Antigen-Specific CD4 Cells Assist CD8 T-Effector Cells in Eliminating Keratinocytes

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Keratinocytes expressing tumor or viral antigens can be eliminated by antigen-primed CD8 cytotoxic T cells. CD4 T-helper cells help induction of CD8 cytotoxic T cells from naive precursors and generation of CD8 T-cell memory. In this study, we show, unexpectedly, that CD4 cells are also required to assist primed CD8 effector T cells in rejection of skin expressing human growth hormone, a neo-self-antigen, in keratinocytes. The requirement for CD4 cells can be substituted by CD40 costimulation. Rejection of skin expressing ovalbumin (OVA), a non-self-antigen, by primed CD8 cytotoxic T cells can in contrast occur without help from antigen-specific CD4 T cells. However, rejection of OVA expressing keratinocytes is helped by antigen-specific CD4 T cells if only low numbers of primed or naive OVA-specific CD8 T cells are available. Effective immunotherapy directed at antigens expressed in squamous cancer may therefore be facilitated by induction of tumor antigen-specific CD4 helper T cells, as well as cytotoxic CD8 T cells.

Journal of Investigative Dermatology (2010) **130,** 1581–1589; doi:10.1038/jid.2010.17; published online 11 February 2010

INTRODUCTION

Development of epithelial malignancy is regulated by the immune system, as chronic immunosuppression after organ transplantation is associated with a greatly increased risk of squamous cancer. Many cancers of epithelial origin express tumor-specific modified self-proteins as neo-self-antigens (Buckwalter and Srivastava, 2008). Epithelial cancer can also arise from skin persistently infected with virus, including human papillomavirus (Al-Daraji and Smith, 2009) and Merkel tumor-associated retrovirus (Feng *et al.*, 2008), and these tumors continue to express virus-encoded antigen. Evidently, neo-self- or viral antigens expressed in squamous epithelial cancer fail to induce an immune response adequate to eliminate cancer. A better understanding of the requirement for effective immune-mediated elimination of keratinocytes expressing viral or neo-self-antigen would therefore assist in the design of appropriate immunotherapeutic interventions for squamous epithelial cancer.

We have developed a murine model to study the requirements for effective immunotherapy directed at antigen expressed in keratinocytes. Skin expressing a non-self- or neo antigen as a transgene in keratinocytes from a keratin promoter is grafted to a syngeneic nontransgenic recipient (Zhong *et al.*, 2004). This allows us to study the requirements for inducing and delivering an effective immune response to antigen expressed only in keratinocytes, and not directly presented to the immune system by professional antigen-presenting cells (APC).

The fate of a graft of skin expressing non-self antigen in keratinocytes depends on the antigen. Some antigens, including papillomavirus nonstructural proteins, fail to invoke spontaneous rejection of grafts (Dunn *et al.*, 1997), allowing the study of the requirements for effective induction of immunity after antigen cross-presentation from keratinocytes. Other antigens, including ovalbumin (OVA) (Holcmann *et al.*, 2009) and human growth hormone (hGH) (Zhong *et al.*, 2004), which is closely related in sequence to mouse growth hormone and therefore a neo-self-antigen in the mouse, induce spontaneous graft rejection, allowing the study of the elimination of keratinocytes by primed T cells in a second graft after immune-mediated elimination of a priming graft.

To prime a cytotoxic CD8 effector T-cell response from naive antigen-specific CD8 T-cell precursors, antigens expressed only in epithelial cells must be cross-presented by professional APC, particularly by dermal dendritic cells in the local lymph node (Bedoui *et al.*, 2009). CD4 helper T cells are required to enable priming of memory and effector CD8 T cells (Schuurhuis *et al.*, 2000; Janssen *et al.*, 2003; Smith *et al.*, 2004). CD4 T cells also assist in the maintenance of a pool of memory CD8 T cells (Sun *et al.*, 2004). However, once they are primed by antigen, CD8 effector T cells are held capable of eliminating their cognate target cells without the need for CD4 T-cell help. Recent *in vitro* evidence

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Abbreviation: OVA, ovalbumin

Received 15 August 2009; revised 29 December 2009; accepted 1 January 2010; published online 11 February 2010

suggests, however, that CD4 T cells can assist in secondary responses to cross-presented but not directly presented antigen (Blachere *et al.*, 2006). We have also previously shown a requirement for CD4 cells in a secondary immune response to hGH in FVB mice of H-2q × H-2b genetic background (Zhong *et al.*, 2008). To extend this observation to mice of a genetic background more commonly used for studies of immune physiology and to therefore enable further dissection of the mechanisms by which CD4 cells contribute to elimination of keratinocytes after priming or immunization, we created K14.hGH transgenic mice on a C57/Bl6 genetic background. We evaluated the role of CD4 T cells in rejection of epithelial cells expressing various nonself-antigens using these mice. Here, we present data showing that CD4 T-cell help improves the efficacy of rejection by already primed CD8 effector T cells of skin expressing either non self-antigen or neo-self-antigen in keratinocytes, and demonstrate that the requirement for CD4 cell responses can be substituted by CD40 costimulation.

RESULTS

Second-set syngeneic skin grafts expressing hGH as a neo-self-antigen are more rapidly rejected by C57Bl/6 recipients than the primary graft

We grafted skin expressing antigen as a transgene in keratinocytes in naive C57Bl/6 (C57) recipient animals, and measured time-to-graft rejection to assess the immune response induced to the expressed antigen. We first studied recall responses to hGH, a near homolog of mouse growth hormone representing modified self (neo-self). Animals that rejected an hGH transgenic graft were considered primed to hGH, and received a further hGH graft. Primary hGH grafts on C57 mice were rejected in a median of 31.5 days (Figure 1a), as was observed for C57 × FVB mice and in contrast to the findings for FVB mice, which rarely reject primary hGH grafts (Zhong *et al.*, 2004). Second hGH grafts placed on graft-primed C57 recipients were rejected with a median survival time of 21 days, significantly earlier than the initial graft ($P = 0.018$). The recall response to hGH by C57 mice is consistent with observations for FVB × C57 mice (Zhong *et al.*, 2004).

Rejection of second-set hGH skin grafts requires CD4 cells

Rejection by FVB × C57 mice of skin expressing hGH antigen in keratinocytes requires both CD4 and CD8 T cells (Zhong *et al.*, 2004, 2008). To establish for C57 mice whether CD4 T cells are required for rejection of hGH transgenic skin following priming, hGH graft-primed C57 animals were depleted of CD4 T cells, and given a second hGH graft (Figure 1b). Rejection of second hGH grafts occurred in 8% (1 out of 13) of CD4-depleted recipients, compared with 87% (7 out of 8) of CD4-replete recipients ($P < 0.0001$). Thus, CD4 T cells are important not only for priming but also for optimal rejection by CD8 T cells of hGH grafts from an hGH graft-primed C57 animal.

CD40 stimulation abrogates the requirement for CD4 cells in rejection of second-set K14hGH.H-2^b skin grafts

CD4 T-helper cells provide costimulatory signals to dendritic cells to assist in priming antigen-specific primary cytotoxic

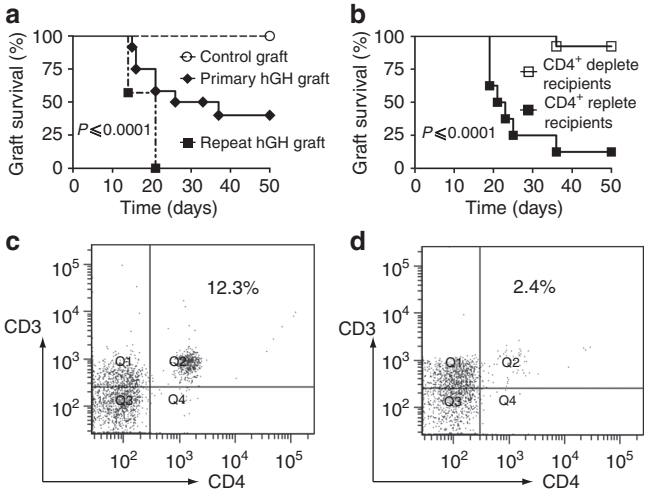

Figure 1. hGH graft-primed animals require CD4 T cells for optimal second graft rejection. (**a**, **b**) Graft survival time is shown for (**a**) naive or K14hGH.C57 graft-primed C57Bl/6 animals ($n = 12$) that received a K14hGH.C57 or a C57Bl/6 graft, and for (**b**) K14hGH.C57 graft-primed animals that received a K14hGH.C57 graft with or without simultaneous depletion of CD4 T cells. Graft survival was compared using the log-rank (Mantel–Cox) test. Multiple mice rejecting grafts on the same day are shown as a single graph point on that day. (**c**, **d**) For control antibody-treated (**c**) and GK1.5 anti-CD4-treated (**d**) animals, efficacy of CD4 cell depletion was assessed by flow cytometry analysis of peripheral blood, using anti-CD4 and anti-CD3mAb. Percentage of CD4 cells at 7 days is shown for each treatment. Results are combined from two experiments.

T-cell responses (Schoenberger *et al.*, 1998). Costimulatory signals delivered by dendritic cells to activated T cells have recently been shown to have a significant role in secondary immune responses (Wang *et al.*, 2007; Salek-Ardakani *et al.*, 2008). We therefore investigated whether CD40 stimulation could replace the requirement for CD4 T cells and enable effector T-cell function and hence graft rejection in an hGH-primed animal. Mice that had rejected an hGH graft were depleted of CD4 T cells, regrafted with a further hGH graft, and treated with agonist anti-CD40 antibody to provide a costimulatory signal, or with control rat serum, at 0, 5, 10, 15, and 20 days after grafting. Rejection of second hGH grafts occurred in 53% (8 out of 15) of mice receiving the CD40 stimulatory antibody FGK45 (Figure 2b), and in 0% (0 out of 6) mice receiving rat IgG, demonstrating that CD40 stimulation could substitute for primed antigen-specific CD4 cells in promoting graft rejection by CD8 T cells from a primed animal.

Primed, OVA-specific CD8 T cells can reject second-set OVA-expressing skin grafts without the need for help

To examine further how help from CD4 T cells enables CD8 T-cell-mediated rejection of skin grafts in antigen-primed recipients, we used an antigen, OVA, for which CD8 and CD4 major histocompatibility complex (MHC) Class I- and II-restricted epitopes are known, and for which mice with T cells transgenic for T-cell receptors recognizing CD4- and CD8-restricted OVA epitopes are available. We first grafted skin from mice expressing a membrane-associated form of

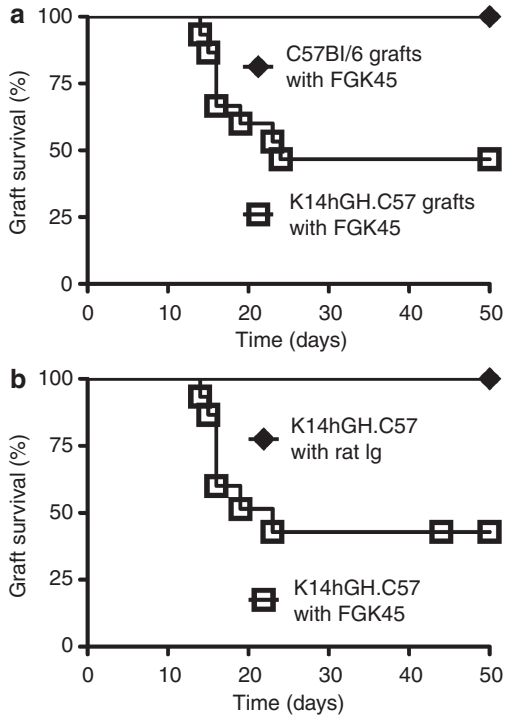

Figure 2. CD40 stimulation abrogates the requirement for CD4 cells to enable rejection of hGH grafts from hGH-primed animals. (**a**) Graft survival over time for K14hGH.C57 grafts ($n=15$) and for C57Bl/6 grafts ($n=6$) placed on hGH graft-primed and CD4-depleted C57Bl/6 recipients treated with agonist CD40 antibody FKG 45. (**b**) Graft survival over time for K14hGH.C57 grafts placed on graft-primed and CD4-depleted C57Bl/6 recipients, treated either with agonist CD40 antibody ($n=15$) or with control antibody ($n=6$). Graft survival differences were assessed for significance using th log-rank (Mantel–Cox) test. Results are from three separate experiments.

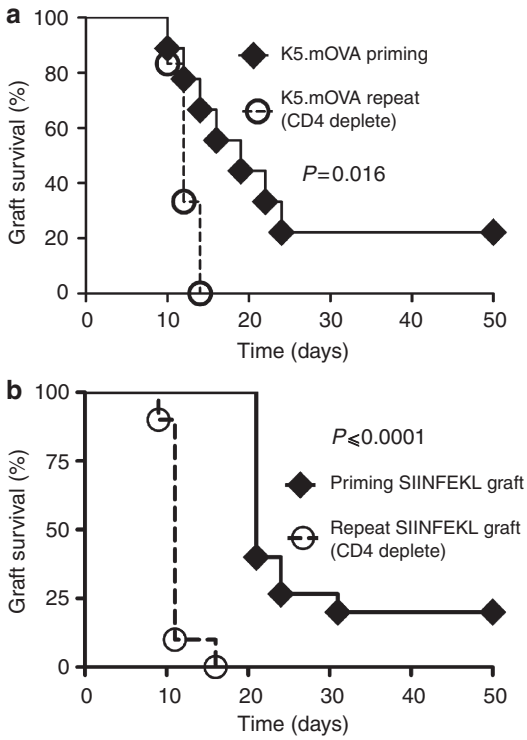

Figure 3. Ovalbumin and SIINFEKL-expressing skin grafts do not require CD4 cells for rejection. (**a**) Graft survival over time is shown for K5.mOVA grafts placed on naive, or OVA graft-primed and CD4-depleted C57Bl/6-recipient animals ($n=12$). (**b**) Graft survival over time is shown for K14 SIINFEKL grafts placed on naive or SIINFEKL graft-primed and CD4-depleted C57Bl/6-recipient animals ($n=12$). Survival differences were assessed for significance using the log-rank (Mantel–Cox) test.

OVA from a keratin 5 promoter (K5-mOVA). K5-mOVA graft recipients successfully rejected their grafts, with a median survival time of 19 days. OVA graft-primed recipients were then depleted of CD4 cells, and received a second K5-mOVA graft on the opposite flank. CD4 T-cell-depleted and OVA graft-primed recipients of a second K5-mOVA graft rejected the second graft with a median survival time of 12 days, significantly shorter than the time to primary graft rejection ($P=0.01$; Figure 3a) and no different than the rejection time (12 days) for OVA graft-primed and otherwise unmanipulated animals.

To confirm that there was no requirement for CD4 cells for rejection of OVA grafts by OVA-specific T cells, we examined the response to a graft expressing a minimal cytotoxic T lymphocyte epitope of OVA (SIINFEKL) expressed from a keratin 14 promoter (K14.SIIN), and hence unable to invoke antigen-specific CD4 cells. K14SIIN skin grafts were rejected by 12 of 15 unprimed recipients in a median of 21 days. Recipients that had rejected a first K14.SIIN graft were depleted of CD4 cells, and regrafted. All 10 recipients rejected a repeat graft in a median of 11 days ($P<0.0001$; Figure 3b), similar to that for OVA grafts. To further investigate whether MHC Class 1-restricted CD8 T cells are sufficient for rejection of K5.mOVA grafts, 1×10^6 $Rag1^{-/-}$ OT-I cells, specific for the MHC-1-restricted SIINFEKL peptide, were transferred to immune-deficient $Rag1^{-/-}$ recipients ($n=4$), which were then grafted with K5-mOVA grafts. All four grafts were rejected, with a median survival time of 10 days (Figure 5a). Thus, CD8 T cells are sufficient to reject skin grafts expressing either OVA or the major MHC Class 1-restricted CD8 epitope of OVA in keratinocytes.

CD4 T cells alone are not sufficient for graft rejection

To establish whether CD4 T cells alone could effect rejection of a K5.mOVA graft, 2.5×10^7 OT-II spleen cells, specific for a CD4-restricted epitope of OVA, were transferred into $Rag1^{-/-}$ recipients ($n=16$), which 3 days later received a K5.mOVA skin graft. FACS staining of peripheral blood on the day of grafting confirmed successful transfer of CD4 T cells (Figure 4). K5.mOVA grafts (13 out of 16) were generally accepted by immunodeficient Rag $1^{-/-}$ animals, irrespective of whether they received OVA-specific CD4 OT-II T cells ($P=0.37$; Figure 4), in contrast to the findings for OVA-specific CD8 T cells described above. Thus, OVA-specific CD4 T cells are unable, in the absence of OVA-specific CD8 cytotoxic T cells, to reject OVA-expressing skin grafts.

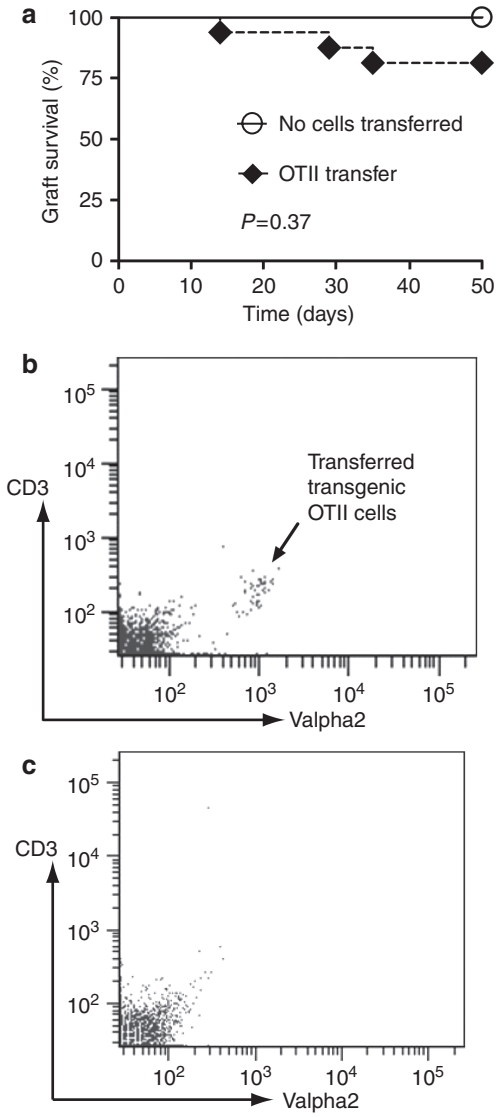

Figure 4. Transfer of CD4 T cells alone does not significantly increase graft rejection. (**a**) Survival over time of OVA grafts placed on immunodeficient Rag1$^{-/-}$ recipients ($n=16$) reconstituted with 2.5×10^7 OVA-specific helper T cells from OT-II transgenic spleen or on control Rag 1$^{-/-}$ animals receiving no cells ($n=8$). (**b**, **c**) Effective transfer of OVA-specific helper T cells from OTII mice was confirmed by staining of peripheral blood from mice receiving OT-II cells (**b**) and control mice (**c**) with anti-CD3 and anti-Vα2 antibodies specific for OT-II cells. Results are from two separate experiments.

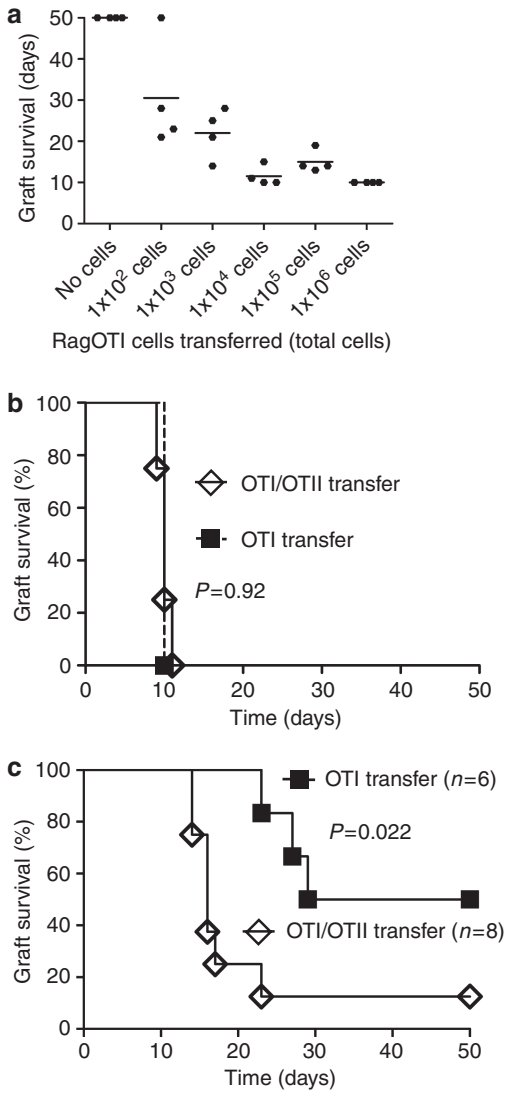

Figure 5. The rate of K5mOVA graft rejection is dependent on CD8 precursor frequency and CD4 T cells. (**a**) Immunodeficient Rag1$^{-/-}$ recipients of K5.mOVA grafts received increasing numbers of Rag$^{-/-}$ OTI OVA-specific CD8 T cells from spleen as shown, together with sufficient irrelevant TCR transgenic T cells to equalize the number of transferred cells, and graft survival was observed ($n \geq 4$ per group). (**b**) Rag-OT-I OVA-specific CD8 T cells from spleen (10^6) were administered with ($n=4$) or without ($n=4$) OVA-specific T-helper (OT-II) spleen cells (10^6) to immunodeficient Rag1$^{-/-}$ recipients. After 3 days, K5mOVA skin grafts were placed and followed up to rejection. (**c**) Rag$^{-/-}$ 2C immunodeficient mice received 10^2 OVA-specific CD8 OT-1 cells, or 10^2 OT-1 cells and 10^6 OVA-specific CD4 OT-2 cells. K5.mOVA skin grafts were placed and followed up to rejection. Graft survival over time is shown. Survival differences were assessed for significance using the log-rank (Mantel–Cox) test. Results are from two separate experiments.

The number of transferred Rag-OT-I spleen cells determines the rate of K5mOVA graft rejection

As CD4 T cells are necessary for rejection of skin expressing hGH but not OVA from naive and antigen-primed animals, and are insufficient to achieve rejection of skin grafts without CD8 T cells, we investigated whether CD4 T cells might accelerate CD8 T cell-mediated K5.mOVA skin graft rejection. OT-I OVA-specific CD8 cytotoxic T cells (10^6) were transferred with or without OT-II OVA-specific CD4 helper T cells (10^6) to immunodeficient recipient animals 3 days before grafting with K5m.OVA skin. Grafts were rejected in a median of 10 days, irrespective of whether OT-II cells were transferred (Figure 5b). To establish whether CD4 cells might contribute to rejection when antigen-specific CD8 T cells were present in limiting numbers, a graded number of Rag-OT-1 OVA-specific CD8 spleen cells were combined with sufficient irrelevant TCR transgenic T cells to equalize the total number of transferred T cells and minimize T-cell activation through homeostatic proliferation, which would

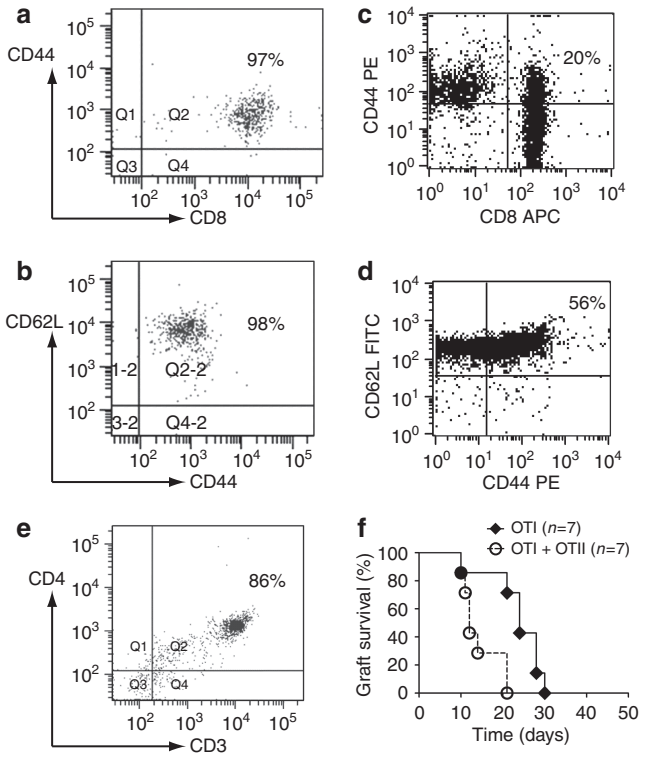

Figure 6. CD4 T cells provide help to activated CD8 T cells. (**a**, **b**) OT-I CD8 Ova-specific T cells, either activated *in vitro* (left panels) or not so activated (''naive''; right panels), were assessed for CD8, CD44 (**a**), or CD62L (**b**) expression by flow cytometry. (**c**) OT-II spleen cells were enriched for OVA-specific T-helper cells using anti-CD4 magnetic beads and enrichment assessed with anti-CD4 and -CD3 antibodies by flow cytometry. (**d**) Mice received 10^2 activated OVA-specific CD8 OT-I cells ($n=7$) or 10^2 activated OT-I and 10^6 CD4-enriched OVA-specific helper OT-II cells ($n=7$). K5.mOVA skin grafts were placed and followed up to rejection. Graft survival over time is shown. Survival differences were assessed for significance using log-rank (Mantel–Cox) test.

otherwise occur in the T cell-deficient Rag $1^{-/-}$ recipient. Rejection of OVA grafts from these immune-reconstituted animals was fastest in animals recipient of larger numbers of transferred OT-1 cells ($P=0.0082$; Figure 5a). To determine whether there was a role of OVA-specific helper CD4 T cells in graft rejection when OVA-specific CD8 cell numbers were limited, 10^2 OVA-specific CD8 T cells were transferred to 2C TCR transgenic recipients, which have normal numbers of T cells without any OVA-specific T-cell repertoire. OVA-specific OT-1 CD8 T cells of ''naive'' phenotype, as shown (Figure 6a and b), were transferred with or without 10^6 OVA-specific OT-II CD4 T cells (Figure 5c). K5.mOVA skin grafts were rejected from recipients of OVA-specific CD8 spleen cells alone, with a median survival time of 40 days. When both OVA-specific CD8 and OVA-specific CD4 T cells were transferred, median graft survival time was significantly shorter at 16 days ($P=0.022$). Thus, in animals with physiological precursor frequencies of antigen-specific CD8 T cells of 100–1000 (Lammermann and Sixt, 2008), antigen-specific CD4 T cells enhance skin graft rejection.

CD4 T-cell help for memory CD8 T cells

OVA-specific CD4 T cells might enhance OVA graft rejection by OVA-specific CD8 cells in the previously described experiments by assisting the priming of naive OVA-specific CD8 T cells. To investigate whether memory CD8 T-cell function could also be enhanced by CD4 T cells, we created CD8 memory T cells specific for OVA *in vitro* by a protocol shown to produce cells predominantly of a central memory phenotype ($CD44CD62L^{hi}$), and 98% of cells demonstrated this phenotype (Figure 6). Memory phenotype OVA-specific CD8 OT-1 cells (100/animal) were then transferred to $Rag1^{-/-}$ C57 mice bearing a K5.mOVA skin graft, with or without 10^6 CD4-enriched OVA-specific cells from OT-II mice (Figure 6c). Grafts were rejected by mice receiving OT-1 CD8 central memory T cells alone, with a median graft survival time of 24 days. Mice receiving both OT-I CD8 central memory T cells and OT-II OVA-specific CD4 cells ($n=7$) rejected grafts more quickly, with a median survival time of 12 days ($P=0.008$; Figure 6d). Thus, OVA-specific CD4 T cells enhanced graft rejection by activated OVA-specific CD8 T cells, confirming that antigen-specific CD4 T cells can assist primed antigen effector and memory CD8 T-cell function in eliminating skin grafts expressing antigen in keratinocytes.

DISCUSSION

The data presented here show that antigen-specific CD4 T cells can enhance the function of antigen-primed effector and memory CD8 T cells, and hence enable, or increase the rapidity of, rejection of keratinocytes expressing non- or neo-self-antigen. Further, the function of CD4 T cells can be substituted by CD40 stimulation, suggesting that the likely mechanism of CD4-mediated enhancement is activation of APCs cross-presenting antigen to CD8 T cells. These findings provide *in vivo* evidence to complement previous *in vitro* generated data (Blachere *et al.*, 2006) that CD8 effector function is enhanced by antigen-specific CD4 T cells, and extend this finding specifically to keratinocyte elimination. Our previous observations for hGH antigen on an F1 (H-2 b × H-2q) genetic background demonstrated that CD8 T cells were necessary for elimination of keratinocytes, that CD4 T cells assisted the process, and that antibody, although induced, was not sufficient to enable rejection (Zhong *et al.*, 2004, 2008). Extension of this observation from H-2q to H-2b mice eliminates the possibility that the previous findings represented a genetically determined limitation of CD8 T-cell function in F1 (H-2q × H-2b) animals, as H-2q mice did not present hGH as a neo-self-antigen for rejection.

As the mechanism by which epithelial cells expressing antigen are eliminated by CD8 T cells is uncertain, it was important to establish that CD4 T cells themselves were not effecting graft rejection in cases in which antigen was expressed in keratinocytes, as it is well recognized that allograft rejection can occur by meansk of an CD4-dependent and MHC class II-restricted mechanism (Rosenberg *et al.*, 1989). Large numbers of antigen-specific CD4 T cells were unable to induce graft rejection in our model, indicating that direct cellular cytotoxicity by CD4 T cells is unlikely to

account for the help provided in the enhancement of skin graft rejection in this system.

CD4 T cells assist priming of naive CD8 T cells (Mintern *et al.*, 2002c), activating DCs to express costimulatory molecules and providing key cytokines for CD8 T-cell expansion, including IL-2. High precursor frequencies of antigen-specific CD8 T cells can overcome the need for CD4 T cells, as evidenced by OVA-specific cytotoxicity *ex vivo* of OT-I T cells generated in MHC II-deficient hosts (Mintern *et al.*, 2002a, b; Wang *et al.*, 2007; Salek-Ardakani *et al.*, 2008). The precursor frequency of antigen-specific CD8 T cells determines the fate of skin grafts, and previous experiments have established that for priming of responses to OVA, expressed constitutively in all cells including APC, higher precursor frequency abrogates the need for CD28- and CD154-mediated costimulation to expand the precursor pool (Ford *et al.*, 2007). However, although priming can occur in the absence of help from antigen-specific CD4 T cells, particularly if CD8 cells generate their own IL-2, availability of antigen-specific CD4 cells at priming seems to be critical for generation of central memory CD8 T cells (Fernando *et al.*, 2002; Castellino and Germain, 2007; Fuse *et al.*, 2009).

More recently, CD4 T cells have also been shown *in vitro* to have a role in the reactivation of memory cells through a mechanism in which IL-2 has a part, although it cannot entirely substitute for CD 4 cells (Blachere *et al.*, 2006). In our experiments, OT-I OVA-specific CD8 T cells were sufficient to reject a K5mOVA graft from a $Rag^{-/-}$ mouse, and the rate of rejection was related to the number of transferred Rag OT-I cells. Thus, at least for OVA, the requirement for CD4 T cells is not absolute, and likely assists in amplification of effector numbers. IL-2-dependent proliferation of CD8 memory cells (Mintern *et al.*, 2002c) is important in increasing cell numbers, and production of IL-2 by OT-1 cells in response to antigen is well described. Proliferation of CD8 cells can occur locally in target tissue (Wakim *et al.*, 2008b; Kim *et al.*, 2009) and CD4 T cells may help activate local dendritic cells (Wakim *et al.*, 2008a), as these have also been shown to have a role in enabling memory T-cell function, or upregulate MHC Class 1 presentation on KC, as these cells can also promote proliferation of antigen-specific T cells *in vivo*, even in the absence of professional APC (Kim *et al.*, 2009). Our data, however, show that, as OT-1 cells are sufficient to enable rejection of well-healed skin grafts from CD4-deficient animals, local dendritic cell activation and CD4 T-cell induction are not mandatory for CD8 effector function for a nonself-antigen, as they seem to be for a neo-self-antigen, but rather assist in amplifying the CD8 effector response. We have previously established in a graft model, in which the target antigen is the E7 protein of human papillomavirus 16, that the inflammation associated with grafting promotes local CD8 effector T-cell function in an antigen-primed host, as well-healed grafts are relatively resistant to CD8 T-cell function (Matsumoto *et al.*, 2004). In the hGH graft rejection model, in which the antigen is a neo-self-antigen, and partial tolerance would be expected (Zhong *et al.*, 2004), inflammation and CD4 cells were required for CD8 effector function, and we now show that CD40 costimulation was able to substitute for CD4 T cells, as has been shown in other systems (Hernandez *et al.*, 2008). Our system differs from previous data in demonstrating the enhancement of a secondary immune response by CD40 costimulation. This suggests a role for CD40 signaling in antigen presentation to memory CD8 T cells. Together, these data demonstrate that the likely *in vivo* mechanism of enhancement of skin graft rejection by CD4 cells and local inflammation involves an effective cross-presentation of antigen by CD4-activated APC in skin to enable amplification of CD8 T-effector cells. Alternatively, although less likely, direct CD40 stimulation of CD8 T cells may enhance cytotoxic T lymphocyte function and could account for rejection mediated by CD40 stimulation in this system (Bourgeois *et al.*, 2002). A further mechanism by which inflammation may promote graft rejection is through chemokine-mediated attraction of T cells (Castellino and Germain, 2007), both antigen-specific and nonspecific (Wakim *et al.*, 2008b), to the site of grafting, enabling selective proliferation of antigen-specific effector cells.

Elucidation of the cellular requirements for an effective immune response to antigen that is cross-presented at the epithelium has significant clinical relevance. Failure to clear chronic human papillomavirus infection leads in some cases to cervical cancer, and many epithelial cancers express tumor-specific antigens. Both nonself-antigen and neo-self-antigen presentations in these clinical scenarios are chronic, invoking regulatory rather than effector CD4 responses, our data demonstrate that absence of antigen-specific helper CD4 T cells may significantly impair the effectiveness of effector or memory CD8 T-cell responses to neo-self-antigen in keratinocytes, whether the immune response is induced by specific immunotherapy or in response to antigen cross-presented from an epithelial tumor. They therefore support the need for induction of appropriate CD4 T-cell responses as part of any immunotherapeutic intervention targeted at the epithelium.

MATERIALS AND METHODS

Mice

All experimental protocols were approved by the animal ethics committee of the University of Queensland. Mice were maintained under conventional conditions in specific pathogen-free holding rooms in the Princess Alexandra Hospital Biological Resources Facility, University of Queensland.

Transgenic mice used in these studies are detailed in Table 1. OT-II and K5m.OVA mice were obtained from Dr W.R. Heath, Walter and Eliza Hall Institute, Parkville, Australia. OT-I and $Rag1^{-/-}$ mice were purchased from the Animal Resource Centre, Perth Australia. 2C mice were obtained from Dr B. Fazekas, Centenary Institute, Sydney. K14hGH.H-2^{b} mice were produced by backcrossing male K14hGH.H-2^{q} mice obtained from Dr Jie Zhong, Diamantina Institute with C57Bl/6 mice for 10 generations. To assess whether backcrossing had successfully generated a C57Bl/6 background, offspring not expressing hGH were used as skin graft donors. Ear skin grafts applied to the flanks of C57Bl/6 recipients were uniformly accepted, indicating histocompatibility.

Table 1. Designation of transgenic animals

Designation	Genetic background	Transgene genotype	Phenotype	Function	Reference
OT-II	H-2b	TCR gene-specific for $OVA_{323-339}$ peptide bound on IA^b	Most T cells are CD4 helper T cells specific for OVA presented in the context of MHC Class II	Source of CD4 helper T cells specific for OVA presented by antigen-presenting cells	Barnden *et al.* (1998)
OT-1	H-2b $Rag^{-/-}$	TCR gene-specific for OVA peptide SIINFEKL bound on H-2 K^b No recombinase activation genes	All T cells are CD8 cytotoxic T cells specific for OVA presented in the context of MHC Class I	Source of CD8 cytotoxic T cells specific for OVA presented by somatic cells	Hogquist *et al.* (1994)
K5.mOVA	H-2b	OVA expressed from a keratin 5 promoter	Squamous epithelial keratinocytes express OVA peptides for direct and cross-presentation	Source of skin-presenting OVA in somatic cells that can also be cross-presented by antigen-presenting cells	Azukizawa *et al.* (2003)
K14.SIIN	H-2b	OVA MHC Class I restricted peptide SIINFEKL expressed from a keratin 14 promoter	Squamous epithelial keratinocytes express OVA peptide for direct presentation to CD8 T cells. Also express low levels of human growth hormone due to leaky transgene construct	Source of skin-presenting OVA peptide in somatic cells that cannot be cross-presented by antigen-presenting cells	McGargill *et al.* (2002)
2C	$H-2^b$	TCR gene-specific for H-2 L^d No recombinase activation genes	All T cells are CD8 cytotoxic T cells specific for an irrelevant foreign MHC molecule	A mouse with normal numbers of T cells, none of which can recognize OVA	$H-2L^d$ Sha *et al.* (1990)
K14hGH	H-2q or H-2b	Human growth hormone expressed from a keratin 14 promoter	Squamous epithelial keratinocytes express human growth hormone peptides for direct and cross-presentation	Source of skin-presenting human growth hormone in somatic cells that can also be cross-presented by antigen-presenting cells	Zhong *et al.* (2004)

Abbreviations: MHC, major histocompatibility complex; OVA, ovalbumin.

Screening of transgenic mice

To screen for the K14.hGH transgene, serum hGH was assayed using the Bioclone Elegance Growth Hormone ELISA Kit (Bioclone Australia Pty Ltd, Sydney, NSW, Australia) according to the manufacturer's instructions. To screen for the K5.mOVA transgene, DNA from tail tips was detected by PCR with forward primer 5′-CTGTGCAGATGATGTAC-3′ and reverse primer 5′-TGGTTGCG ATGTGCTTG-3′.

Skin grafting

Whole-thickness ear skin grafting was performed as previously described (Frazer *et al.*, 1998). Grafts were examined every second day and were classified as rejected if there was >80% loss of epithelium, and as accepted if there was no rejection by day 50. Rejection or acceptance of transgenic skin was confirmed genetically and histologically in a random sample of mice. In cases in which repeat grafting was undertaken, it was performed on the opposite flank.

CD4 cell depletion

CD4 cells were depleted by intraperitoneal injection with anti-CD4 mAb (GK1.5) at 1 mg per mouse at 3 days before grafting. An equal amount of rat IgG was used as a control. Efficacy of depletion was evaluated by FACS staining of 100–200 μl peripheral blood. Briefly, peripheral blood mononuclear cells were prepared and washed with FACS buffer (1% FCS in phosphate-buffered saline (PBS)). Cells were stained with FITC-conjugated anti-CD4 (RM4-4, BD Biosciences, San Jose, CA; RM4-4 does not cross-compete with GK1.5) and phycoerythrin-conjugated anti-CD3 (BD Biosciences) mAbs and visualized with flow cytometry.

mAb production

Anti-mouse CD40 agonist Rat IgG2a antibody was isolated from FGK45 hybridoma cells used with permission from Professor Antionius Rolink (Basel Institute, Switzerland). Briefly, FGK45 cells were cultured, and the protein produced was isolated with a protein G column and quantified by the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Rat IgG was prepared by separating an Ig-enriched fraction of rat serum by ammonium sulfate precipitation. The product was dialyzed against PBS, filtered (0.2 μm filter), and the protein concentration was measured by the BCA Protein Assay kit (Pierce, Thermo Scientific, Rockford, IL). Antibody was administered according to a published schedule (Fischbein *et al.*, 2000) that was shown to be effective in our laboratory.

Preparation and transfer of spleen cells

A single cell suspension of spleen cells in DMEM (Invitrogen, Carlsbad, CA) was prepared. Red cells were lysed by addition of 2 ml ACK lysing buffer (Invitrogen), and cells were washed in sterile PBS.

Cell viability was determined by Trypan blue (Invitrogen) dye exclusion.

In vitro activation of OT-1 T cells

OT-I T cells were *in vitro* activated as described by Kenna *et al.* (2008). Briefly, OT-I lymph node cells were harvested and cultured in complete RPMI (RPMI 1640 supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM 2-ME) with 1% normal mouse serum, 0.1 μg ml^{-1} $OVA_{257-264}$, and 10 ng ml^{-1} IL-2. After 3 days, cells were harvested and washed (3 ×) with RPMI 1640 and recultured in six-well plates at 2×10^6/ml in the absence of antigen but with 10 ng ml^{-1} IL-15 for an additional 5 days. Cells were harvested, washed, and resuspended in sterile PBS before transfer to recipients.

Purification of OT-II cells by positive selection

A single cell suspension of OT-II spleen cells was resuspended at 90 μl 4% fetal bovine serum in sterile PBS per 10^7 cells and incubated with MACS anti-CD4 beads (Miltenyi Biotech, Bergisch Gladbach, Germany) (10 μl beads per 10^7 total cells) for 15 minutes at 4 °C. Magnetic separation was performed with the autoMACS Separator (Miltenyi Biotec, 51429 Bergisch Gladbach, Germany) using the Possel positive selection program as per the manufacturer's instructions (Miltenyi Biotec). Purity was assessed by FACS staining with anti-CD4 (FITC conjugated, BD Pharmingen, North Ryde, NSW, Australia), and anti-CD3 (PE conjugated, eBioscience, Jomar Bioscience, Kensington, SA, Australia). Cells were washed twice in sterile PBS before transfer.

Statistics

Statistical comparisons were determined by Mantel–Cox analysis of Kaplan–Meier survival curves. Differences with $P<0.05$ were considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

IHF was funded by a Queensland Government Premiers Fellowship. JB was funded by an NHMRC postdoctoral studentship. This study was funded by an NHMRC Program Grant.

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