A combination of local inflammation and central memory T cells potentiates immunotherapy in the skin

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Abstract

Adoptive T cell therapy utilises 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. Here, we utilise a murine model of immunotherapy to optimise 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 antigen 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 of *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 that 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.

Keywords

Rodent; T Cells; cytotoxic; Inflammation; Memory; Skin

Introduction

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, antigen-specific T cells is mooted as potential therapy. Adoptive T cell transfer, which utilises the specificity of the T cell receptor, conveys an advantage over immunisation, as individual antigen 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

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immunotherapy suggests a need to better understand the requirements for optimisation of *in vivo* outcomes after adoptive transfer of T cells.

Once activated, naïve CD8$^+$ T cells differentiate into short-lived CD44^{hi} CD62L^{lo} effector and effector memory T cells (T$_{EFF}$/T$_{EM}$), or lymphoid organ-residing CD44^{hi} CD62L^{hi} central memory T cells (T$_{CM}$) (3). Previous work investigating the means by which to optimise T cell therapy has shown that, whilst 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 subcutaneous immunisation, for example, induces activated T cells that home to skin (9). Thus, optimisation 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 utilised a system in which murine skin expressing a model antigen is grafted onto a naïve host to study the requirements for effective skin-targeted immunotherapy (10–13). In this system a model antigen, such as ovalbumin 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 ovalbumin, this system models the immune consequences of antigen expression in epithelial cells without the confounding effects of tumour-related immune modulation. Rejection of the transgenic graft is easily visualised and is a measure of local immune effector function (12). This system allows investigation of induction of immune responses by antigen expressed in skin and effector functions elicited within this site by endogenous or adoptively transferred naïve T cells (10–13). Here, we investigate the capacity of different populations of *ex vivo* activated memory T cells to elicit immune effector functions in 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). T cell receptor transgenic mice specific for H-2k^b-bound SIINFEKL peptide (OT-I mice) were originally sourced from William R. Heath (University of Melbourne, VIC, Australia). K5mOVA transgenic C57BL/6 mice, in which ovalbumin (OVA), is expressed under control of the *keratin 5* promoter, were provided by H. Azukizawa (Osaka Univeristy, Osaka, Japan) (14). CD11c.OVA mice, in which OVA expression is under the CD11c promoter, were bred at the Biological Resources Facility (BRF; QLD Australia). All mice were housed under specific pathogen-free conditions, used at 6–10 weeks 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 antigen staining

Monoclonal antibodies (mAb) to murine CD8, CD3, CD44, CD62L, interferon-γ, T-bet, IL-2 and associated immunoglobulin controls were purchased from BD Bioscience (CA, USA) and eBioscience (CA, USA) and used as per manufacturer protocol. For intracellular cytokine staining, cells were stimulated with 25ng/ml phorbol 12-myristate 13-acetate (Sigma; NSW, Australia) and 1µg/ml ionomycin (Sigma; NSW, Australia) and monensin (BioLegend; CA, USA). Permeabilisation, fixation and staining were performed as per

manufacturer's instructions (Mouse Intracellular Cytokine Staining Kit; BD Biosciences). Staining of the transcription factor T-bet was performed with fixation and permeabilisation reagents from the Foxp3 staining kit as per manufacturer's instructions (BD Biosciences). Data was acquired using the FacsCalibur flow cytometer (BD Bioscience) and analysed using FlowJo version 8.7.3 (Tree Star; CA, USA).

Skin grafting

Ear skin was grafted onto the flanks as previously described (12, 15). Briefly, donor ear epidermis was placed onto graft beds of $1cm^2$ on the flanks of anaesthetised mice and fixed with Elastoplast fabric strips (Beiersdorf; NSW, Australia). Bandages were removed 7 days later and grafts allowed to heal completely for 6 weeks 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 30mg of 5% imiquimod cream (Aldara™; iNova Pharmaceuticals; NSW, Australia) under occlusive dressing for 10 consecutive days or until rejection occurred (15). Aqueous cream (Sorbolene; Redwin Skincare, 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 central (T_{CM}) and effector memory-phenotype cells (T_{EM}) from wild-type, antigen-naive OT-I mice at 6–8 weeks of age (18). For differentiation of T_{EM}, splenocytes from naïve OT-I mice were cultured overnight with 100ng/ml of SIINFEKL peptide, washed and then grown with 20ng/ml of recombinant IL-2 (Peprotech; NJ, USA). T_{CM} were differentiated similarly using lymphocytes from naïve OT-I mice and, following peptide exposure, were cultured with 10ng/ml of recombinant IL-7 and IL-15 (Peprotech; NJ, USA). All cells were grown in RPMI Medium 1640 (Gibco; CA, USA) supplemented with 10% foetal calf serum (Bovogen; VIC, Australia). Prior to tail vein adoptive transfer, non-viable cells were eliminated by centrifugation with Histopaque 1077 as per manufacturer's instructions (Sigma; NSW, Australia).

Lymphocyte purification from skin and liver

Skin grafts were excised and incubated in 1mg/ml collagenase at 37°C for 3 hours with mechanical dissociation. Cells were then strained and resuspended in buffer containing 5% foetal calf serum. Single cell suspensions of liver cells were purified with a 32% Percoll gradient (GE Healthcare; Uppsala, Sweden) as per manufacturer's instructions and red blood cells removed with the use of ACK (Ammonium-Chloride-Potassium) lysis buffer (Life Technologies; CA, USA).

Monoclonal antibody production and treatment

Anti-CD8β (antibody 53-5.8) was produced from hybridoma cell lines using a sequential serum dilution technique as previously described (15). For grafting experiments 100μg of 53-5.8 in PBS was injected intraperitoneally on the day prior to skin grafting. On days 2 and 7 post-grafting animals were treated with a further 150μg of 53-5.8. On day 9 post-grafting, CD8^{+} T cell depletion was assessed by analysis purification of leukocytes from a peripheral eye bleed stained with CD3 and CD8β. Anti-IL-2 rat IgG monoclonal antibodies from clones S4B6 and Jes6-1A12 were sourced from BioXcell (NH, USA) a fermentation and purification service which produces endotoxin-negative, *in vivo* verified antibodies. 100μg

of both S4B6 and Jes6-1A12 were injected daily for 10 days from the day of cell transfer as previously described (19).

In vitro cytotoxicity assay

Cytotoxicity assay was performed as described previously (20). EL4 H-2K^b expressing thymoma cells were labelled with 100μCi of Chromium^{51} with or without 1μM SIINFEKL peptide. Labelled cells were then incubated for 2.5 or 5 hours with T_{EM}, T_{CM}, media (spontaneous release control), or 5% Triton-X100 (maximum release control). Cells were then spun down and supernatant dried onto Lumaplate membrane overnight (Packard; CT, USA) and scintillation detected by TopCount NXT (Perkin Elmer; 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 (NJ, USA). The chemokine antagonist, CCL5$_{(9–68)}$, and inert control peptide, CCL2$_{(4Ala)}$, were synthesized as previously described (21). Mice were treated with 100μg of CCL5$_{(9–68)}$ or the inert control peptide CCL2$_{(4Ala)}$ via i.p. injection every other day following day 4 post-grafting. The *in vivo* activity of this antagonist has been validated as previously described (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 analysed by Prism Version 4 (Graphpad Software; CA, USA).

Results

Antigen specific CD8$^+$ T cells are necessary and sufficient for K5mOVA skin graft rejection

Rejection of skin grafts expressing non-self antigen 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 ovalbumin from the *keratin 5* promoter (henceforth, K5mOVA mice) (14). Newly-placed K5mOVA skin grafts were rejected spontaneously by naïve, immunocompetent C57BL/6 animals but not by lymphodeplete *Rag1*$^{−/−}$ animals, nor by wild-type animals depleted of cells expressing the CD8β chain, confirming that K5mOVA graft rejection is dependent on CD8$^+$ T cell effector function (Figure 1A). Transfer of TCR transgenic T cells specific for the H-2K^b-bound dominant ovalbumin epitope, SIINFEKL (Rag.OT-I), but not of TCR transgenic T cells specific for the H-2K^b allo-epitope, SIYRYYGL (Rag.2C), induces graft rejection in *Rag1*$^{−/−}$ animals bearing healed K5mOVA grafts, confirming that antigen-specific CD8$^+$ T cells are sufficient for induction of graft rejection (Figure 1B).

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

Using a papillomavirus antigen, HPV16 E7 protein, expressed as a transgene in skin grafts, we have previously demonstrated that healed grafts are less effectively rejected by transferred antigen-specific T cells than newly-placed grafts (12). To investigate whether local inflammation alters the efficiency of antigen-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 CD8β$^+$ T cells, and given two further 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 (Figure 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 Figure 1).

To investigate the mechanism by which local inflammation could enhance CD8+ effector function, we first studied whether activated, adoptively transferred, antigen-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 weeks. Following healing, grafted animals received an adoptive transfer of OT-I splenocytes and subcutaneous immunisation 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 (Figure 1D–F). The effect of imiquimod on graft rejection was most pronounced at the lowest number (10^5) of OT-I transferred cells indicating the capacity of imiquimod to enhance suboptimal responses. In these immunised animals, two distinct populations of activated CD8+ T cells could be demonstrated: CD44^{hi} CD62L^{lo} T cells of effector memory T (T_{EM}) phenotype, and CD44^{hi} CD62L^{hi} T cells of central memory T cell (T_{CM}) phenotype (Supplementary Figure 2).

Local TLR7 ligation augments cutaneous immunotherapy elicited by T_{CM} but not T_{EM}

We next aimed to determine which subset of adoptively transferred, activated CD8+ T cells, (T_{EM} or T_{CM}-phenotype cells) were responsible for the accelerated rejection induced by topical imiquimod. Modification of an existing protocol (18) using non-immunised wild-type OT-I lymphocytes was implemented for the *ex vivo* production of T_{CM} and T_{EM}. OT-I cells differentiated in the presence of IL-7 and IL-15 were CD44^{hi} CD62L^{hi} and expressed interferon-γ and tumour necrosis factor-α but failed to express granzyme B after stimulation with PMA/ionomycin, consistent with a T_{CM}-like phenotype (Figure 2A–E). In contrast, cells differentiated in the presence of IL-2 showed a morphology consistent with fully differentiated effector/effector memory T cell (compare Figure 2G and H) and were CD44^{hi} and CD62L^{lo} T_{EM}-like cells and expressed high levels of interferon-γ, granzyme B and tumour necrosis factor-α (Figure 2A–E). The putative T_{EM} population expressed high levels of T-bet, whilst the T_{CM} and naïve cell populations expressed intermediate and low levels of T-bet, respectively (Figure 2F). *In vitro* differentiated T_{EM} exhibited enhanced *in vitro* killing capacity as compared to T_{CM} (Figure 2I–J). *In vitro* differentiated memory T cell populations, therefore, demonstrated the functional and phenotypic attributes held characteristic of T_{EM} and T_{CM}-like cells.

To compare the capacity of T_{EM} and T_{CM} 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 grafts received *in vitro* differentiated T_{CM} or T_{EM}, and one of the two grafts was treated with topical imiquimod. Rejection of the K5mOVA skin grafts was significantly quicker in recipients of T_{CM} than in recipients of equivalent numbers of T_{EM} (Figure 3A–C). Further, grafts treated with topical imiquimod on animals recipient of T_{CM} showed accelerated rejection compared with untreated grafts, whereas this was not observed for animals recipient of T_{EM}. The effect of rapid T_{CM}-mediated graft rejection augmented by imiquimod was consistently observed across a range of transferred OT-I T_{CM} and T_{EM} frequencies.

T_{CM} persist to a greater extent than T_{EM} in both lymphodeplete and lymphoreplete environments

We next investigated whether T_{CM}-mediated graft rejection was a consequence of prolonged OT-I T cells survival *in vivo*. *In vitro* differentiated T_{CM} and T_{EM} 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 (25), T_{CM} and T_{EM} were also transferred into lymphoreplete Rag.2C mice bearing well-healed K5mOVA skin grafts – Rag.2C mice possess a complement of functional T cells solely specific for the SIYRYYGL alloepitope. 45 days following transfer spleen, lymph nodes and liver were harvested, processed into single cell suspensions, stained for CD8, Vα2 and CD62L and analysed by flow cytometry. Figure 4A , C and E show that T_{CM} are present in larger numbers than T_{EM} 45 days following transfer into either lymphodeplete $Rag1^{-/-}$ or lymphoreplete Rag.2C mice. Consistent with memory T cell phenotype, transferred T_{CM} and T_{EM} 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 (Figure 4 B, D and F).

Inhibition of IL-2 mitigates enhanced rejection by T_{CM}

Autocrine production of IL-2 has been shown to drive the secondary expansion and survival of central memory T cells (26). Figure 5A and B show that *in vitro* differentiated T_{CM} but not T_{EM} produce large amounts of IL-2 upon stimulation with PMA/Ionomycin. We thus hypothesised that IL-2 played a role in enhancing T_{CM} mediated graft rejection. Treatment with IL-2 inhibiting monoclonal antibodies JES6-1 and S4B6 was associated with significantly delayed well-healed K5mOVA graft rejection in recipients of T_{CM} but not T_{EM} (Figure 5C). Furthermore, inhibition of IL-2 binding to the high affinity IL-2 receptor α, CD25, by JES6-1 alone also delayed T_{CM}-mediated skin graft rejection to that same extent as combination of JES6-1 and S4B6 which blocks IL-2 completely (Figure 5D). This confirms a role for autocrine IL-2 signalling through the trimeric high affinity IL-2 receptor complex in the acquisition of effector function by T_{CM} but not T_{EM}.

Lymphoreplete environments have been reported to inhibit adoptive cell therapy by decreasing the availability of γ-chain cytokines to adoptively transferred, effector-phenotype cells (25). Given that T_{CM} are able to produce the γ-chain cytokine, IL-2, we proposed that T_{CM} but not T_{EM} would reject skin grafts in lymphoreplete environments. Figure 5E shows that T_{CM} but not T_{EM} 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 T_{CM}-mediated graft rejection

We next examined the mechanism by which imiquimod enhances T_{CM}-mediated graft rejection. Imiquimod treatment of the flank of C57BL/6 mice induces a dense inflammatory infiltrate into the epidermis and dermis (Figure 6A–B). To assess if imiquimod increased trafficking of T_{CM}-derived cells into inflamed skin, $Rag1^{-/-}$ mice bearing two well-healed K5mOVA skin grafts were adoptively transferred with 10^5 CFSE-labelled T_{CM} 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 post-transfer showed imiquimod increased T cell traffic into imiquimod-inflamed skin. All cells analysed were CFSE negative indicating that these cells were divided progeny of transferred T_{CM}, as $Rag1^{-/-}$ mice have no endogenous T cells (Figure 6C).

We next hypothesised that local inflammation could enhance graft rejection in part via inflammatory chemokines We have previously shown in a microarray screen that local

imiquimod application up regulates the expression of the chemokines CCL2, CXCL2 and CCL4 all of which are known to induce T cell trafficking (15, 27, 28). To determine whether chemokines alone could enhance graft rejection, one of two grafts on $Rag1^{-/-}$ animals bearing well-healed K5mOVA grafts that received *in vitro* differentiated T_{CM}, were injected subcutaneously with recombinant CCL2, CXCL2 or CCL4, and the other with vehicle control. Only CCL4 accelerated rejection of grafts mediated by T_{CM} and this was in a dose-dependent fashion (Figure 6D–G).

If CCL4 was the only determinant of imiquimod-enhanced T_{CM}-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 T_{CM}-mediated graft rejection (Figure 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* (Figure 6I–J).

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

Therapy mediated by adoptively transferred antigen specific T cells may be inhibited by tolerance (29–34). To investigate whether local inflammation might overcome tolerance induced in adoptively transferred T cells, we utilised CD11c.OVA mice, in which expression of OVA in DC from the CD11c promoter in DC induces tolerance in adoptively transferred OVA specific memory T cells (35–37). K5mOVA skin grafts were not rejected by CD11c.OVA mice consistent with the impaired response of this transgenic mouse strain to ovalbumin (Figure 7A) (35). CD11c.OVA mice bearing bilateral well-healed K5mOVA skin grafts received an adoptive transfer of either T_{CM} or T_{EM} and one graft was treated daily with imiquimod. Animals that received OVA-specific T_{CM} rejected only the grafts treated with imiquimod whereas animals that received OVA-specific T_{EM} did not reject grafts, whether or not treated with imiquimod.

OVA specific T cells transferred to CD11c.OVA mice more than 4 weeks previously are fully tolerised (35). To demonstrate whether such tolerance could be overcome by local pro-inflammatory signals, we grafted CD11c.OVA mice with K5mOVA skin. When grafts were healed, animals received OVA specific T_{CM} by adoptive transfer. After a further 30 days grafts were treated with imiquimod, and no rejection was observed (Figure 7B). Thus, once tolerance is established, local inflammation can no longer establish a successful adaptive immune response mediated by T_{CM}.

Discussion

In this study we demonstrate that cells differentiated *in vitro* to acquire T_{CM} characteristics exhibit more rapid *in vivo* cutaneous immune effector function, manifest as rejection of transgenic skin, than those differentiated to T_{EM}. Rapid T_{CM}-but not T_{EM}-mediated graft rejection is mitigated by the inhibition of IL-2. We further show that T_{CM}-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 T_{EM} is not significantly enhanced by TLR7 ligation. Local inflammation in skin allows T_{CM} effector capacity in a tolerogenic environment that is otherwise induced by presentation of cognate antigen 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 (38). The ability of local inflammation to enhance the development of fully competent T effector cells from

memory T cell populations, as demonstrated here using parallel transgenic graft targets, has only recently been recognised, yet no studies have investigated the effect of local inflammation on T_{CM} and T_{EM}-mediated immune responses (39–42). Local TLR7 ligation augments rejection of human growth hormone (hGH) 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). Here we show that time to effective T_{CM}-mediated immune response in skin is decreased by local inflammation, whereas T_{EM}-mediated immune responses are not affected. Traditionally, T_{EM}, which express cytotoxic molecules, such as granzyme B, and demonstrate enhanced *in vitro* cytotoxicity, have been considered the ideal cell type for adoptive immunotherapy (43, 44). Yet, T_{CM} have been reported to mediate disease in mouse model of skin graft versus host disease (GVHD) (45), and central memory T cells mediate regression of transplanted melanoma in mice more effectively than effector memory T cells (24), which is considered a product of their prolonged *in vivo* persistence (46). Likewise, recently described human and murine T memory stem cells (T_{SCM}) possess prolonged *in vivo* persistence that has been attributed to comprehensive regression of established tumours 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 $CD8^+$ T_{CM}-mediated attack in immunodeficient mice. Although the effect of imiquimod in enhancing T_{CM} but not T_{EM}-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 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 pro-inflammatory signalling through TLRs might also reflect increased recruitment of effectors (42), 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 here that only intradermal injections of CCL4 but not CCL2 nor CXCL2 is 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 chemo-attractive molecules dictating effector T cell migration to the skin (58). Further analyses of the differential expression of chemokines and chemokine receptors seen on T_{CM} effector progeny and T_{EM} may further elucidate such redundancy.

Enhancement of graft rejection by local inflammation was specific to T_{CM}, and inhibited by blockade of IL-2 signalling. A role for IL-2 in augmenting T_{CM} but not T_{EM}-mediated effector function has been proposed but not experimentally shown (59). Here we show that $CD8^+$ T cells differentiated in the presence of IL-15 and IL-7 but not IL-2 produce IL-2 upon stimulation. Blocking IL-2 mediated signalling with monoclonal antibodies prolongs T_{CM}-mediated effector function, manifest as delayed graft rejection where the time to rejection is similar that observed in recipients of T_{EM}. Given that autocrine production of

IL-2 has been shown to drive secondary expansion of memory T cells (26), the likely effect of IL-2 in this model is to induce T_{CM} proliferation. Signalling through the trimeric high affinity IL-2 receptor complex has been shown to cause proliferation as opposed to maintain T cell number (19). This is of consequence given that we have shown there that inhibition of signalling through the high affinity trimeric IL-2 receptor complex prolongs T_{CM}-mediated graft survival.

T_{CM}-phenotype cells induce more complete regression of transplantable melanoma than T_{EM} (24), and it has been postulated that enhanced *in vivo* reactivity against tumours by T_{CM} is a product of lymph node homing and prolonged *in vivo* survival (24, 46). In this study we confirm that T_{CM} persist to a greater extent *in vivo* than T_{EM}. We further previous observations in showing that persistence of T_{CM} is seen across multiple lymphoid organs. This is significant given that the liver has been shown to be a niche for T_{EM}/T_{EFF} T cells (60), yet in our study the number of T_{CM} in liver at day 45 following transfer is greater than that of T_{EM}. We further show that 45 days following transfer into grafted hosts, T_{CM} possess the capacity to differentiate into $CD62L^{hi}$ central memory phenotype cells. Maintenance of large populations of *in vitro* derived T cells following transfer is thought to be a product of homeostatic gamma-chain cytokines present in lymphopaenic hosts (25). We transferred T_{CM} into both lymphodeplete (*Rag1*$^{-/-}$) and lymphoreplete (Rag.2C) hosts and failed to see a significant difference in the persistence of T_{CM}, whilst the number of persisting T_{EM} was too small to conclude any significant difference *Rag1*$^{-/-}$ and Rag.2c mice. Moreover, we show that T_{EM} failed to reject grafts in Rag.2C lymphoreplete mice. Thus the capacity of T_{CM} to persist and reject skin grafts in both lymphodeplete and lymphoreplete environments may be a consequence of intrinsic T_{CM} programming and cytokine production.

Jensen and colleagues (2011) recently proposed that host-produced IL-15 contributes to persistence of adoptively transferred human T_{CM} in a lymphodeplete mouse model (8). In our study, T_{CM} differentiated in the presence of IL-15 produced IL-2, whereas T_{EM} 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 T_{CM} progeny. 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 T_{CM} 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 (29, 30, 32–36, 62, 63). To address the effects of tolerance induction we utilised the CD11c.OVA murine model. This mouse strain lacks a functional OVA-responsive repertoire and induces tolerance in adoptively transferred naïve and memory $CD8^+$ T cells (35–37). In this model, we show that adoptive transfer of antigen specific T cells alone did not reject K5mOVA skin grafts. Rather, only skin grafts that were inflamed by imiquimod in animals that had received T_{CM} were rejected. Imiquimod treatment could not overcome established tolerance as treating skin grafts with imiquimod after T_{CM} 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 pro-inflammatory signals and T cell transfer may complement these (39, 64–66) and provides a means to enhance the efficacy of skin-directed adoptive immunotherapy in a potentially tolerogenic environment. Holcmann et al. (2009) reported that skin inflammation alone does not break tolerance and cause skin disease when naïve $CD8^+$ T cells are adoptively transferred into animals that express tamoxifen-inducible K5mOVA (68). This disparity may be accounted for by the observation

that memory and naïve CD8$^+$ T cells display differing sensitivities to tolerance induction (35, 36, 62).

The mechanisms by which imiquimod breaks tolerance in the skin are currently under investigation. Imiquimod-inflamed skin tumours in humans possess decreased number of T$_{Reg}$ cells (57). Furthermore, we have shown recently that T$_{Reg}$ expression of CD25 limits memory CD8$^+$ T cell expansion by limiting availability of IL-2 (68). We show here that CD25 plays a crucial role in the rapid response of T$_{CM}$ in *Rag1*$^{-/-}$ model system shown here. Therefore, in the CD11c.OVA mice imiquimod may decrease T$_{Reg}$ 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, whilst injection of the mast cell stimulating compound 48/80 induces rejection of skin grafts in CD11c.OVA mice recipient of T$_{CM}$ (S.F. unpublished observations).

To our knowledge, this work presents the first comparative analysis of T$_{CM}$ and T$_{EM}$-mediated regression directed against skin and provides a mechanism underlying the rapid rejection of skin grafts expressing a foreign antigen by T$_{CM}$. Furthermore, this is the first work we are aware of that provides a simple and clinically available intervention that differentially optimises *in vitro* derived T$_{CM}$ but not T$_{EM}$ 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 viral infection in the skin and the potential of adoptive cell therapy to address this clinical problem (1, 3). This work posits an effective strategy for the use of *ex vivo* differentiated T$_{CM}$ in combination with a local inflammatory cue for immunotherapy in the skin in tolerogenic hosts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used in this article

T$_{CM}$	Central memory T cell
T$_{EM}$	Effector memory T cell
T$_{Reg}$	Regulatory T cell

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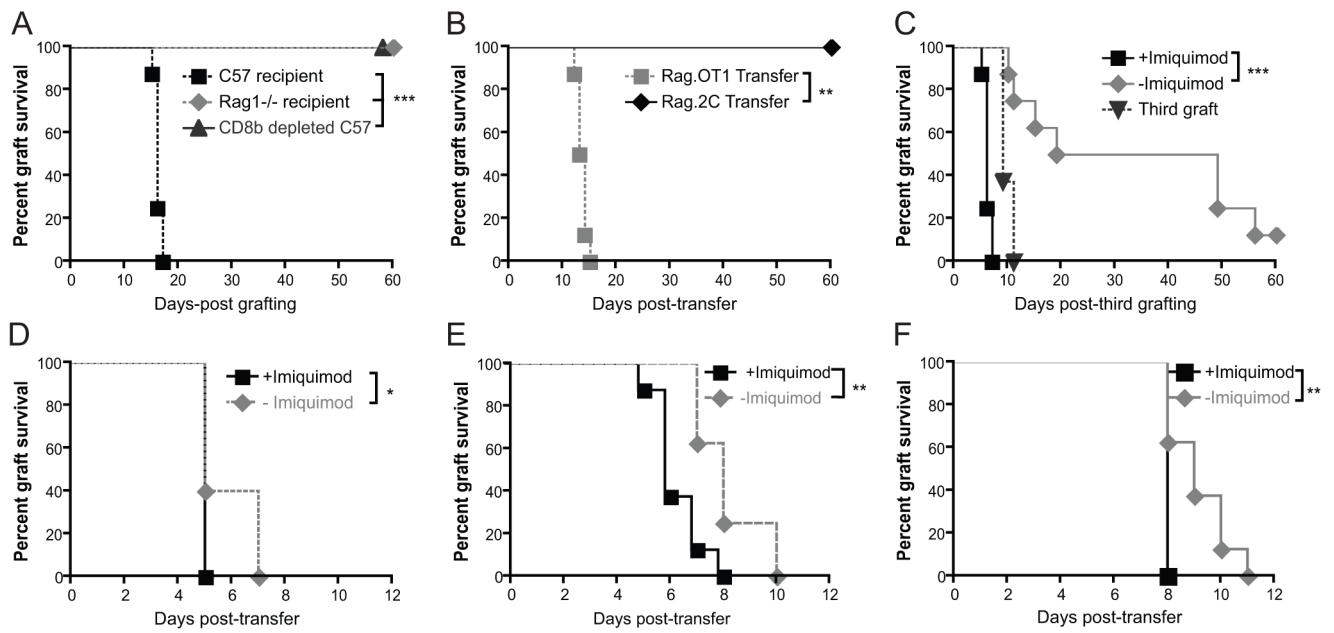
Figure 1. K5mOVA skin graft rejection is CD8+ T cell dependent and is enhanced by local TLR7 ligation

Kaplan-Meier survival curves of membrane-bound ovalbumin-expressing skin grafts (K5mOVA). **A**. C57BL/6, *Rag1*−/− mice and C57BL/6 mice treated with anti-CD8β monoclonal antibody were grafted with K5mOVA expressing skin and grafts monitored for rejection. n=8, ***p<0.001 by log rank test. **B.** *Rag1*−/− mice bearing K5mOVA skin grafts received an intravenous transfer of 10^6 SIINFEKL-specific Rag.OT-I splenocytes or SIYRYYGL-specific Rag.2C splenocytes. Grafts were then monitored for rejection. n=7, **p<0.01 by log rank test. **C.** C57BL/6 mice were depleted of CD8β+ T cells by treatment with the monoclonal antibody 53–5.8 and grafted with two K5mOVA skin grafts. 10 weeks later, animals received a third, contralateral graft, and one of the two original grafts were treated for 10 days with imiquimod whilst the other received control cream. n=7, ***p<0.001 by log rank test. **D–F.** *Rag1*−/− mice bearing bilateral, well-healed K5mOVA skin graft were adoptively transferred with 10^7 (**D**), 10^6 (**E**), or 10^5 (**F**) naïve Rag.OT-I splenocytes. At the same time all animals received subcutaneous immunisation with the adjuvant OVA in Quil A, whilst one of the two grafts was treated with imiquimod and the other with the control cream. n=8 for all groups, *, p < 0.05, **p<0.01 by log rank test.
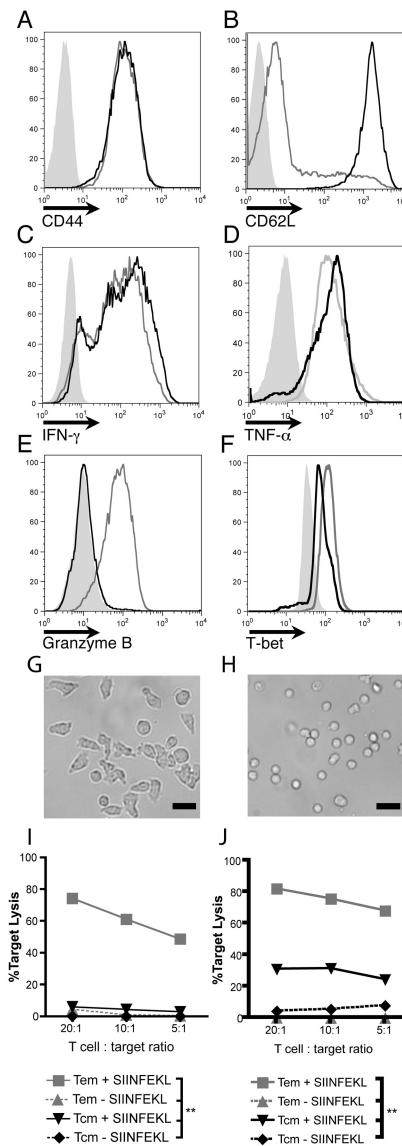
Figure 2. *Ex vivo* **differentiated T$_{CM}$ and T$_{EM}$ exhibit extracellular markers, intracellular molecules and killing capacity characteristic of T$_{CM}$ and T$_{EM}$**
A&B. CD44 and CD62L staining of OT-I *in vitro* derived T$_{CM}$ (black lines) and T$_{EM}$ (grey lines) cultured as per materials and methods. Isotype control shown in filled grey histograms. **C–E**. Intracellular Interferon-γ, tumour necrosis factor-α, granzyme B expression in *in vitro* differentiated T$_{CM}$ and T$_{EM}$ following stimulation with PMA/ionomycin. T$_{CM}$ shown as black lines, T$_{EM}$ as grey lines and unstimulated cells shown as filled grey histograms. **F.** Intracellular expression of the T-box transcription factor T-bet. T$_{CM}$ shown as black lines, T$_{EM}$ as grey lines and naïve cells shown as filled grey histograms. All vertical axes in histograms are frequency of cells as percentage of total cells analysed. Flow cytometry plots represent one three individual experiments conducted with pooled lymphocytes from OT-I mice. **G&H.** Micrograph of OT-I T$_{EM}$ (G) and T$_{CM}$ (H). Photos represent one of two individual experiments using pooled lymphocytes. Scale bar = 20μm. **I & J.** *In vitro* cytotoxic killing assays of T$_{CM}$ (black lines) and T$_{EM}$ (grey lines) demonstrating chromium release following 2.5 hours (K) and 5 hours (L) of exposure to SIINFEKL-pulsed (sold lines) and non-pulsed 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%.
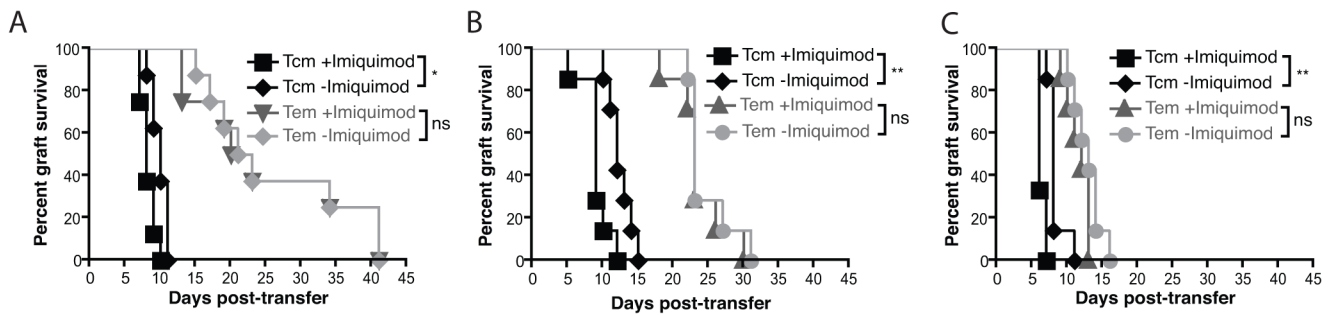
Figure 3. *In vitro* differentiated central but not effector memory CD8^{+} T cell rejection of skin grafts is enhanced by a local inflammatory stimulus

Rag1$^{-/-}$ mice bearing two well-healed K5mOVA grafts received either *in vitro* differentiated T_{CM} (black lines) or T_{EM} (grey lines). Grafts were then treated with either imiquimod or control cream and graft rejection plotted on a Kaplan-Meier survival curve. **A.** 10^4 adoptively transferred T_{CM} and T_{EM} CD8 T cells. Data analysed by log rank test, n=7 for each group, *p<0.05, ns = not significant. **B.** 10^5 adoptively transferred T_{CM} and T_{EM} CD8 T cells. Data analysed by log rank test, n=7 for each group, **p<0.01, ns = not significant. **C.** 10^6 adoptively transferred T_{CM} and T_{EM} CD8 T cells. Data analysed by log rank test, n=8 for each group, **p<0.01, ns = not significant.
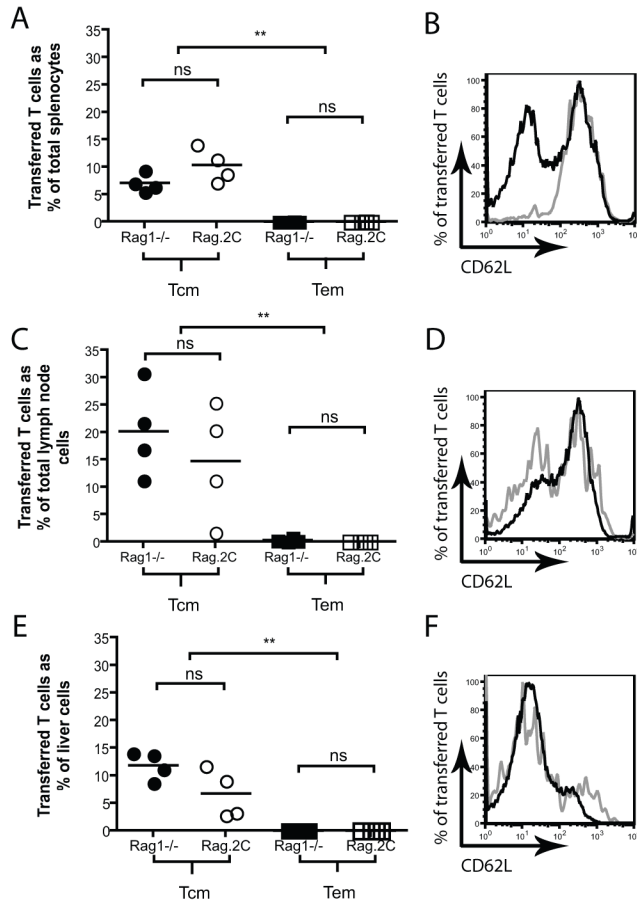
Figure 4. T$_{CM}$ persist to greater extent than T$_{EM}$ in both *Rag1*$^{-/-}$ and Rag.2C mice
Rag1$^{-/-}$ (filled symbols) and Rag.2C (open symbols) mice bearing well-healed K5mOVA grafts were adoptively transferred with 10^6 T$_{CM}$ (circles, black lines on histogram) or T$_{EM}$ (squares, grey lines on histogram). 45 days following transfer spleen (panels A&B), pooled inguinal, brachial, axillary and mesenteric lymph nodes (panels C&D) and liver (panels E&F) were harvested and cells stained for CD8, Vα2, and CD62L. **A**, **C** and **E**. CD8$^+$ Vα2$^+$ cells as percent of total cells in each organ. n=4per group, **p<0.01 when comparing *Rag1*$^{-/-}$ to *Rag1*$^{-/-}$ and Rag.2C to Rag.2C, ns = not significant; analysed by one-way ANOVA analysis with post-hoc multiple comparison test. **B**, **D** and **F**. Representative CD62L expression of CD8$^+$ Vα2$^+$ CD44$^+$ in various organs analysed by flow cytometry 45 days following transfer into Rag1−/− mice.
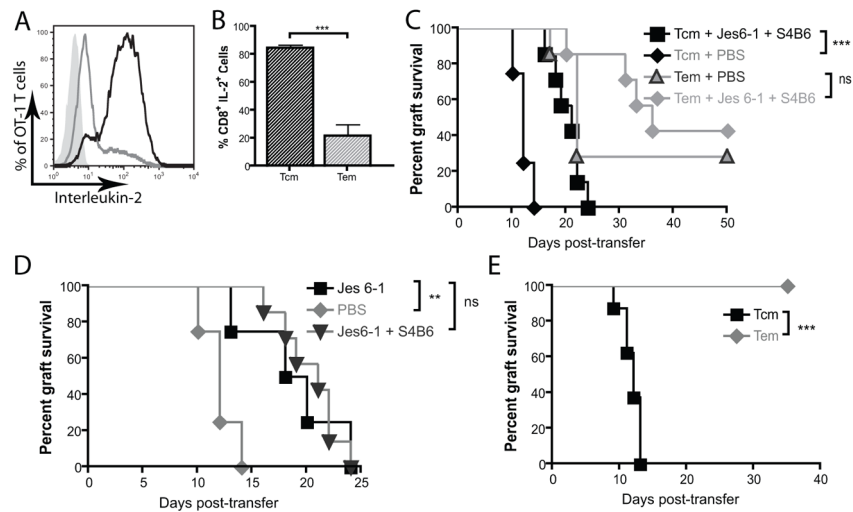
Figure 5. IL-2 inhibition delays T_{CM} mediated-rejection of well-healed K5mOVA grafts
A. Intracellular expression of interleukin-2 by flow cytometry following stimulation of *in vitro* differentiated central memory T cells (T $_{CM}$, black line) and effector memory T cells (T_{EM}, grey line) with PMA/Ionomycin. **B.** Quantitation of percent of IL-2-expressing *in vitro* differentiated T_{CM} and T_{EM}. Data from three individual experiments. Data analysed by Student's t-test. Error bars represent S.E.M. ***p<0.001. **C & D.** Kaplan-Meier survival curve of well-healed K5mOVA skin grafts on *Rag1$^{-/-}$* mice which received an adoptive transfer of either 10^5 T_{CM} or T_{EM} treated for 10 days with intraperitoneal injections of PBS or 100μg of the IL-2-blocking monoclonal antibodies Jes6-1 and S4B6 (**C**); or Jes6-1 alone or PBS (**D**). Animals were then monitored for graft rejection. n=8 for T_{CM} and n=7 for T_{EM}. **p<0.001, ns = not significant. Data analysed by log rank test. **E.** Rag.2C mice bearing well-healed K5mOVA skin grafts were adoptively transferred with 10^5 T_{CM} or T_{EM}. Following transfer, grafts were monitored daily for graft rejection. n=8 per group, ***p<0.001. Data analysed by log rank test.
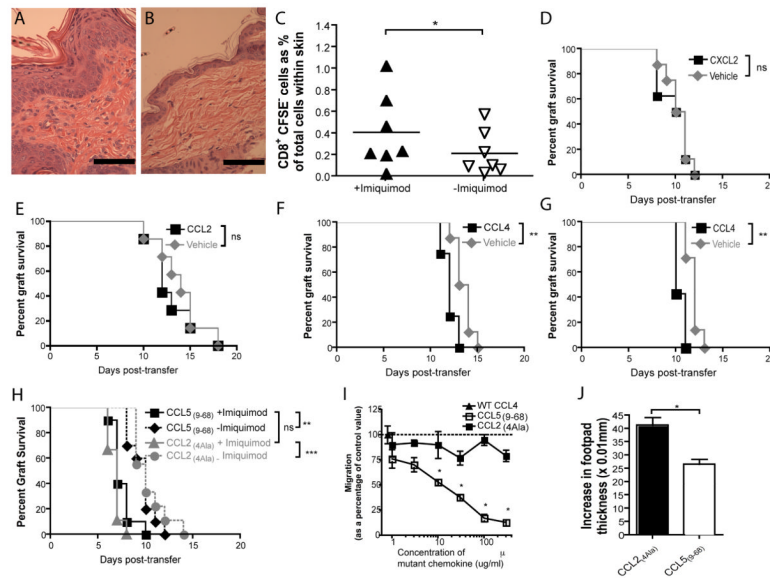
Figure 6. Imiquimod enhances inflammatory infiltrate and traffic of adoptively transferred CD8$^+$ T cells into inflamed grafts. The effects of imiquimod on T$_{CM}$-mediated graft rejection are recapitulated by the chemotactic molecule, CCL4

A & B. Haematoxylin and eosin micrograph of flank skin of C57BL/6 mice treated for 5 days with imiquimod (**A**) or control cream (**B**). Scale bars represent 50µm. **C**. *Rag1$^{-/-}$* mice bearing two, bilateral K5mOvA received an adoptive transfer of 10^5 CFSE-labelled, *in vitro*-derived T$_{CM}$ 6 weeks following grafting. At time of transfer one of the two grafts was treated with imiquimod daily for 5 days, whilst the other received control cream. Grafts were removed, processed into single cell suspension. Cells were then analysed by flow cytometry for CD3, CD8 and CFSE staining. n=7 per group, *p<0.05 by paired Student's t test. **D–G.** *Rag1$^{-/-}$* bearing two well-healed K5mOVA skin grafts were adoptively transferred with 10^5 T$_{CM}$ and received intradermal injections of 200ng of recombinant murine CCL2 (**D**) or CXCL2 (**E**) or CCL4 (**F**) or 1µg of CCL4 (**G**) or vehicle control on days 4, 5, 7 and 9 post-transfer. Grafts were then monitored for rejection. n=8 for each experiment, **p<0.01, ns. not significant by log rank test. **H.** Two groups of *Rag1–/–* mice bearing bilateral, well-healed K5mOVA skin grafts were adoptively transferred with T$_{CM}$ and treated with the CCR5 inhibiting peptide CCL5$_{(9–68)}$ or the non-functional mutated chemokine CCL2$_{(4Ala)}$ whilst 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<0.01, ***p<0.001, ns = not significant by log rank test. **I**. Transwell migration assay of purified CD8$^+$ T cells stimulated with CD3 and CD28 monoclonal antibodies, 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 percent of migrated cells relative to wild-type CCL4. Non-functional 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 immunisation of C57BL/6 mice with Ovalbumin in complete Freund's adjuvant. 7 days later, recall was conducted in footpad and increase in footpad thickness was assessed by callipers. To assess for *in vivo* activity of CCL5$_{9–68}$, one day prior to recall animals were treated with CCL5$_{9–68}$ or control peptide CCL2$_{4Ala}$. Data are presented as mean + S.E.M. Data analysed by Student's t-test. n = 12 per group, *p<0.05.
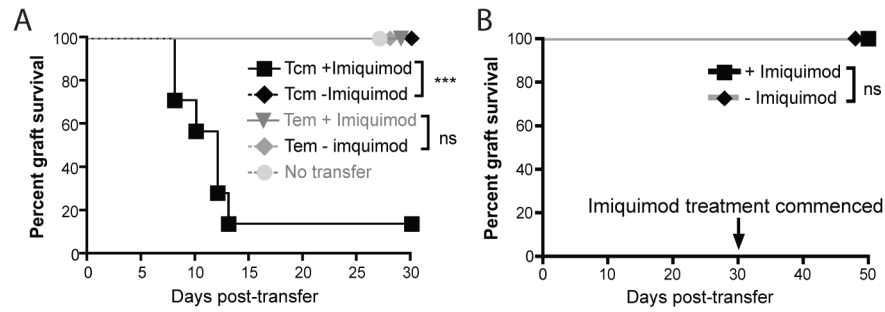
Figure 7. Local TLR7 ligation and T_{CM} transfer is able to overcome tolerogenic environments and instate cutaneous immunotherapy

A. CD11c.OVA mice bearing two well-healed K5mOVA skin grafts were adoptively transferred with 10^5 T_{EM} or T_{CM} 6 weeks following grafting. At time of transfer one of the two grafts was treated with imiquimod whilst the other was treated with control cream. Data analysed by log rank test, n=7 for T_{CM}, n=8 for T_{EM}. ***p<0.001, ns = not significant. B. CD11c.OVA mice bearing well-healed K5mOVA skin grafts were adoptively transferred with 10^5 T_{CM}. 30 days later grafts were treated with either imiquimod or control cream for 10 days. Data analysed by log rank test, n=8, ns = not significant.