The intraepithelial T cell response to NKG2D-ligands links lymphoid stress-surveillance to atopy

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Abstract

Epithelial cells respond to physico-chemical damage by upregulation of Major Histocompatbility Complex (MHC)-like ligands that can activate the cytolytic potential of neighbouring intraepithelial T cells by binding the activating receptor, NKG2D. The systemic implications of this lymphoid stress-surveillance response, however, are unknown. We found that antigens encountered at the same time as cutaneous epithelial stress induced strong primary and secondary systemic, T-helper-2 (Th2)-associated atopic responses in mice. These responses required NKG2D-dependent communication between dysregulated epithelial cells and tissue-associated lymphoid cells. These data are germane to uncertainty over the afferent induction of Th2 responses, and provide a molecular framework for considering atopy as a significant component of the response to tissue damage and carcinogenesis.

A conserved feature of T lymphocytes is their subdivision into two main types. One, composed of CD4+ and CD8+ αβ T cells specific for complexes of peptides and MHC molecules, has been termed "conventional", and is largely responsible for clonal, pathogen-specific memory responses that are the hallmark of adaptive immunity. The second T cell type, composed of cells expressing the γδ T cell receptor (TCR), is not generally specific for peptide-MHC complexes, and may instead recognize cell surface microbial and/or self-encoded moieties, some of which are upregulated by cellular dysregulation. Although γδ T cells exhibit some properties characteristic of adaptive immunity, a predominant function of these "unconventional" T cells is thought to be lymphoid stress-surveillance because they do not require major clonal expansion; because their functional potentials are pre-programmed during development; and because some may be activated *in vivo* independently of the TCR, by cytokines or by ligands for activating "natural killer" (NK) receptors, such as NKG2D *(1)*. Such cells are major components of large but enigmatic tissue-resident T cell compartments, which include murine dendritic epidermal T cells (DETC) and human or murine intraepithelial lymphocytes (IEL), among which CD4-CD8β- αβ T cells may also contribute to rapid lymphoid stress-surveillance.

Because NKG2D ligands such as Rae-1 (murine) and MICA (human) are activated by DNA damage *(2)* and are frequently expressed by tumour cells, such T cell responses, which have cytolytic potential, have been associated with local tumour surveillance. Indeed, DETC-deficient mice show increased susceptibility to chemical carcinogenesis (*3, 4*). However, whether lymphoid stress-surveillance describes a purely local response

or whether it affects the systemic immune compartment remains a key question. To investigate this, transgenic Rae-1 expression was induced as described *(4)* specifically in keratinocytes by a doxycycline (dox)-dependent, bitransgenic (BTg) molecular switch (Fig S1A). This mode of Rae-1 induction avoids pleiotropic effects of applying agents that induce a stressed state within the epidermis. Importantly, Rae-1 induction on otherwise normal epithelium promoted rapid morphological rearrangements of DETC and of epidermal Langerhans cells (LC), which are a tissue-associated dendritic cell (DC) subset lacking NKG2D (Fig S1B; Fig S2 *(4)*). Such changes were not seen in single transgenic (STg) mice that cannot induce dox-dependent Rae-1 upregulation. While subject to acute Rae-1 upregulation, patches containing highly purified ovalbumin (ova, a nominal non-self, non-microbe-associated antigen) or vehicle controls were placed onto skin of mice that had been gently shaved.

As in previous studies *(5, 6)* this provoked low but reproducible T helper 2 (Th2) immune responses in STg mice, characterized by low levels of interleukins (IL)-4 and -13; and IL-13-dependent induction of ova-specific and generalized immunoglobulin (Ig)G1 and IgE *(5)*. In contrast, BTg mice showed significant increases in responsiveness, as judged by ova-specific proliferation of lymph node (LN) and splenic T cells (Fig 1A; Fig S3); ova-dependent LN IL-2 and IL-13 production (Fig 1B); ova-specific IgG, IgG1, and IgE; and total IgE, that is customarily and substantially upregulated in the context of antigen-specific IgE responses (Fig 1C). Thus, localized epithelial upregulation of a single self stress-antigen (Rae-1) can powerfully enhance the response to coincidentally encountered antigen. Of note, this “adjuvant-effect” amplified but did not dysregulate the immune response, as there was no atypical induction of IgG2a.

The implication of these data is that lymphoid stress-surveillance of tissue damage may be a significant driver of Th2 responses developing during cutaneous antigen exposure. To investigate this, mild epidermal abrasion was induced in wild type (WT) mice by tape-stripping, which transiently removes the outer dead cell layer, the stratum corneum, while leaving underlying keratinocytes largely intact. The epidermis returns to normal within 48h (Fig S4). Such treatment mimics shaving and depilatory treatments in humans. Tape-stripping is known to activate LC *(6, 7)*, but whether it also induces a lymphoid stress-surveillance response is unknown. Rae-1 RNA was upregulated in the epidermis within 2h, peaking at 18-24h, and returning toward baseline by 48h, consistent with morphological tissue repair (Fig S5). Rae-1 upregulation was comparable in WT and TCRδ-deficient (*Tcrd-/-*) mice, as expected for an event upstream of DETC activation (Fig 2A). Consistent with the consequences of Rae-1 upregulation seen in BTg mice, tape-stripping was followed by coincident rounding of DETC and LC and an aggregate loss of DETC from the suprabasal and basal layers of the epidermis (Fig 2B). Of note, the cellular changes in tape-stripped and BTg mice were rapidly reversed as Rae-1 expression declined (Fig 2B, C). In sum, mild cutaneous abrasion provokes the initial events of lymphoid stress-surveillance.

To define abrasion-induced lymphoid stress-surveillance, we analyzed local gene expression changes in sets of age- and gender-matched WT and *Tcrd-/-* mice. As reported following epithelial stress (*8, 9)*, several cytokine mRNAs commonly attributed to epithelial cells were upregulated, including IL-1, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) (Fig 3A; Fig S6). mRNAs for some T cell-associated interleukins,

notably IL-4, IL-13, IFNγ and IL-10 were also upregulated. Such upregulation of Rae-1 and Th2 cytokines was likewise observed after careful shaving of mice (Fig S7).

The response to tape-stripping was radically different in *Tcrd-/-* mice: whereas IL-10 and TSLP upregulation were unaffected, IL-4, IL-13, and IL-25 RNAs were not induced, and those of IL-33 and IL-1β less durable (Fig 3A; Fig S6). Collectively, these data establish that γδ T cells are required for the normal local induction of specific cytokines commonly associated with Th2 responses to antigens encountered at epithelial surfaces. This conclusion was in part supported by newly-generated lines of BTg mice with dox-inducible expression of either Rae-1 or H60c, another NKG2D ligand *(10)*. When TCRγδ+ DETC, LC, and keratinocytes were purified from post-induction epidermis, rapid increases in IL-13 mRNA were specifically shown by DETC (Fig S8), attesting to the rapid functional activation of the local T cell compartment *in vivo*. Moreover, because NKG2D ligands are in this case only transiently upregulated on suprabasal keratinocytes in the absence of any other perturbation, local IL-13 upregulation can be attributed to intrinsic lymphoid stress-surveillance, rather than to obligate disruption of the epidermal barrier as is often invoked *(7, 11)*.

To determine whether such responses of γδ T cells translated to effects on systemic immunity, shaved mice were exposed to ova-containing patches, as described. As before, there was significant induction of ova-specific IgG, IgG1, and IgE, and of total IgE. However, whereas such responses were enhanced in BTg mice, they were significantly reduced in *Tcrd-/-* mice that cannot mount epidermal lymphoid stress-surveillance (Fig 3B). Again, there was no atypical induction of IgG2a.

Consistent with this, skin-draining LN cells from *Tcrd-/-* mice produced significantly less ova-dependent IL-13 than WT (Fig 3C). Parallel treatment of other strains showed as predicted that αβ T cells were required for IgG and IgE specific for antigen encountered during mild cutaneous abrasion, whereas less predictably congenital LC deletion had negligible impact (Fig 3B; Fig S9). As more experiments were undertaken, it became clear that the dependence of ova-specific IgG and IgG1 on γδ T cells was less than the dependence on αβ T cells, and did not always reach statistical significance (see Figs 3D; Fig S10 below): conversely, γδ T cells were invariably important for IgE induced in this manner. Moreover, the characteristic induction of non-specific IgE was fully ablated in *Tcrd-/-* mice, and in *Tcrb-/-Tcrd-/-* double-knockout mice (Fig S9). That γδ T cells are a driving force for such IgE production was evident in the finding that *Tcrb-/-* mice carry abnormally high levels of non-specific IgE, as was previously reported for *Tcra-/-* mice *(12)*. Thus, the γδ T cell compartment has a profound effect on the production of antigen-specific and total IgE following antigen exposure at mildly stressed epithelium.

Th2 by comparison with Th1 cell differentiation is often regarded as *de facto* irreversible, because of the positive feedback loop established by GATA3 expression *(13)*. Hence, the impact of stress-surveillance on primary Th2-IgE responses may have long-term implications for Th2-immunity. To examine this, WT and *Tcrd-/-* mice were exposed to ova as before, and then re-exposed 70 days after initial challenge. Again, γδ T cells were required for WT levels of ova-specific IgE induction (Fig 3D). After 70 days, ova-specific and total IgE levels in WT mice had returned to normal. Although they were substantially boosted in mice re-exposed to antigen at day 70 and reexamined 10 days later, this secondary response was greatly impaired in *Tcrd-/-* mice (Fig 3D). By contrast,

ova-specific IgG1 had remained elevated 70 days after challenge, and was boosted comparably in WT and *Tcrd-/-* mice, again segregating the impact of lymphoid stress-surveillance on IgE and IgG1, respectively. In sum, the magnitude of antigen-specific IgE responses to antigen repetitively encountered at the skin is regulated by γδ T cells.

To test whether this dependence of IgE upregulation on γδ T cells reflects its origin in lymphoid stress-surveillance, the studies were repeated in mice deficient in TCRVδ1Vδ5 that lack only the normal DETC repertoire *(4)*. Such mice showed substantially impaired ova-specific and total IgE, consistent with which almost no ova-dependent IL-13 production could be evoked from skin-draining LN cells (Fig 4A, B). In these studies, there was no significant impact on IgG1. Moreover, germane to the greater impact of local T cells on IgE *versus* IgG1, total IgE was already greatly upregulated in WT mice (but not *Tcrd-/-* mice) by day 7, whereas IgG1 upregulation was not seen until day 10 (Fig S10). Consistent with IgE induction being substantially a product of lymphoid stress-surveillance, its upregulation was significantly impaired in mice lacking the NKG2D receptor that mediates DETC responses to local Rae-1 upregulation. Again there was less impairment of IgG and IgG1 responses. Interestingly, there was an intermediate impairment of IgE responses in mice heterozygous for the gene that encodes NKG2D (*Klrk1*) (Fig 4C). IL-13 production from skin-draining LN was also greatly reduced in *Klrk1-/-* mice (Fig 4D). Complementing these deficiencies in IgE production in NKG2D-deficeint mice, IgE was readily detected in WT mice in squamous cell carcinomas that over-express Rae-1 (*3, 4*; Fig S11).

While epithelial dysregulation has previously been linked to atopy, as in the genetic association of atopic dermatitis with mutations in the epidermal structural protein, filaggrin *(14)*, this has most often been attributed to the effects of microbes gaining access to local DC, such as LC, by way of barrier disruption *(7, 11)*. However, shaving and ova-patching of WT mice and mice lacking MyD88, that transduces signals from bacteria-responsive Toll-like receptors [TLRs], induced comparable levels of Rae-1, IL-25, IL-13, and IgE (Fig S12; Fig 4E), consistent with the proposal that these responses are initiated by epithelial dysregulation *per se*.

Given the critical role of IL-13 in epicutaneously induced Th2 responses, and its strong association with clinical allergies, it is noteworthy that by comparison to conventionally polarized TCRαβ+ Th2 cells, DETC activated *in vitro* or *in vivo* express an excess of IL-13 mRNA relative to IL-4 mRNA (Fig S13). Moreover, such cells' regulation of stress-dependent epithelial expression of IL-25, IL-33, and IL-1 seems also germane to the afferent promotion of Th2 responses. Given that NKG2D-expressing murine and human systemic γδ cells and NKT cells also co-produce IL-13 and IFNγ (*15, 16*), and have been locally implicated in human allergic disease (*17*, *18*), there may be multiple cellular mechanisms for stress-initiated Th2 responses.

Although the same factors regulate B cell class-switching from IgM to IgE or to IgG1 (mouse) or IgG4 (human), an alternative, recently elucidated pathway favouring IgE class-switching (*19*) might accommodate the preferential induction of IgE by NKG2D-dependent stress-surveillance shown here, and the germinal centre-independent induction of IgE in αβ T cell deficient and germ-free mice (*12*, *20*). In parallel, increasing tissue damage would expose DC to IL-1 cytokines and/or to microbes, promoting conventional

CD4+ αβ T cell help for antigen-specific IgG1 responses. This can explain prior implications of TLRs in atopy (*11*, *21*); the γδ T cell-independence of IgG responses in multiply shaved and tape-stripped mice repeatedly exposed to antigen (*22*); and the reduced induction of antigen-specific IgG in shaved and ova-patched *Myd88-/-* mice (Fig S14).

The humoral component of stress-surveillance may limit tissue damage by targeting foreign moieties, such as toxins, that are root causes of tissue dysregulation. The IgE effector response could promote toxin expulsion and (by reducing blood pressure) limit their systemic dissemination, the collateral cost being the IgE response to benign antigens co-encountered with tissue disruption (*23*). This linkage of lymphoid stress-surveillance and atopy evokes a long-standing implication in allergy of tumour-responsive NKT cells *(24)*, and a large epidemiologic meta-analysis asserting an inverse relationship, albeit weak, between IgE production and carcinogenesis *(25)*. It also raises the possibility that increasing frequencies of atopic allergy might result from increased exposure to environmental toxins, rather than simply a paucity of environmental microbes.

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Figure Legends

Fig. 1. Acute epidermal Rae-1 expression induces systemic immune effects. (A-C) Adjuvant-free ova was administered in PBS in epicutaneous patches to shaved areas of BTg mice (red) and STg mice (blue) dox-treated for 7 days, and 10 days later, measurements were made of (A) skin draining LN ova-specific lymphocyte proliferation by [^{3}H] thymidine incorporation; (B) of ova-dependent production of IL-2 and IL-13 by cells from draining LN, as measured by Luminex assay; (C) of levels of serum ova-specific Ig and of total IgE. Black bars/lines show data for mice exposed to patches containing only PBS. Data expressed as mean ± 1 SEM; n = 6-8 mice/group with experiment repeated on 4 independent occasions with comparable results.

Fig. 2. Epidermal Rae-1 expression and local immune activation characterize the response to mild tissue abrasion. (A) Expression of endogenous Rae-1 mRNA in WT (black) and *Tcrd-/-* (red) epidermis following tissue abrasion by tape-stripping. qRT-PCR was performed on isolated epidermis; each bar represents an individual mouse; data expressed relative to the control gene cyclophilin. 6 WT and 4 *Trcd-/-* mice were analysed per time-point in 3 separate experiments. (B) Representative confocal images show freshly isolated epidermal sheets from WT FVB mice at various time-points post tape-stripping: DETC (red) and LC (green). (C) Confocal images show DETC (red) and LC (green) after 5 days on dox (left panel) and 3 days after withdrawal of dox (right panel). Original magnification x63 for (B) and (C). Micrographs are representative of analysis of 4-10 mice per condition.

Fig. 3. Local stress-surveillance responses to mild abrasion are linked to γδ T cell-dependent initiation of Type-2 responses. (A) qRT-PCR analysis of mRNA expression for the indicated cytokines over a 48h period following tape-stripping in WT (black) and *Tcrd-/-* epidermis (red). Data expressed relative to the control gene cyclophilin; p-values refer throughout the figure to statistically significant differences between WT and *Tcrd-/-* mice. 2 representative mice of each genotype are shown per time-point. The entire experiment was repeated 3 times for WT (n=6 per time-point) and 2 times for *Tcrd-/-* (n=4 per time-point). (B), (C) Following shaving, ova was applied as in Fig. 1 to dorsal skin of WT (black), *Tcrd-/-* (red), or LC-deficient (blue) (LC-/- = Langerin-DTA mice) mice. The Ig response (B) was assayed 10 days after primary immunzation and IL-13 secretion by skin draining LN (C) after further 72h *in vitro* re-stimulation with ova; all by

ELISA. Data expressed as mean ± 1 SEM and n=6-8 mice/group. Experiments (B & C) were repeated independently six times for *Tcrd-/-* and WT, and twice for LC-/- mice with comparable results. (D) *Tcrd-/-* (red) and WT (black) mice were rested for 70 days after primary epicutaneous antigen exposure; were re-bled; were re-exposed to an ova-containing patch as described, and re-bled 10 days later. Ig responses were measured by ELISA. Data expressed as mean ± 1 SEM and n=8 mice/group.

Fig. 4. IgE is regulated by intraepidermal γδ T cells and is dependent on NKG2D. (A), (B) Total and ova-specific serum Ig induction (A) and IL-13 secretion in the skin draining LN (B) of *TcrgV5-/-TcrdV1-/-* (orange), relative to WT mice (black) after epicutaneous immunization with ova as described in Fig 1. (C) Serum ova-specific Ig and total IgE responses after epicutaneous immunization with ova, as described in Fig. 1, in WT (black) and *Klrk1-/-* (green) mice as measured by ELISA. (D) ova-dependent IL-13 production in skin draining LNs from *Klrk1-/-* (green) and WT (black) mice, after epicutaneous immunization and 72h *in vitro* re-stimulation with ova; determined by ELISA. Data expressed as mean ± 1 SEM and n=6-10 mice/group. All experiments (A-D) were repeated twice with comparable results. (E) Total serum IgE response in WT C57BL/6 (black) and *Myd88-/-* (blue) mice 10 days after primary epicutaneous immunization as described in Fig. 1 or in un-immunized mice. Data expressed as mean ± 1 SEM and n=4 for un-immunized mice and n=7 for immunized mice/group.