors in humans possess decreased number of regulatory T cell (TReg) cells (57). Furthermore, we have shown recently that TReg expression of CD25 limits memory CD8+ T cell expansion by limiting availability of IL-2 (68). We show in this study that CD25 plays a crucial role in the rapid response of TCM in Rag1−/− model system shown in this paper. Therefore, in the CD11c.OVA mice, imiquimod may decrease TReg number, thus liberating available IL-2 for local memory T cell expansion. Mast cells may also play an important role in breaking tolerance in skin. Imiquimod increases mast cell number in the skin, whereas injection of TCM- and TEM-mediated regression directed against skin and unpublished observations).

To our knowledge, this work presents the first comparative analysis of TCM and TEM-mediated regression directed against skin and provides a mechanism underlying the rapid rejection of skin grafts expressing a foreign Ag by TEM. Furthermore, this is the first work we are aware of that provides a simple and clinically available intervention that differentially optimizes in vitro-derived TEM and TEM function in the skin in a tolerogenic environment. The clinical implications of this work for future approaches to immunotherapy are significant. Understanding the in vivo function of adoptively transferred memory T cells in the skin is particularly important given the significant morbidity and mortality posed by cutaneous chronic disease when naive CD8+ T cells are adoptively transferred into animals that express tamoxifen-inducible K5mOVA (68). This provides a mechanism underlying the rapid rejection of skin grafts expressing epitope-cell derived antigen. Immun. 184: 1242–1250.


