Acute NKG2D ligand upregulation promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis

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Abstract

The self-encoded NKG2D ligands MICA (human) and Rae-1 (mouse), are highly expressed in carcinomas and inflammatory lesions, and are strongly implicated in immunosurveillance and graft rejection. However, whether NKG2D ligands possess an intrinsic capacity to acutely regulate tissue-associated immune compartments is not known. Here we show that epidermal-specific Rae-1 up-regulation induced rapid, coincident and reversible changes in the organization of tissue-resident $V_\gamma5V_\delta1$ TCR$\gamma\delta^+$ intraepithelial T cells and Langerhans cells (LC), followed by epithelial infiltration by unconventional $\alpha\beta$ T cells. Local $V_\gamma5V_\delta1^+$ T cells limited carcinogenesis, whereas, unexpectedly, LC promoted carcinogenesis. These results provide unique insight into the early phases of tissue immunosurveillance, and indicate that acute changes in NKG2D ligands may alone initiate a rapid, multifaceted immunosurveillance response *in vivo*.

Introduction

The immunosurveillance theory of Burnet and Thomas proposed that adaptive lymphocytes could respond to and reduce tumour growth by recognizing tumour antigens. Myriad approaches have provided experimental data in support of this theory. For example, B and T cell reactivity to tumours is observed in tumour-bearing patients, whereas increased susceptibility to spontaneous and chemically-induced carcinogenesis has been reported for mice lacking various T cell subsets ($\alpha\beta$ T cells, $\gamma\delta$ T cells, natural killer T (NKT) cells), effector molecules, (perforin, TRAIL, interferon-γ), and associated signalling molecules (interferon-γ receptor, STAT-1)[1].

This notwithstanding, the generality and clinical applicability of tumour-specific adaptive responses has remained conspicuously controversial with some questioning whether immune systems can detect the very early signs of cellular dysregulation that precede tumorigenesis and that occur in the absence of non-self signals and/or inflammation[2]. At the same time, it has become increasingly clear that anti-tumour potential exists in lymphoid cells such as NKT and $\gamma\delta$ T cells, that may sit between innate and adaptive immunity. Like adaptive cells, they bear receptors encoded by somatically rearranged genes yet, like innate cells, they lack clear potential for establishing antigen-specific clonal memory and thus may sit between innate and adaptive immunity. This type of response has been referred to as "transitional immunity", and its significance in tumour-surveillance is evident in the anti-tumour activity of the NKT cell agonist, alpha galactosyl ceramide (α-GalCer)[3,4], and the observation that mice deficient in $\gamma\delta$ T cells are substantially more susceptible to several regimens of skin carcinogenesis.[5,6]. Indeed, adjuvants for $\gamma\delta$ T cells are now being employed clinically as part of tumour immunotherapy regimens[7,8].

The carcinogenesis regimens to which $\gamma\delta$ T cells respond are commonly characterised by over-expression of the MHC Class Ib protein, Rae-1. Murine Rae-1, H60 and MULT and their human orthologs MICA and MICB[9-12] are particularly attractive candidates for triggering immunosurveillance, because these proteins engage the activating receptor NKG2D, which is expressed by cells of the innate (NK cells), transitional ($\gamma\delta$ and NKT cells), and adaptive ($CD8^+$ T cells) immune responses. Moreover, NKG2D ligand expression appears to be activated very early following cellular dysregulation. Transcripts encoding Rae-1 and H60 are upregulated in the skin of mice within 24h of carcinogen treatment, and this expression is sustained throughout papilloma and carcinoma formation[5], evoking the common expression of MICA and MICB by human carcinomas[13]. Rae-1 induction occurs within hours of DNA damage[14], although NKG2D ligands are also

upregulated during viral infection[15], in transplant scenarios[16], and in numerous non-malignant inflammatory lesions such as Type I diabetes (in the NOD mouse)[17] and rheumatoid arthritis (in humans)[18]. Indeed, a recent paper argued that antibodies generated against MICA had prognostic value in renal graft rejection[16]. Consistent with a substantive role of Rae-1 and MICA in immune activation, it was reported that MCA-induced fibrosarcomas develop more readily in mice in which antibodies are used to neutralize NKG2D *in vivo*[19]. Not surprisingly then, viruses and tumours collectively possess several mechanisms to evade NKG2D-mediated recognition[15,20], and tumors that develop in the absence of perforin-mediated cytotoxicity express higher amounts of Rae-1[19]. These observations are consistent with the hypothesis that NKG2D-expressing cells 'immunoedit' tumor phenotypes[21].

At the same time, one immunoevasion mechanism attributed to tumours is chronic expression of Rae-1 or MICA, that downregulates NKG2D expression on effector cells[20]. By modelling this chronic NKG2D ligand expression in transgenic mice, an intrinsic capacity of NKG2D ligands to affect immune cells was established[22,23]. Despite these findings, experiments assessing *in vivo* the intrinsic immunoregulatory capacity of acute alterations in NKG2D ligand expression, which would seem to be a prerequsite for its efficacy as a generic sentinel of cellular dysregulation, have not been undertaken. Indeed, no existing experimental system provides a framework for understanding early events in tissue immunosurveillance *in vivo*, which may occur prior to the gross dysregulation that characterises tumorigenesis, infection, and inflammation (e.g. altered cell growth, pertubations in tissue organization, increased oxidative stress, and the potential expression of neo-antigens). Thus, the key question of whether NKG2D ligands might initiate immunosurveillance and/or inflammation by acting as primary activators of an immune compartment remains unresolved. Indeed, it has been hypothesised that stimulation *de novo* of lymphocytes requires cell necrosis and/or activation of antigen presenting cells *via* microbial sensors such as Toll-like receptors (TLRs)[24]. Furthermore, were NKG2D ligands to be sufficient to promote responses among immune cells, there is as yet no appreciation of the extent of those responses. For example, are early responses exclusively demonstrated by $NKG2D^+$ cells or can cells respond indirectly? These are critical issues speaking not only to the intrinsic capacity of the immune system to respond to non-microbial dysregulation, but also to the potential of manipulating NKG2D ligands to clinical effect.

Much attention has recently been paid to the fact that stromal immune cells including macrophages[25], and T cells[6,26], may *de facto* promote rather than inhibit tumor growth. These observations notwithstanding, the initial responses of local, tissue-associated immune cells to acute activating signals are generally assumed to

be protective. In part, this assumption owes itself to the widely-accepted hypothesis that during infection and tumor growth, tissue-resident dendritic cells such as Langerhans cells (LC) may promote the expansion of host-protective antigen-specific T cell populations following their migration to draining lymph nodes[27]. Added to this, evidence that γδ T cells compose a component of host resistance to carcinogenesis[5] has fuelled the widespread belief that intraepithelial lymphocytes (IELs), often rich in γδ T cells, are themselves immunoprotective. Nonetheless, the relative contributions to carcinogenesis of local *versus* systemic γδ T cells (and likewise of local dendritic cells, such as LC) have never been tested *in vivo*. Indeed, the established immunoregulatory capacities of TCRγδ$^+$ IELs[28,29] and LC[30] have raised questions about their respective immunoprotective potentials.

Here we present an experimental system that provides a novel framework in which to determine how the early events in tissue immunosurveillance may unfold. By divorcing Rae-1 expression from any other aspect of tumorigenic or inflammatory dysregulation, our experiments revealed that tissue-associated immune cells compose an extremely dynamic compartment that is sensitive to upregulation of self-encoded MHC-like stress molecules, in the absence of any other overt molecular signals. The local immune reorganization induced by acute expression of these stress molecules was unexpectedly multifaceted, and featured an unanticipated infiltration by unconventional αβ T cells that proved predictive of the phenotype of tumor-infiltrating lymphocytes (TILs). Moreover, although local T cells exhibited strong immunoprotective potential, local LC promoted carcinogenesis.

Results

Local immunoregulation by Rae-1

Whereas the intrinsic immunoregulatory potential of microbial ligands for host receptors such as TLRs has been established[24,31], there has been no such assessment of acute exposure to the self-encoded ligands for the activating NKG2D receptor. To examine the intrinsic capacity of acute Rae-1 upregulation to regulate immune responses within a tissue, we designed a bi-transgenic (BiTg) mouse (**Supplementary Fig. 1**, online) wherein Rae-1 mRNA expression could be routinely induced specifically in the epidermis, simply by administration of doxycycline (dox) (**Fig 1a**). Under normal conditions, the resident immune cells in the epidermis compose an interdigitating network of LC and TCRγδ$^+$ IELs, known as dendritic epidermal T cells (DETC). Both cell types were highly dendritic in morphology and seemed sufficient in number and area to monitor most (and possibly all) basal keratinocytes (**Fig 1b**, **Supplementary Video 1**, online). DETC, but not LC, constitutively expressed NKG2D (**Fig 1c**).

Three days after addition of dox to control (single transgenic) mice, there was no change in the characteristic pattern of immune cells in the epidermis (**Fig 2a**). As dox is administered to food that is provided *ad libitum*, specific time points reflect the maximum time of induction. There was also no change in the immune compartment of dox-treated BiTg mice in which the expression of the human epidermal protein corneodesmosin (CDSN), instead of Rae-1, was dox-inducible (data not shown). Collectively, these controls establish that neither dox, nor the epidermal upregulation of a transgenic protein substantially affect LC or DETC.

Conversely, by the same time point, BiTg mice displayed substantial changes in response to dox-induced epidermal expression of Rae-1. First, the DETC became rounded (**Fig 2a**; **Supplementary Video 2**, online), consistent with the established Vav1-mediated regulation of cytoskeleton induced by NKG2D signalling[32]. We also detected other phenotypic changes, such as the consistent, albeit modest, upregulation of CD69 on DETC, and the appearance of cells with markedly reduced TCRγδ expression (**Fig 2b, c**). Conversely, the surface expression of NKG2D on DETC was not decreased over the time of induction studied here (**Supplementary Fig. 2**, online).

Overt and co-ordinate changes also characterised the LC compartment, which was perhaps surprising given that LC did not express NKG2D (**Fig 1d**). These cells too became rounded, and displayed fewer and longer dendrites, consistent with the morphologic changes reported after more complex epidermal stimuli[33] (**Fig 2a**). LC

also showed modest but consistent CD86 upregulation (Fig 2d). It was common for DETC and LC cell bodies to become juxtaposed (Fig 2a, arrows); as a result of these collective changes, large areas of the epidermal sheets were no longer proximally contacted by DETC or LC. These changes were particularly accentuated by 120h after dox administration, when in the BiTg Rae-1 mice, but not in single Tg mice or BiTg CDSN mice, all LC and DETC cell bodies seemed fully rounded, and the residual dendrites of the LC were very narrow (Fig 2a, inset and data not shown). In short, the resident local immune compartment is highly dynamic in response to acute changes in the expression of a single, self-encoded, 'stress-associated' gene product.

Unconventional αβ T cells

By 72h after dox treatment, occasional αβ T cells were detected in the epidermis of BiTg Rae-1 mice, whereas they were very rare in single Tg or BiTg CDSN mice (Fig 3a, b and data not shown). By 120h, we detected large clusters of αβ T cells in BiTg Rae-1 mice (Fig 3c). These αβ T cells did not intersperse with DETC; instead these two cell populations occupied almost mutually exclusive regions (Fig 3d). In focal areas, the αβ T cells assumed the dendritic morphology normally associated with DETC (Fig 3e), indicative of intimate molecular associations with keratinocytes and akin to those displayed by αβ T cells that fill the epidermal space vacated by DETC in *Tcrd*$^{+/-}$ mice[34] (Fig 3f). These findings demonstrated that such morphological changes to αβ T cells occur rapidly, and may compose a normal aspect of acute immunosurveillance within epithelia.

Notably, despite the complete reconfiguration of the epidermal immune compartment evident by 120h, all changes were fully reversible within three days of dox withdrawal (Fig 3g, h), revealing a rapid "resetting" of the local immune compartment upon removal of the epithelial stress ligand. Flow cytometric analysis of the epidermis of 15 BiTg Rae-1 mice permitted us to establish that the increased representation of αβ T cells in the induced BiTg Rae-1 mice was statistically significant relative to that of either single Tg mice (P = 0.003) or induced BiTg Rae-1 mice from which dox had been withdrawn (P = 0.024) (Supplementary Fig. 3, online).

Because overt epidermal infiltration by αβ T cells following Rae-1 upregulation occurred rapidly and in the absence of obvious antigen it seemed unlikely that it was attributable to conventional αβ T cells recruited from the local lymph nodes. Infiltrating αβ T cells were uniformly NKG2D^{+}NK1.1^{+}CD4^{-}CD8^{-} (Fig 4a), thus refuting the hypothesis that these cells may have been conventional NKG2D^{+}CD8^{+} memory T cells percolating through the skin. Consistent with an effector phenotype,

infiltrating αβ T cells expressed surface CD44 but not CD62L (Fig 4a). Staining with a set of antibodies recognizing specific TCR V_β regions showed that the TCR repertoire of infiltrating αβ T was clearly distinct from that of splenic αβ T cells (Fig 4b). For example, although $V_\beta6^+$ cells were consistently detected in both skin and spleen, there was negligible representation in the skin of other TCR V_β regions (e.g. $V_\beta14$ and $V_\beta7$) that were invariably present among splenic T cells of wild-type mice and among the epidermal αβ T cells in *Tcrd*$^{+/-}$ mice. Conversely, many of the skin cells in the BiTg Rae-1 mice expressed $V_\beta2$ which is rare among systemic cells of wild-type mice, but which frequently pairs with $V_\alpha14$ on NKT cells[35,36]. The mean fluorescence intensity of TCR staining on infiltrating αβ T cells was consistently lower than that of systemic cells. However, because the diverse repertoire of αβ T cells that fills the epidermal space in *Tcrd*$^{+/-}$ mice commonly displayed high amounts of surface TCR complexes (Fig 4b), we could exclude that low TCR expression in BiTg Rae-1 mice was an artefact of the isolation procedure. Instead, it more likely reflects the cells' activated state and/or a characteristic feature of the cells, such as has been reported for NKT cells[36]. Consistent with this, the majority of T cells infiltrating the epidermis of BiTg Rae-1 mice bound specifically to α-GalCer-CD1d tetramers, by contrast to the majority of splenic T cells, DETC, or resident epidermal αβ T cells in *Tcrd*$^{+/-}$ mice (Fig 4c).

$CD4^-CD8^-$ αβ T cell infiltration may be a previously unrecognized aspect of tissue immunosurveillance. Indeed, by individual analysis of 4 mice, we found consistently that tumor-infiltrating lymphocytes from papillomas and carcinomas induced by 2-stage chemical carcinogenesis were approx 10-fold-enriched in $CD4^-CD8^-$ αβ T cells, relative to systemic lymphocytes (7.68% ± 2.50% $CD4^-CD8^-$ in TIL vs. 0.70% ± 0.31% $CD4^-CD8^-$ in peripheral blood lymphocytes; $P = 0.03$).

Protective role of skin IEL

As Rae-1 RNA is rapidly upregulated during chemical carcinogenesis[5], the coordinated response to acute local Rae-1 expression may be considered a model for the early phases of tumor immunosurveillance. Indeed, in carcinogen-treated mice, we detected rounded-up $V_\gamma5^+$ T cells juxtaposed with upregulated Rae-1 protein expression, primarily in basal and follicular areas out of which squamous cell tumors most often develop[37,38] (Supplementary Fig. 4, online). Collectively, these findings prompted us to ask whether the immune cell populations coordinately responsive to Rae-1 upregulation are universally host-protective. Although it is established that *Tcrd*$^{+/-}$ mice are more susceptible to two stage chemical carcinogenesis[5], the relative contributions of systemic versus intraepithelial T cells have not been established in this or any other tumor surveillance system.

We exploited the fact that the over 90% of the natural DETC population expresses $V_\gamma5V_\delta1$ TCRs that bind to the clonotypic TCR-specific antibody, 17D1[39]. Such cells develop in the fetal thymus and in the adult are found only in the skin. By intercrossing FVB *Tcrg-V5*$^{-/-}$ and *Tcrd-V1*$^{-/-}$ mice we generated *Tcrg-V5*$^{-/-}$ *Tcrd-V1*$^{-/-}$ double knockout as well as *Tcrd-V1*$^{-/-}$ and *Tcrg-V5*$^{-/-}$ single knockout mice. *Tcrd-V1*$^{-/-}$ but not *Tcrg-V5*$^{-/-}$ mice contained $V_\gamma5^+$ DETC (~60% of epidermal γδ T cells) (**Fig. 5a**), whereas (as observed previously[39]), some DETC in *Tcrg-V5*$^{-/-}$ mice expressed TCRs that bound to 17D1 (~40% of epidermal γδ T cells). As expected, neither $V_\gamma5^+$ nor 17D1$^+$ cells were represented in double knockout mice, although such mice contained substantial numbers of 'replacement γδ TCR$^+$ DETC' (**Fig. 5a**). Notably, double knockout mice resembled *Tcrd*$^{-/-}$ mice in their susceptibility to tumors induced by a two stage carcinogenesis regimen, thereby establishing a key host-protective role played by local T cells (**Fig. 5b**). Nonetheless, given that *Tcrg-V5*$^{-/-}$ *Tcrd-V1*$^{-/-}$ mice contain replacement DETC, local T cell compartments *per se* are insufficient to provide protection. Instead, intraepidermal T cells with appropriate TCR specificities and/or responses are required, as are clearly retained in the *Tcrd-V1*$^{-/-}$ and *Tcrg-V5*$^{-/-}$ mice, which showed no significant increase in tumor susceptibility relative to wild type mice (**Fig. 5b**).

In short, the results establish a reliance of tumour resistance on certain types and/or TCR specificities of intraepidermal T cells. Consistent with the involvement of local $V_\gamma5V_\delta1^+$ DETC in the early stages of immunosurveillance, double knockout mice (like *Tcrd*$^{-/-}$ mice) displayed ~2.2-fold greater susceptibility than wild-type mice to papilloma formation (**Table 1**). Conversely, tumor progression, as measured by the ratio of carcinomas to total tumors, was similar in double knockout (average 0.634), wild-type (average 0.665) and single knockout mice (average 0.619).

Tumor-promoting role of Langerhans cells

Analogous studies were undertaken on mice rendered LC-deficient by the use of a diphtheria toxin (DT) transgene regulated by the Langerin promoter[30]. Of note, comprehensive LC ablation in these mice is observed without exogenous addition of DT, and is highly selective with both dermal DC and splenic and LN Langerin$^+$ DC populations remaining intact[30]. In addition, the number and morphology of γδTCR$^+$ DETC was normal[30] and the epidermis was completely devoid of αβ T cells (**Supplementary Fig. 5**, online). Whereas all wild-type mice subjected to low doses of chemical carcinogens displayed tumors by 14 weeks post initiation, over 50% of LC-deficient mice remained tumor-free by 16 weeks (**Fig. 6a,b**). Even when high doses of chemicals were applied to the highly susceptible FVB strain, most LC-deficient mice remained tumor-free at 7 weeks post-initiation, by which time-point all wild-type mice displayed multiple tumors (**Fig. 6c**). Moreover, LC-deficient mice

rarely contained more than one or two tumors per animal, whereas there was often more than 20 tumors per wild-type mouse (Fig. 6a-c). This degree of resistance far exceeded that previously reported for *Cd8*$^{-/-}$ mice[26], strongly suggesting that the resistance could not alone be explained by a failure to prime tumor-promoting T cells, such as CD8^{+} tumor infiltrates in chemically-induced carcinomas, that express IFNγ, TNFα and cyclooxygenase-2, but which lack cytolytic effector molecules. Indeed, the resistance to carcinogenesis of LC-deficient mice was independent of the presence or absence of either some or all αβ T cells (Fig. 6d,e). In sum, the two tissue-resident immune cell types that rapidly and co-ordinately responded to acute Rae-1 upregulation had, on aggregate, diametrically opposed effects on tumor incidence, thus establishing the pleiotropy of local immunosurveillance.

Discussion

Tissue immunosurveillance, with particular reference to tumor immunology and inflammatory diseases, has been the subject of much speculation. Interest in determining whether or not immune cells can respond to dysregulated self, in addition to foreign pathogen-associated molecular patterns (PAMPs), is compounded by the clinical potential of enhancing anti-tumor responses or inhibiting excessive inflammatory tissue surveillance. In this regard, the prospect that immunosurveillance may be underpinned by the recognition of self-encoded MHC-related stress molecules such as Rae-1 and MICA, that are upregulated in a spectrum of tissue inflammation events associated with both malignant and non-malignant lesions, is of great interest. Nonetheless, few experimental data depict either the events that compose tissue immunosurveillance or their temporal sequence. At least part of the problem is the complexity of myriad events presented by full-fledged malignant or inflammatory lesions, coupled with the difficulty in identifying and examining early lesions.

In contrast, here we exploited a transgenic system to establish that acute Rae-1 upregulation is itself an axis of immunoregulation. Our data revealed the dynamic nature of a tissue-associated immune compartment (in this case the skin) in response to acute alterations in only a single self-encoded molecule. We observed a complete re-organization of tissue layout and cellular composition, including bulk coincident changes in DETC and LC. The changes in the structure and the apparent migration of DETC are predicted by the known regulation of Vav-1 downstream of NKG2D[32]. However, the bulk changes in LC are intriguing in light of the fact that LC do not express NKG2D; these findings imply a potential of local T cells to dictate the behaviour of tissue-resident myeloid cells. The mechanism underpinning this indirect NKG2D-mediated regulation is now under study, and may be germane to increasing evidence that T cells can profoundly regulate monocytes and dendritic cells, as well as *vice versa.*

The aggregate consequence of this study is to extend primary responsiveness of immune compartments from engagement of microbial pattern recognition receptors on myeloid and epithelial cells to the recognition of dysregulated self-encoded molecules by lymphoid cells. Because it has been established in transgenic mice that human MICA and murine Rae-1 have largely equivalent capacities to engage murine NKG2D[22], it is reasonable to extrapolate from our data to the effects of MICA upregulation in humans. Further refinements of our system to allow upregulation of different molecules in the skin and in different tissues will permit us

to classify other self-encoded, MHC-related molecules according to their capacity to acutely regulate local immune compartments.

Although we have not established whether or not NKG2D-ligand upregulation alone is sufficient to promote a fully-fledged adaptive immune response, the changes described are overt and make it difficult to accept the proposal that the immune system cannot "see" cellular dysregulation and hence cannot naturally mount tumor immunosurveillance[2,40]. Indeed, the response of local T cells to Rae-1 upregulation and their contribution to protection at seemingly an early stage in carcinogenesis jointly adds weight to other experimental evidence for the existence of a natural tumour immunosurveillance potential[41].

The primary responsiveness of the epidermal immune compartment to acute NKG2D ligand upregulation that is described here is a logical model for the early phases of immunosurveillance, because upregulation of Rae-1 and MICA *in vivo* can occur early after infection or carcinogen exposure; in the latter case it is upregulated prior to palpable tumour formation[5]. Indeed, upregulated Rae-1 protein decorated basal and follicular areas out of which tumours most frequently emanate[37,38]. Thereafter it was generally sustained throughout the formation of papillomas and carcinomas, evocative of the sustained expression of MICA by many human cancers. As sustained chronic expression of Rae-1 and/or MICA ultimately downregulates T and NK cell responses[20,22,23], there is strong weight to the notion that the response to acute expression of Rae-1 and/or MICA expression is a key component of immunosurveillance and exerts a high pressure for immunoevasion.

This work provided much new insight into the events occurring during early tumor immunosurveillance. First, we documented a hitherto unrecognized enrichment of $CD4^{-}CD8^{-}$ TCR$\alpha\beta^{+}$ NKT cells in tumor infiltrates. It will be interesting to resolve the origin of these cells (e.g. the dermis or the blood). Furthermore, these cells may compose a common feature of tissue immunosurveillance that is quite distinct from the antigen-driven infiltration of tissues by cognate conventional $\alpha\beta$ T cells following their clonal expansion in local lymph nodes. Of note, independent reports indicate that NKT cells are greatly enriched among IELs in human psoriatic epidermis[42,43], where they are associated with cells expressing CD1d, that is likewise upregulated within 24 hours of inducing contact dermatitis[42]. The NKT cells within these lesions were not obviously the classical, invariant type, related to which, future studies will clarify the composition and heterogeneity of the infiltrating $CD4^{-}CD8^{-}$ $\alpha\beta$ T cell repertoire in the system described here. Importantly, the intriguing possibility exists that $CD4^{-}CD8^{-}$ T cells and/or NKT cells may form a significant commonality between epidermal immunology in mice and in humans, which has

heretofore been obscured by the lack of an obvious DETC compartment in humans. Rather than accepting that the two species possess radically different mechanisms, we might now hypothesize that infiltration of $CD4^{-}CD8^{-}$ T cells and/or NKT cells (witnessed here in mice, and previously reported in humans[42,43]) may be a critical conserved feature of epidermal surveillance.

$CD4^{-}CD8^{-}$ T cells and NKT cells cells can display significant pleiotropy, and whereas human skin-derived NKT cells can produce potentially pathogenic IFNγ[42], NKT cells from irradiated mice appear to exert immunosuppressive functions[44]. In yet another system, different subsets of NKT cells enhanced and suppressed tumor surveillance respectively[36,45,46]. Clearly the primary functions of the skin-infiltrating $CD4^{-}CD8^{-}$ T cells cells detected here merit further study that may clarify the cells' aggregate contributions in human tissue surveillance. However, in practical terms, this may require specific deletion of the $CD4^{-}CD8^{-}$ NKT cell population, as $CD4^{-}CD8^{-}$ and $CD4^{+}$ NKT cells have been proposed to have different functions[36].

The second insight provided by this study is into the functional potential of local T cells. Whereas DETC can display aggregate immunosuppressive functions[29], the present study unequivocally establishes their immunoprotective function *vis-à-vis* carcinogenesis. Given that DETC act early (on papilloma formation), these data seem emphatically to uphold the long-held hypothesis that IELs directly monitor surrounding tissue dysregulation to the host's benefit, a hypothesis that has nonetheless not previously been tested experimentally. And yet, the data strikingly refute the related hypothesis that "any IEL will do the job". *Tcrg-V5*$^{-/-}$ *Tcrd-V1*$^{-/-}$ mice are more susceptible to carcinogenesis, yet harbor many "replacement" skin IELs that clearly are insufficient to protect. In contrast, the IELs in *Tcrg-V5*$^{-/-}$ and *Tcrd-V1*$^{-/-}$ single knockout mice did provide protection. By examining genes differentially expressed in these different subsets, we may be able to identify key mechanisms of local T cell protection. Moreover, were the results to extend to other tissues, such as the gastrointestinal tract, these data might hold promising clinical implications for targeted immunotherapy.

The third insight provided by this work is that LC, on aggregate, promoted carcinogenesis. This result was surprising for several reasons. First, LC are ordinarily viewed as contributing to the early activation of protective immune responses. Second, LC deficiency reverted tumor susceptibility even to high dose carcinogen in the FVB strain, which is particularly susceptible to carcinogenesis. The detailed mechanism(s) underpinning LC-mediated tumor promotion is under study, but this paper already rules out that it is purely *via* acting with $\alpha\beta$ T cells, which is the primary *modus operandi* by which LC exert their known biological effects. Indeed,

these data emphasise that tissue-associated myeloid cells likely have profound biological roles that may *de facto* be obscured by the experimental emphasis on their interactions with lymphocytes.

By establishing that Rae-1 upregulation can promote major reorganization of an immune compartment, we provoke the question as to whether Rae-1 and/or MICA dysregulation might indeed be a primary cause of inflammatory pathology, without the need to implicate tissue damage or infection. In this regard, MICA is strikingly polymorphic, with many variants occurring in areas of the molecule that are more likely to affect the level of its expression rather than its engagement of ligands[47]. As the changes provoked by Rae-1 upregulation were rapidly reversible, this system provides an experimental tool by which to identify secondary events, such as specific cytokine dysregulation, that may prevent this reversibility and thereby promote chronic inflammation, as is seen in psoriasis and myriad other pathologies.

We and others previously showed that enforced chronic expression of human and mouse NKG2D ligands seemingly opposes chronic inflammation by promoting immunosuppression[22,23]. This was largely interpreted to occur *via* NKG2D receptor downregulation promoted in part by shedding of MICA and/or Rae-1. Consistent with this hypothesis, Dranoff and colleagues observed a positive association of human tumor immunotherapy with the development of antibodies that would target secreted MICA[48]. Nonetheless, the situation may be more complex. In particular, a future experimental comparison of the effects of constitutive Rae-1 expression with the effects of long-term inducible expression will hopefully permit resolution of the "tipping point" that defines the transition of NKG2D-mediated immuno-activation into immunosuppression. This may clarify whether the transition requires a particular amount and/or duration of NKG2D ligand expression, or a particular form (soluble versus cell–bound) of NKG2D ligand. It is also possible that the greatest suppressive effects of chronic MICA and/or Rae-1 expression occur during T cell and NK cell development, where it may "tune" their response thresholds for activation, as was originally considered by Grossman and colleagues in relation to TCR signalling[49]. The experimental system used here will likewise permit analysis of how responses to dysregulated self interact with responses to microbes (e.g. through TLRs). Collectively, the data have the promising potential to aid the design of immunotherapeutic and prophylactic protocols.

Methods

Mice. FVB/N mice (Jackson Laboratories) were bred for use as controls for FVB/N transgenic mice with inducible expression of the NKG2D ligand Rae-1[23] or CDSN gene (T. Silberzahn and A.H., manuscript in preparation). Mice expressing the reverse tetracylin-responsive transactivator domain (rtTA) under control of the epidermis-specific involucrin promoter were intercrossed with mice bearing Rae-1 or CDSN transgenes under control of a tetracycline-dependent response element (pTRE). Expression of Rae-1 or CDSN expression was induced in rtTA/pTRE bi-transgenic (BiTg) offspring by 3mg/g of doxycyclin (dox) (Lillico) in solid food. For other mutant mice, see Supplementary methods. All studies complied with institutional guidelines and the UK Home Office or American Association for Laboratory Animal Care regulations.

RT-PCR. Induction of transgenic Rae-1 in skin was determined by RT-PCR on tail biopsies that were snap-frozen and homogenized directly into Trizol (Invitrogen). RNA was purified, treated with RNase-free DNase (Promega) and reverse-transcribed with SuperScript II RT polymerase (Invitrogen), before amplification using a forward primer in the pTRE vector and a reverse primer in Rae-1. pTRE F, 5'-GGTCGAGTAGGCGTGTACGG-3'; Rae-1 R, 5'-GGTCAAGTTGCACCTAAGAGAGTG-3'. PCR amplifications were compared to amplification of β-actin with primers: β-actin F 5'-CAGCTTCTTTGCAGCTCCTT-3'; β-actin R 5'-CACGATGGAGGGGAATACAG-3'.

Epidermal cell isolation. Epidermal cell suspensions were prepared from shaved body wall skin by trypsinization, as described[50] or ears were split into dorsal and ventral sides and floated dermal side down in 20mM EDTA for 2h at 37°C. Whole epidermal sheets were gently lifted from the dermis and incubated 15 min in trypsin and DNAse, while shaking at 37°C. Single cell suspensions were collected following filtering through a 90μm mesh.

Tumor and peripheral blood lymphocyte isolation. Cell isolations were performed as previously described[26]. For peripheral blood lymphocytes, 200μl of blood was obtained by capillary pipette of the retroorbital plexus. The blood was mixed with 30μl of 1,000 units/ml heparin (Sigma) and 5 ml D-PBS and leukocytes purified by Lympholyte-M (Accurate Chemical) as per manufacturer's instruction. All mice were processed individually. For tumor infiltrating lymphocytes (TIL), tumors were excised and minced on ice in RPMI 1640 medium supplemented with Hepes, 2-mercaptoethanol, sodium pyruvate, antibiotics, 2.5 mg/ml collagenase I and 1.5 mg/ml collagenase II (both from Worthington), 1 mg/ml collagenase IV and 0.25 mg/ml hyaluronidase IV-S (both from Sigma), 300 μg/ml DNase I and 0.06 μg/ml

soybean trypsin inhibitor (both from Roche). Suspensions of tumor pieces were incubated at 37°C for 2 h. The pieces were then gently pressed between the frosted edges of two sterile glass slides, and the cell suspension passed through sterile 100µm Nylon mesh to remove debris and to separate cell clumps. RPMI medium was added to stop the digestion. Cells were washed three times in HBSS before Lympholyte-M gradient separation, then resuspended in RPMI medium for overnight incubation at 37°C, 5% CO2. The following day, the TIL were washed twice in HBSS and filtered through 30µm Nytex.

Flow cytometry and antibodies. Cell suspensions were blocked with 2.5µg anti-FcR (CD16/CD32) in PBS containing 2% FCS for 15 min on ice. Optimally diluted antibodies were added and cells were stained on ice for 45 min, washed twice, and, where appropriate, stained with secondary antibodies for 45 min on ice. Following washing, cells were analyzed on a FACScalibur (Becton Dickinson) using FlowJo (TreeStar) or CellQuest (BD) software. Electronic gates were set on live cells using a combination of forward and side scatter properties and 7-AAD (Calbiochem) or propidium iodide exclusion. Antibodies to CD16/CD32 (2.4G2), TCRγδ (GL3), $V_\gamma5$ (536), CD3 (145-2C11), I-A/I-E (2G9), CD86 (GL1), CD4 (RM4-5), CD44 (IM7), CD62L (MEL-14), NK1.1 (PK136), CD49b (DX5), IFN-γ (XMG1.2), hamster Ig and rat IgG, as well as isotype control antibodies, conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), CyChrome or allophycocyanin (APC) were purchased from BD PharMingen. Antibodies to TCRαβ (H57-597), NKG2D (CX5), CD69 (H1.2F3), CD8α (53-6.7), CD8β (CT-CD8b), FASL (MFL3), Perforin (eBioOMAK-D), hamster IgG and isotype control antibodies conjugated to FITC, PE or APC were purchased from eBioscience. Antibody against the prototypic $V_\gamma5V_\delta1$ DETC TCR (17D1[39]) was additionally used (author R.E.T). FITC or PE-conjugated monoclonal antibodies to $V_\beta2$, $V_\beta3$ $V_\beta4$ V_β□□□□□□□□V_β□□ V_β□□ V_β□□□□□□ V_β□□□□ V_β□□ V_β□□□□ V_β□□□ V_β□□□ V_β□□□ V_β□□□ V_α□□ V_α□□□□□□□ V_α□□□□□□□ kindly provided by J. Dyson (Imperial College, London, UK) and PE-conjugated CD1d tetramers unloaded or loaded with α-GalCer were provided by F. Nestle (Kings College London, UK).

Fluorescence microscopy. Epidermal sheets were prepared from mouse ears that had been split into dorsal and ventral sides and floated dermal side down in 20mM EDTA for 2h at 37°C. 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 with 2% BSA in PBS for 1h at room temperature and stained with optimally diluted monoclonal antibodies or isotype controls at 4°C overnight. Sheets were washed thoroughly in PBS and bound antibodies were detected with goat anti-hamster 546 or 633 and goat anti-rat 555 or 633 (all from Invitrogen) in PBS containing 1% BSA for 90 min at 37°C. After extensive washing, epidermal

sheets were mounted onto slides using anti-fade mounting medium (Vector) and examined using a Leica TCS SP2+AOBS confocal laser scanning microscope with digital processing using LCS v2.02 (Leica). Sheets were stained with antibodies specific for TCR$\gamma\delta$ (GL3), $V_\gamma 5$ (536), MHCII (I-A/I-E; 2G9), TCR$\alpha\beta$ (H57-597), CD49b (DX5) or Rae-1 (186107; R&D Systems). For microscopic analysis of tumors, see Supplementary methods.

Two-stage chemical carcinogenesis. Chemicals were obtained from Sigma. DMBA was dissolved in acetone (4 mM), and TPA was dissolved in 100% ethanol (0.2 mM). Application of DMBA and TPA and tumor monitoring were performed as previously described[5,26]. Briefly, initiation by pipette application of DMBA was performed 1 week after shaving dorsal skin and was followed by twice-weekly application of TPA. Cutaneous tumors were counted, measured, and scored weekly as clinically apparent papillomas (typically well demarcated, symmetrical, pedunculated or dome-shaped papules without erosion or ulceration) or clinically apparent carcinomas (poorly demarcated, asymmetrical, sessile or dome-shaped papules with erosion or ulceration). Tumors were evaluated by visual inspection by an observer blinded to the experimental groups. At the conclusion of experiments, tumors were excised for TIL isolation or formalin-fixed and paraffin-embedded, and sections stained with hematoxylin and eosin and examined by a certified dermatopathologist for histologic confirmation.

Statistical evaluation. The statistical significance of difference between experimental groups was determined using two-tailed Student's t-test for unpaired data, with significance at $P < 0.05$.

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Figure Legends

Figure 1. Inducible transgenic expression of Rae-1 in the epidermis where NKG2D is constitutively expressed by resident DETC. **(a)** Expression of the Rae-1 transgene in the skin of bi-transgenic (BiTg) and single transgene (SingleTg) mice after 120h on dox. Induction of transgenic Rae-1 was determined by RT-PCR on RNA from tail biopsies and compared to amplification of β-actin in the same sample. **(b)** LC and DETC form a contiguous interdigitating network in the epidermis. Confocal microscopy image of epidermal sheet isolated from ear skin of a wild-type FVB mouse showing the highly dendritic $MHCII^+$ LC (green) in non-overlapping association with $TCR\gamma\delta^+$ DETC (red). Original magnification x63. **(c)** Epidermal cells from body wall skin were stained with NKG2D-specific (red) or isotype control (blue) antibodies. Histograms depict gated $CD3^+TCR\gamma\delta^+$ DETC or $CD3^-MHCII^+$ LC.

Figure 2. Acute upregulation of Rae-1 in the epidermis induces morphological and activational changes in both LC and DETC compartments *in vivo*. **(a)** Representative confocal microscopy images of epidermal sheets freshly isolated from indicated mice treated with dox for defined time periods. $MHCII^+$ LC are depicted in green and $TCR\gamma\delta^+$ DETC in red. A minimum of 10 BiTg mice were analyzed per condition. Original magnification x63. **(b-d)** Flow cytometry of epidermal cell suspensions from ear skin of mice after 120h on dox (n=6). In graphs each dot represents an individual mouse; histograms and dot plots depict one representative mouse. **(b)** CD69 expression on $CD3^+TCR\gamma\delta^+$ DETC. *, $P = 0.002$ **(c)** TCR expression on $CD3^+TCR\gamma\delta^+$ DETC. **(d)** CD86 expression on $CD3^-MHCII^+$ LC. **, $P < 0.001$

Figure 3. $TCR\alpha\beta^+$ cells rapidly infiltrate the epidermis upon upregulation of Rae-1. **(a-f)** Representative confocal images of epidermal sheets freshly isolated from indicated mice treated with dox for defined time periods, showing $TCR\gamma\delta^+$ DETC in red and $TCR\alpha\beta^+$ cells in green. A minimum of 10 BiTg mice were analyzed per condition and images were taken of representative fields following analysis of whole epidermal sheets. **(g, h)** Reversibility of changes induced by Rae-1 expression within the epidermis. **(g)**_$TCR\gamma\delta^+$ DETC are depicted in red and $MHCII^+$ LC in green **(h)** $TCR\gamma\delta^+$ DETC are depicted in red and $TCR\alpha\beta^+$ cells in green.

Figure 4. Epidermal infiltrating $TCR\alpha\beta^+$ cells differ from conventional circulating αβ T cells and all express NK markers. **(a,b)** Flow cytometry of epidermal cell suspensions prepared from ears of BiTg mice after 120h on dox. Stringent isolation procedure ensured no dermal contamination was present and no $TCR\alpha\beta^+$ cells were found in SingleTg or WT controls. Zebraplots show representative individual mice. A minimum of 10 BiTg mice were analyzed per condition. **(a)** Plots were gated on

$CD3^+TCR\alpha\beta^+$ epidermal cells. **(b)** Comparison of TCRβ repertoire in BiTg and *Tcrd*$^{-/-}$ epidermis and WT spleen. Plots were gated on $CD3^+TCR\gamma\delta^-$ cells. **(c)** α-GalCer-CD1d tetramer binding to epidermal T cells. Plots were gated on $CD3^+TCR\gamma\delta^-$ splenic or epidermal cells. A total of 7 BiTg mice were analyzed.

Figure 5. Mice selectively deficient in the prototypic $V_\gamma5V_\delta1^+$ DETC show increased susceptibility to tumor development. **(a)** Flow cytometric analysis of intraepidermal T cell populations from indicated mice. Plots in top panel gated on all epidermal cells and stained with antibodies against TCRγδ (GL3) and $V_\gamma5$ (536). Plots in lower panel gated on $TCR\gamma\delta^+$ cells and stained with antibodies against $V_\gamma5V_\delta1$ (17D1) and $V_\gamma5$ (536) **(b)** Tumor development in indicated mice subjected to low-dose two-stage chemical carcinogenesis (200nmol DMBA initiation, 10nmol weekly TPA promotion). For statistical analysis see Table 2.

Figure 6. Langerhans cell-deficient (Langerin-DTA) mice are protected from tumor development. **(a, b)** Tumor formation induced by a low-dose (200nmol DMBA initiation, 10nmol weekly TPA promotion) two-stage chemical carcinogenesis protocol in wild-type and Langerin-DTA mice (5.67±1.63 versus 1.00±0.47 tumors/mouse at wk 16, respectively, $P < 0.005$). **(b)** Photographs of representative Langerin-DTA and WT mice from the low-dose experiment in **(a)**. **(c)** Tumors induced by a high-dose two-stage chemical carcinogenesis protocol (400nmol DMBA, 40nmol TPA weekly) in Langerin-DTA and WT mice (20.20±0.59 versus 2.39±0.59 tumors/mouse at wk 16, respectively, $P < 0.0000001$). **(d, e)** Deficiency in all αβ T cells or in $CD4^+$ T cells did not abrogate the LC requirement for robust tumor development. Tumor formation induced by the low-dose two-stage chemical carcinogenesis protocol in the indicated mice (9.20±1.80 in *Tcrb*$^{-/-}$ versus 0.36±0.28 in *Tcrb*$^{-/-}$Langerin-DTA, $P < 0.0005$; 6.14±1.48 in *Cd4*$^{-/-}$ versus 0.36±0.20 in *Cd4*$^{-/-}$ Langerin-DTA, $P < 0.001$).

Table I

Mouse Strain	Tumors/Mouse[1]	P-value[2]	Carcinomas/Mouse[1]	P-value[2]
Tcrd$^{-/-}$	11.08 ± 1.74	*≤ 0.002*	5.46 ± 1.24	*≤ 0.05*
Tcrg-V5$^{-/-}$	5.83 ± 1.09	*N.S.*	3.58 ± 0.99	*N.S.*
Tcrd-V1$^{-/-}$	6.07 ± 0.95	*N.S.*	3.79 ± 0.73	*N.S.*
Tcrg-V5$^{-/-}$ *Tcrd-V1*$^{-/-}$	11.17 ± 1.36	*≤ 0.0004*	7.08 ± 1.05	*≤ 0.01*
WT	5.08 ± 0.84	-	3.38 ± 0.75	-

[1]*Mean values at week 17 post-DMBA initiation.*
[2]*P-values versus WT; N.S., not significant.*

Table I. Tumor development and progression in mice lacking components of the prototypic DETC $V_\gamma5V_\delta1$ TCR.