Supporting Online Material for

**The Intraepithelial T Cell Response to NKG2D-Ligands Links Lymphoid Stress Surveillance to Atopy**

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Materials and Methods

Mice. Mice expressing the reverse tetracycline-responsive transactivator domain under control of the epidermis-specific involucrin promoter were intercrossed with mice bearing Rae-1 or H60c transgenes under control of a tetracycline-dependent response element. Expression of Rae-1 or H60c was induced in offspring bi-transgenic (BTg) for the reverse tetracycline-responsive transactivator domain and tetracycline-dependent response element by administration of 3 mg of doxycyclin (Lillico) per gram of solid food (4). FVB/N LC-deficient (Langerin-DTA) mice were generated by using a transgene encoding the human Langerin promoter to drive expression of diptheria toxin, as previously described (26). Tcrg-/- (27), Tcrb-/- (28), Tcrg-V5-/- (29) and Tcrg-V1-/- (30) mice were backcrossed onto the FVB/N background for >10 generations. Tcrg-V5+/Tcrg-V1+ and Tcrgb-/ mice were generated by intercrossing the respective mutants. FVB/N mice (Jackson Laboratories) were bred for use as controls. Klrk1-/ mice were generated as previously described (31) and Klrk1+/ and C57BL/6 wild type mice used as controls. Myd88/- mice were generated as described (32) and C57BL/6 wild type mice used as controls. Mice were maintained under specific pathogen-free conditions and food and water provided ad libitum. Mice were used at ≥6 weeks of age. All studies complied with institutional guidelines and the UK Home Office for Laboratory Animal Care regulations.

Epicutaneous immunization. The upper backs of mice were closely shaved with surgical blades and the mice rested for 24h. A single patch (Johnson & Johnson) containing 100 μg endotoxin-free OVA (extracted from chicken eggs as described in (33)) in PBS, or PBS alone for control mice, was then fixed to the mouse back using Mastisol liquid adhesive. Patches detached naturally, usually within 4 days. Primary immune responses following epicutaneous immunizations were assayed on day 10 after application of the patch. For assay of memory responses, a second patch was applied similarly after re-shaving 70 days after the primary patch and secondary immune responses determined 10 days later.

Tape-stripping. The stratum corneum was removed from both sides of the earlobe by application and removal of cellophane tape (ScotchTM) five to eight times. Skin was collected for examination at various time-points after tape-stripping.

Microscopy. Epidermal sheets were prepared from mouse ears that had been split into dorsal and ventral sides and floated dermal side down for 2h at 37°C in 20 mM EDTA. Epidermal sheets were gently lifted from the dermis, washed in PBS, and fixed in cold acetone for 20 min at −20°C. After washing in PBS, epidermal sheets were incubated for 1h at room temperature with 2% (wt/vol) BSA in PBS and were stained overnight at 4°C with antibodies specific for TCRγδ (GL3; BioLegend), MHCII (I-A/I-E; 2G9; BD PharMingen), Rae-1 (186107; R&D Systems) or isotype controls. Sheets were washed thoroughly in PBS and bound antibodies were detected for 90 min at 37°C with goat anti-hamster 546 and goat anti-rat 555 or FITC (all from Invitrogen) in PBS containing 1% (wt/vol) BSA. After extensive washing, epidermal sheets were mounted onto slides with antifade mounting medium (Vector). For microscopic analysis of
epidermal Rae-1 induction and skin histology, whole ear or back skin was embedded in OCT medium (Sakura Finetek) and snap-frozen in liquid nitrogen and t-Butanol (Sigma). 6 μm cross-sections were prepared using a Leica JUNG CM1800 cryostat and stored at -80°C. When defrosted, slides were air-dried for ~15 min, the sections encircled with an ImmEdge pen (Vector) and left to dry. Sections were then fixed in acetone for 5 min at -20°C and subsequently rehydrated for 7 min in 0.02M PBS containing 0.15M NaCl. Following blocking with 0.02M PBS containing 3% BSA and 0.15M NaCl for 1h at room temperature, sections were incubated with monoclonal antibodies against Rae-1 (186107) for 1h at room temperature. Sections were washed three times for 5 min in 0.02M PBS containing 0.15M NaCl and antibody binding was visualized by incubating the sections with goat anti-rat 555 (Invitrogen) for 1h at room temperature. Sections were washed three times for 5 min in 0.02M PBS containing 0.15M NaCl and mounted with a cover slip using anti-fade mounting medium with DAPI (Vector). Analysis of whole epidermal sheets and sections was performed using a Leica TCS SP2+AOBS confocal laser scanning microscope with digital processing using LCS v2.02 (Leica). Skin sections were additionally stained for hematoxylin and eosin and histology examined using a Nikon Microphot-sa microscope (Nikon) with Openlab (Improvision) software.

**T cell proliferation and cytokine production.** Draining axillary LN and spleen cell suspensions were obtained by mechanical disaggregation, and 2×10^5 cells cultured in 96-well flat-bottom plates (Nunc) in a total volume of 200 μl RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-ME and 5 μg/ml gentamycin. OVA (Grade V, Sigma) was added at concentrations ranging from 5 to 450 μg/ml. Control responses to an irrelevant antigen (BSA) or concanavalin A at 1 μg/ml were also determined. Cultures were incubated at 37°C for 90h and pulsed with 1 μCi [3H]thymidine (Amersham Pharmacia) for the last 16h. Cells were harvested and thymidine incorporation determined by liquid scintillation counting on a MicroBeta (Wallac).

Supernatants were assayed initially using a Luminex multiplex kit (Millipore) according to manufacturer’s specifications, and samples analysed on a Luminex100 machine (cytokine data in Fig. 1 assayed using Luminex). Cytokines were analysed in duplicate from all supernatants (2x10^5 cells) and standard curves for each cytokine were generated using the reference cytokine concentrations supplied by the manufacturer. Raw data (mean fluorescent intensity) were then analysed using Star-Station software to obtain concentration values. The detection limit of the Luminex assay was 1.8 pg/ml. IL-13 cytokine analysis was additionally routinely performed using a DuoSet ELISA Development System (R&D Systems) according to manufacturer’s instructions (IL-13 data in Fig. 3 and 4 assayed using DuoSet ELISA with supernatants collected from 5x10^6 (Fig. 3) or 2x10^6 (Fig. 4) cells). The detection limit of the IL-13 ELISA was 15 pg/ml.

**Antibody responses.** At the end of each experiment, mice were bled by cardiac puncture and sera prepared for specific antibody determinations. For IgG, IgG1 and IgG2a antibodies, 96-well Maxisorb plates (Nunc) were coated with OVA protein at 250 μg/ml in carbonate-bicarbonate buffer at 4°C overnight. The plates were then blocked with 1% goat (total IgG) or rat (IgG1 and IgG2a) serum for 1h at 37°C. Following washing, appropriately diluted sera (100 μl in PBS) were added and the plates incubated at 37°C for 90 min. After washing, alkaline phosphatase-conjugated polyclonal goat anti-
mouse IgG Fc (Sigma), rat monoclonal anti-mouse IgG1 or IgG2a (also detects IgG2c in C57BL/6 mice) (both PharMingen) were added for 1h at 37°C. The alkaline phosphatase substrate pNPP (Sigma) was then added following further washing and absorbance measured at 405 nm. Antigen-specific and total IgE was measured by an IgE capture method. Sera to be tested and IgE standard were added to Maxisorb microtiter plate wells coated with 1 μg/ml rat monoclonal anti-mouse IgE (PharMingen) and blocked with 3% milk (Marvel) (for specific IgE) or 1% rat serum (for total IgE). Biotinylated OVA at a concentration of 100 μg/ml or biotinylated rat monoclonal anti-mouse IgE (PharMingen) at 1 μg/ml were then added and incubated for 2h at 37°C. After washing, alkaline phosphatase-streptavidin (PharMingen) was added for 1h followed by pNPP substrate.

**Quantitative real-time PCR.** Ears or back skin were collected and epidermis was isolated by floating skin dermal side down on 0.5M NH₄SCN in PBS for 40 min at 37°C. Epidermis and dermis was then separated using a dissection microscope and stored in RNA-later. The tissue was later lysed by a Qiagen TissueLyser II (Retsch) and total RNA isolated using RNAeasy kit (Qiagen). Purified RNA was treated with RNase-free DNase (Promega) and reverse-transcribed with SuperScript II RT polymerase (Invitrogen) before amplification with primer pairs described below. Real-time quantitative PCR was performed on duplicate cDNA samples using SYBR Green Master Mix (Applied Biosystems) with forward and reverse primers at a final concentration of 300 nM in a reaction volume of 20 μL. Gene-specific PCR products were continuously measured by the 7900HT Fast Real-Time PCR System (Applied Biosystems) during 40 cycles at 60°C. Following primers were used: Rae-1: F TGGACACTCAACAAGACCAATG, R CCCAGGTGACTAAGGAGT, IL-1α: F TTGGTTAAATGACCTGCAACA, R GAGCGCTCAGAACACAGTTG, IL-1β: F TTGACGGACCCAAAAGAT, R GAAGCTGGATGCTCTCATCTG, IL-4: F CATCGGCAATTTTGAGACGAG, R CGAGCTCACTCTCTTGAGTGA, IL1-β: F TTGACGGACCCAAAAGAT, R GAAGCTGGATGCTCTCATCTG, IL-10: F GCCCAGAAATCAAGGAGCATT, R TGCTCCACTCCTTCTCTTTGA, IL-13: F ACCATGACCATCTGCAAGG, R CAGAGCTTCTTCAGACTACCTACATC, IL-25: F TGGAGCTCTGCATCTGTGTC, R GATTCAAGTCTCTTCCATACATC, IL-33: F CACATTGACCATCCAAGGAA, R AACAGATTGTTGCTATTGTATCTAC, TSLP: F TGGACACTCTCAGAGCAGG, R TGGTGTTCATGAGCTCCTGGTA, cyclophilin: F CAAATGTACACTACGAGATCATC, R CAATGGGAGACCAACACAA, R CCATGCCAGCATTCTGCTTT, and GAPDH: F ACTCCACTCAGGCAAATTCA, R GCCTCACCCCGATTTTGAGTGT. Data were evaluated relative to these reference genes using the 2^ΔΔCT method.

**Flow cytometry and cell sorting.** Epidermis of BTg and STg mice was collected as above and dissociated into a single-cell suspension as previously described (4). Cell suspensions were blocked for 15 min on ice with 2.5 μg antibody to FcR (anti-FcR; CD16-CD32) in PBS containing 2% (vol/vol) FCS. The cell suspensions were then labeled with anti-MHCII and anti-CD45 antibodies (BD PharMingen) on ice, and sorted for DETC (CD45 single-positives), LC (CD45 and MHCII double-positives) and keratinocytes (double-negatives) on a MoFlo cell sorter (Beckman Coulter). Electronic gates were set on live cells with combination of forward- and side-scatter properties and
7-amino-actinomycin D (Calbiochem) exclusion. The sorted cells were immediately lysed and the RNA extracted for qRT-PCR analysis using the Qiagen RNAeasy Mini Kit (Qiagen).

**DETC culture.** Epidermal cell suspensions were prepared from shaved body-wall skin by trypsinization as described (4) and stimulated with 1μg/ml anti-TCRδ antibody (GL3; BioLegend) for 48h. The cells were then removed from stimulation and cultured for a further 12 days in complete RPMI supplemented with IL-2. The cultures were analyzed by FACS for purity, and then stimulated at 200,000 cells/well with varying concentrations of Rae1-Fc fusion protein, Fc fusion control (both R&D Systems) and/or anti-TCRδ antibody (GL3). After 48h stimulation, supernatants were collected and analyzed by ELISA and the cells lysed and RNA extracted for qRT-PCR analysis.

**Statistical evaluation.** The statistical significance of differences between most experimental groups was determined with a two-tailed Student’s t-test for unpaired data. For analysis of differences between Klrk1/−, Klrk1+/− and WT C57BL/6 mice a nonparametric Mann Whitney test was performed. For analysis of differences between the WT and Trcd/− RT-PCR data, groups were compared using ANCOVA with the response modeled as polynomial functions of time. For each PCR product the significance of differences between groups was estimated using the smallest p-value for the differences between the polynomial coefficients. Calculations were performed in R (34). For all statistical evaluation, differences were considered significant at the p<0.05 level.
Fig. S1. Acute epidermal Rae-1 expression causes local immune activation
(A) Photomicrographs of transgenic Rae-1 expression [red] in dorsal epidermis of STg (control) mice [left] and BTg mice [right], both after doxycyclin (dox) treatment and co-stained for nuclei using DAPI (blue). (B) TCRγδ+ DETC (red) and MHCII+ LC (green) in freshly isolated whole epidermal sheets of STg control mice [left] and BTg mice [right], both treated with dox for 5 days. Original magnification x63. Micrographs are representative of analysis of ≥ 10 mice per condition.
Fig. S2. DETC are the only NKG2D expressing cells in the epidermis

Whole epidermal cell-suspension were prepared and co-stained for CD45, TCRγδ, MHCII and NKG2D. Electronic gates were set on live cells with a combination of forward- and side-scatter properties and 7-amino-actinomycin D exclusion. Further gating on CD45+ cells shows that only TCRγδ+ DETC express NKG2D.
Fig. S3. Acute epidermal Rae-1 expression induces systemic immune effects with enhanced splenic T cell proliferation

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 ova-specific lymphocyte proliferation from spleen by [³H] thymidine incorporation. Black line shows 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. S4. Mild tissue abrasion removes the stratum corneum but leaves the epidermis and dermis intact**

(A) H&E staining of WT mouse ears before [left panel] and immediately after tape-stripping (0h) and at 24h and 48h thereafter. The stratum corneum (deep pink) is transiently removed, causing mild damage to the epidermis but not to the dermis, akin to resting atopic dermatitis skin in humans. The epidermis is fully repaired by 48h. Cross-sections of frozen ear skin was investigated after staining and images are representative of analysis of ≥ 5 mice per condition. Original magnification x20.
Fig. S5. Epidermal Rae-1 expression characterize the response to mild tissue abrasion

Expression of endogenous Rae-1 mRNA in WT epidermis at various time-points following 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 mice were analysed per time-point in 3 separate experiments.
Fig. S6. Local stress-surveillance responses to mild abrasion induce a variety of cytokines in the skin

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 is expressed relative to the control gene cyclophilin; p-values refer to statistically significant differences between WT and Tcrd-/ mice as a function of time. 2 mice of each genotype are shown per condition. The entire experiment was repeated 3 times for WT (n=6 per time-point) and 2 times for Tcrd-/ (n=4 per time-point).
Fig. S7. Skin expression of Rae-1 and Th2 cytokines following shaving

The upper backs of WT mice were closely shaved and skin collected at various time-points after shaving. qRT-PCR was run on RNA extracted from isolated epidermis with each bar representing an individual mouse. Results are expressed relative to a reference gene, cyclophilin.
Fig. S8. Epidermal stress-surveillance is linked to DETC-induced Type-2 responses
LC, DETC and keratinocytes (KC) were FACS sorted from isolated epidermis of BTg Rae-1 and H60c inducible mice (red) or STg control mice (blue) 72h after commencing
dox treatment. Each bar represents mRNA pooled from 2 mice with two independent experiments shown for each strain. Results are expressed relative to the reference gene cyclophilin ((-) indicates sample below detection).
Fig. S9. Induction of ova-specific immunoglobulins is dependent on \( \alpha \beta \) T cells but total non-specific IgE is not

Following shaving, ova was applied to dorsal skin of WT (black), Tcrd-/- (red) or Tcrb-/- (grey) mice. The Ig response was assayed 10 days after primary immunzation by ELISA. Total IgE levels were also assayed by ELISA and compared to un-immunized WT and Tcrd/-/Tcrb/- double-knockout mice. Data expressed as mean ± 1 SEM and n=6-8 mice/group. Experiments were repeated independently six times for Tcrd-/- and WT and twice for Tcrb-/- mice with comparable results.
Fig. S10. IgE is rapidly induced and regulated by γδ T cells
Serum ova-specific IgG1 and total IgE in WT (black) and Tcrd-/- (red) mice 7 and 10 days after epicutaneous immunization. Controls include serum Ig measured 7 and 10 days post s.c. immunization of WT mice with ova in incomplete Freund’s adjuvant (IFA).
**Fig. S11. IgE is readily-detected in chemically-induced skin tumours**

Confocal images showing cross-sections of skin tumours, from WT mice subjected to low-dose two-stage chemical carcinogenesis, stained against IgE (red) and cell nuclei (blue). Both skin papillomas (A) and carcinomas (B) show high levels of IgE staining. Micrographs are representative of analysis of several separate papillomas and carcinomas from 4 individual mice. Original magnification x20.
Fig. S12. Mice lacking MyD88 induce comparable levels of Rae-1, IL-13 and IL-25 in the epidermis following mild epicutaneous stress

qRT-PCR analysis of mRNA expression for Rae-1 and the indicated cytokines over a 48h period following tape-stripping in WT C57BL/6 (black) and Myd88-/- epidermis (blue). Data expressed relative to the control gene cyclophilin; each bar represents an individual mouse.
Fig. S13. DETC are distinguished from conventional Th2 cells by more overt IL-13 than IL-4 production

(A) RNA from cultured DETC lines (black), that were stimulated with Rae-1, CD3 or left unstimulated for 48h, were compared with conventionally polarized αβ Th2 cells (green), stimulated with CD3 or left unstimulated (-), in their capacity to produce IL-13 and IL-4. qRT-PCR analysis of IL-13 and IL-4 mRNA is expressed relative to the reference gene cyclophilin and data is here shown as the ratio of IL-4 to IL-13 and *vice versa*. (B) qRT-PCR analysis as in (A) of IL-13 and IL-4 mRNA expressed by DETC isolated from Rae-1 inducible BTg mice given dox for 5 days. Expression of BTg DETC (red) IL-4 and IL-13 is analysed relative to cyclophilin and is here expressed as the cytokine ratio and compared to similarly analysed conventional Th2 cells (green). DETC from STg control mice do not express IL-4 or IL-13.
Fig. S14. Contribution of MyD88 to antigen-specific IgG responses following epicutaneous stress-immunization

WT C57BL/6 (black) and Myd88-/- (blue) mice were epicutaneously immunized with ova by applying a patch to shaved back skin. Ten days after such primary immunization, induced serum levels of ova-specific total IgG, IgG1 and IgG2c were assayed by ELISA and compared to un-immunized mice. Data is expressed as mean ± 1 SEM; n=4 for un-immunized mice and n=7 for immunized mice.
References and Notes


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unized mice per group.