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Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity

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Natural killer (NK) cells attack many tumour cell lines, and are thought to have a critical role in anti-tumour immunity[1–7]; however, the interaction between NK cells and tumour targets is poorly understood. The stimulatory lectin-like NKG2D receptor[8–13] is expressed by NK cells, activated CD8$^+$ T cells and by activated macrophages in mice[11]. Several distinct cell-surface ligands that are related to class I major histocompatibility complex molecules have been identified[11–14], some of which are expressed at high levels by tumour cells but not by normal cells in adults[11,13,15,16]. However, no direct evidence links the expression of these 'induced self' ligands with tumour cell rejection. Here we demonstrate that ectopic expression of the murine NKG2D ligands Rae1β or H60 in several tumour cell lines results in potent rejection of the tumour cells by syngeneic mice. Rejection is mediated by NK cells and/or CD8$^+$ T cells. The ligand-expressing tumour cells induce potent priming of cytotoxic T cells and sensitization of NK cells *in vivo*. Mice that are exposed to live or irradiated tumour cells expressing Rae1 or H60 are specifically immune to subsequent challenge with tumour cells that lack NKG2D ligands, suggesting application of the ligands in the design of tumour vaccines.

As demonstrated by staining with a tetramerized derivative of the extracellular portion of NKG2D, NKG2D ligands are expressed by most of the tumour cells tested, including various lymphoid, myeloid and carcinoma cell lines (ref. 11; and A. Diefenbach and D. Raulet, unpublished data). Northern blot analysis showed that many of the positive cell lines express Rae1 transcripts, whereas H60 transcripts were limited to only one or two of the cell lines tested (data not shown). Rae1 transcripts have not been detected in normal cells from adult mice[15], suggesting that these genes are specifically upregulated in tumour cell lines.

To investigate whether tumour cells that express NKG2D ligands stimulate anti-tumour immune responses, we used a retrovirus expression system to ectopically express high levels of Rae1β or H60 in EL4 (a thymoma), RMA (a T-cell lymphoma) and B16-BL6 (a melanoma). These cell lines are all from C57BL/6 (hereafter termed B6) mice and do not normally express NKG2D ligands[11]. Ligand-transduced cells were selected on the basis of staining with NKG2D tetramers. To serve as controls, tumour cells that were transduced with empty retrovirus vector (designated as EL4/–, B16/– and RMA/–) were selected by genomic polymerase chain reaction (PCR) (see Methods).

For analysis of the response to EL4 and B16-BL6 tumour cells, groups of five B6 mice were inoculated subcutaneously with syngeneic tumour cell transductants. Control-transduced EL4 or B16-BL6 cells grew progressively at a rate similar to that of untransduced cells (Fig. 1a, c, d, and data not shown), leading to uniform terminal morbidity by about 28 days. Notably, Rae1β- or H60-transduced tumour cells of both types were rejected rapidly and completely, as they failed to yield detectable tumours at any time point (Fig. 1a, c, d). A tenfold increase in the dose of Rae1β- or H60-transduced EL4 cells (to 5×10^7 cells) did not change the outcome, whereas a higher dose (1×10^5) of Rae1β- or H60-transduced B16-BL6 cells resulted in progressive, although substantially delayed, tumour growth in all the mice, compared with the control-transduced tumour cells (data not shown). Ligand-transduced tumour cells of both types also failed to grow in B6 mice that had been depleted of CD8$^+$ T cells or in B6-Rag1$^{-/-}$ mice—which lack all T and B cells—but grew progressively in normal and B6-Rag1$^{-/-}$ hosts that had been depleted of NK1.1$^+$ cells (Fig. 1a–e). Thus, these doses of Rae1β- or H60-transduced EL4 cells and B16-BL6 cells are rejected rapidly by conventional NK cells without a requirement for T and B cells, including NK1.1$^+$ T cells or γδ T cells. Interestingly, Rae1β- or H60-transduced B16-BL6 cells reproducibly exhibited retarded growth in NK-depleted B6-Rag1$^{-/-}$ mice (Fig. 1e). It is possible that a residual response against these cells is mediated by non-lymphoid cells such as macrophages, or by small numbers of NK cells that survive antibody treatment.

Rae1β or H60 expression by B16-BL6 cells reduced the frequency of lung metastases by over tenfold after intravenous injection (Fig. 1f). In another experiment where mice were examined at a later time point, control-transduced B16-BL6 cells formed massive contiguous lung metastases, but ligand-transduced B16-BL6 cells were almost completely rejected (Fig. 1g). NK1.1 depletion before tumour cell inoculation markedly depressed the rejection of the metastases.

Rae1β- or H60-transduced RMA tumour cells were also rejected by B6 mice (Fig. 2). Unlike the responses to the other tumour cells, however, the primary rejection of ligand-transduced RMA cells was mediated by both CD8$^+$ T cells and NK cells, although the specific outcome depended on the dose of tumour cells. Depletion of both NK1.1$^+$ cells and CD8$^+$ T cells was necessary to abrogate rejection of the smallest inoculum of 10^4 ligand-transduced tumour cells, whereas depletion of either population allowed tumour growth in at least some animals that were injected with the largest dose (10^6) of tumour cells (Fig. 2a). With the intermediate dose of 10^5 tumour cells, depletion of CD8 cells allowed tumour cell growth, but NK cell depletion did not (Fig. 2a). Thus, either subset is sufficient for

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tumour rejection at the lowest tumour cell dose, CD8 cells (but not NK cells) are sufficient at the intermediate dose, and the two cell types must cooperate to achieve consistent rejection at the highest tumour cell dose. In B6-Rag1$^{-/-}$ mice, NK cells were sufficient to reject the Rae1β- or H60-transduced RMA cells at the two lower tumour doses (Fig. 2b), suggesting that NK cells are more active or effective in these B6-Rag1$^{-/-}$ mice than in B6 mice. Parallel analysis of mice that were inoculated with the RMA/S cell line—a major histocompatibility complex (MHC) class I^{low} version of RMA cells—confirmed previous reports that these cells are rejected by NK cells and not by CD8^{+} T cells[2], and also demonstrated the efficacy of our NK cell-depletion procedure (Fig. 2a, b).

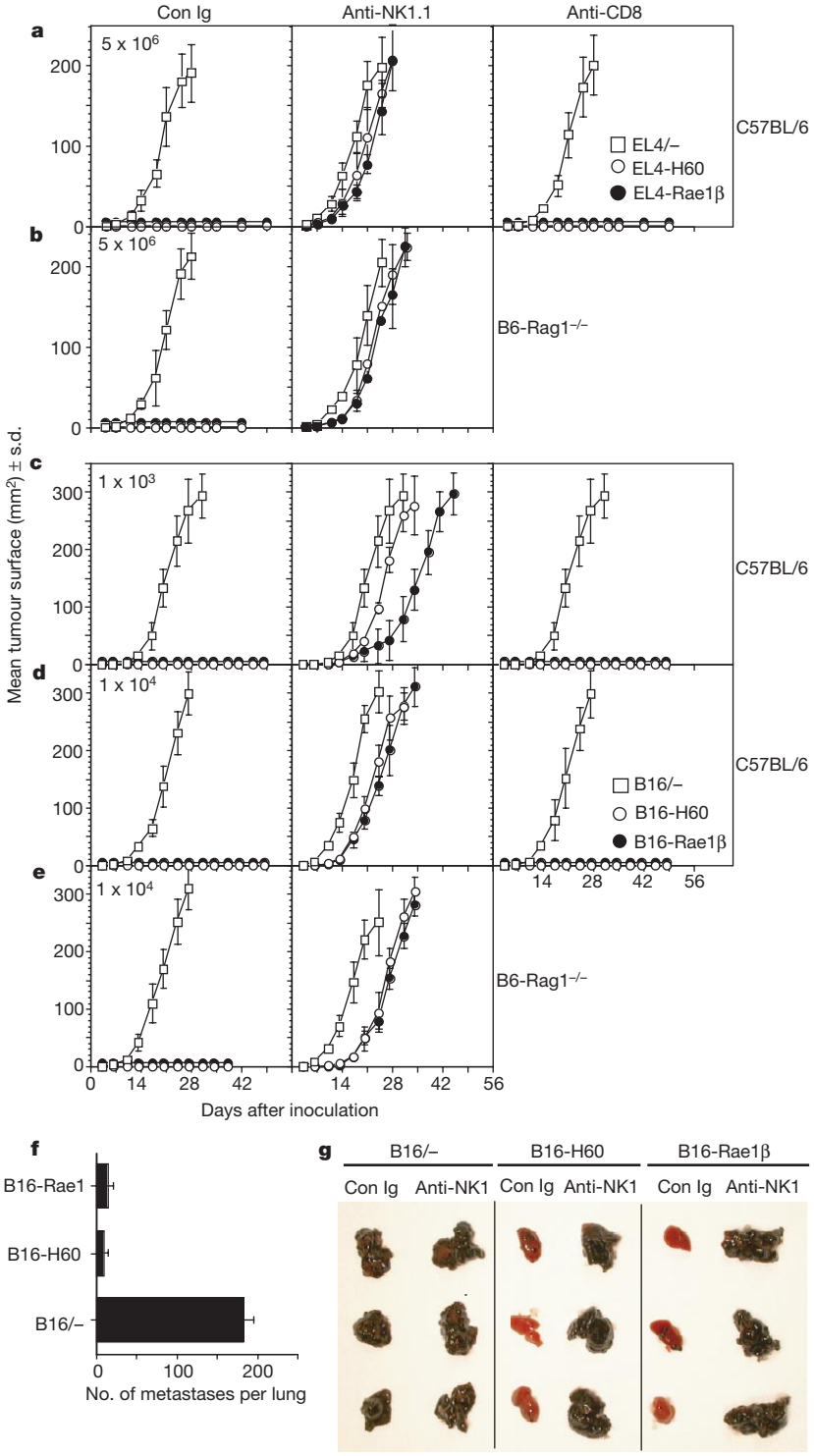

Figure 1 EL4 and B16-BL6 tumour cells that express NKG2D ligands are rejected by NK cells in syngeneic mice. Con Ig, control immunoglobulin. **a**–**e**, C57BL/6 or B6-Rag1$^{-/-}$ mice were treated with the indicated antibodies before inoculation with the indicated number of EL4 (**a**, **b**) or B16-BL6 (**c**–**e**) tumour cell transductants. Tumour growth was monitored thereafter. Data are representative of at least three experiments. **f**, **g**, B6 mice were injected intravenously with 3×10^5 B16-BL6 transductants. Lung metastases were counted two weeks after the tumour challenge (**f**), or the lung lobes from representative mice in a separate experiment were examined three weeks after tumour challenge (**g**).

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Why does NKG2D ligand expression not cause the rejection of most of the tumour cell lines that naturally express these ligands[11]? In some cases, the host response to the ligands may be overwhelmed by an excess tumour load. Furthermore, the response may critically depend on the levels of NKG2D ligands expressed by tumour cells, especially if the NK cells are also subject to inhibitory signalling by means of MHC-specific receptors. Notably, the levels of Rae1β and H60 expression in our transductants were substantially higher than the levels of endogenous NKG2D ligands on most of the naturally expressing tumour cell lines that we tested (Fig. 2c). Indeed, a comparison of varying ligand levels showed that RMA transductants that had intermediate ligand levels, comparable to most tumour cells[11] (Fig. 2c, data not shown), were less efficiently rejected than were transductants with higher ligand levels (Fig. 2d). Therefore, the antitumour response to naturally arising tumour cells probably varies depending on the level of NKG2D ligands that are expressed.

To address whether prior immunization with tumour cells that express NKG2D ligands induces protective immunity to ligand-negative tumour cells, mice that had previously rejected Rae1β- or H60-transduced tumour cells (EL4, B16-BL6 or RMA cells) were re-challenged with corresponding ligand-negative tumour cells 8–12

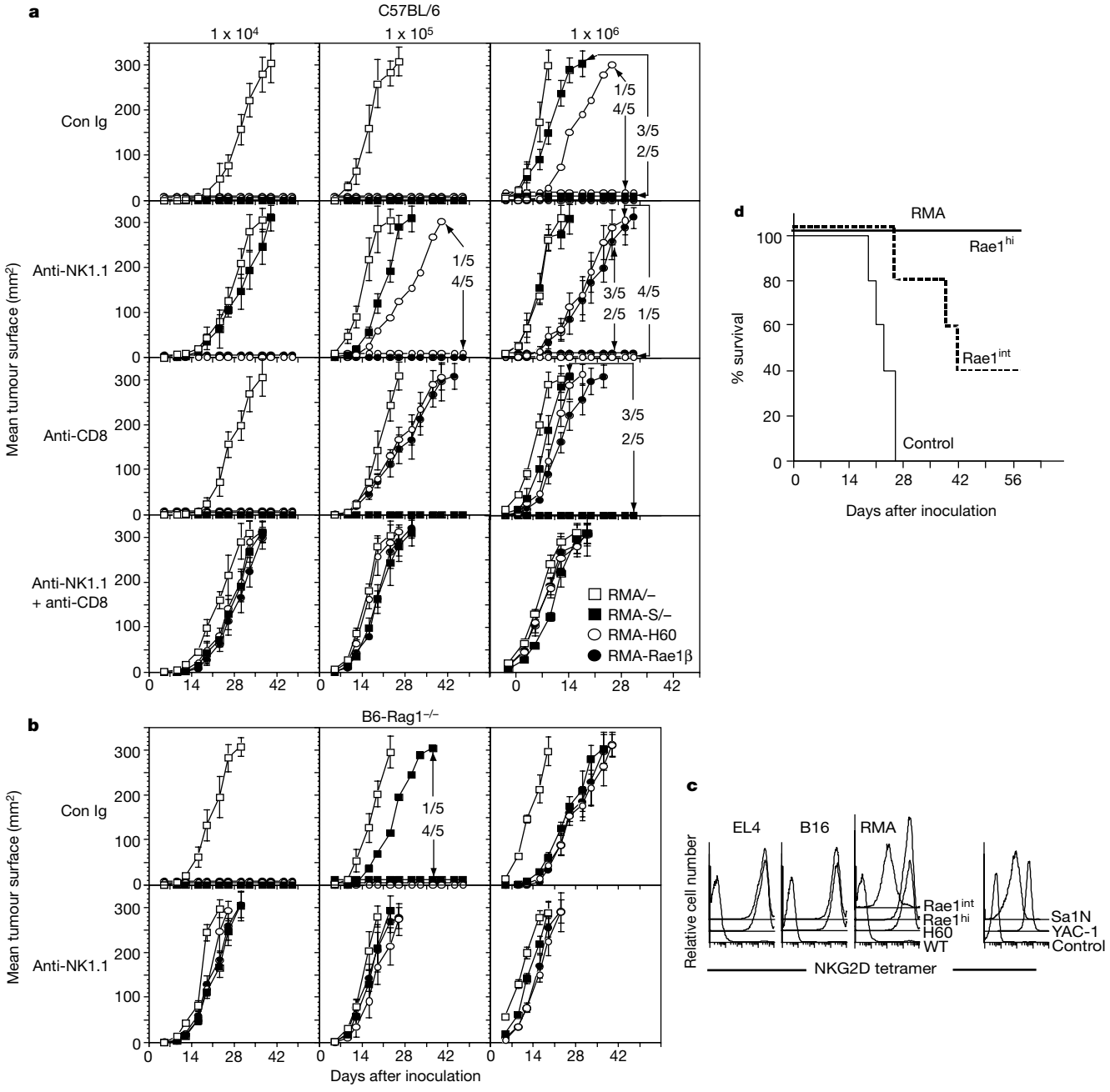

Figure 2 RMA cells expressing NKG2D ligands are rejected by NK cells and CD8 T cells in syngeneic mice. **a**, **b**, B6 (**a**) or B6-Rag1$^{-/-}$ (**b**) mice were pretreated with the indicated antibodies and inoculated subcutaneously with the indicated numbers of RMA transductants or RMA/S cells. Fractions are indicated for instances where tumours grew in some, but not all, mice. Data are representative of four experiments. Con Ig, control immunoglobulin. **c**, Levels of NKG2D ligands determined by staining with fluorescently labelled tetrameric NKG2D. Tumour cell transductants, the YAC-1 lymphoma and the Sa1N fibrosarcoma are compared. WT, wild type. **d**, The anti-tumour response depends on Rae1β levels. Survival of B6 mice that had been inoculated with 1×10^5 RMA cells, or RMA cells expressing high (Rae1^{hi}) or intermediate (Rae1^{int}) levels of Rae1β, is shown. Terminally moribund mice were killed. Data are representative of two experiments.

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weeks after the first exposure. The ligand-negative tumour cells grew progressively in naive B6 mice, but were rejected by the mice that had been exposed previously to the corresponding Rae1β- or H60-transduced tumour cells (Fig. 3a). The immunity was specific, as mice that had previously rejected ligand-transduced tumour cells of each type were immune only to untransduced tumour cells of the same type (Fig. 3b). Some delay was observed in the rejection of RMA cells by mice that were primed with ligand-transduced EL4 cells (Fig. 3b), and this may be in line with evidence indicating a common origin of EL4 and RMA cells[17,18]. However, the cross-protection was only partial, and was absent or very weak in the reciprocal situation, suggesting that our cell lines have an independent origin, or that most of the response is focused on antigens that are unique to each cell line. We conclude that ligand-expressing tumour cells specifically vaccinated the mice against ligand-negative tumour cells of the same type.

Primary rejection of ligand-transduced EL4 and B16-BL6 cells by naive mice was mediated by NK cells (Fig. 1), but it was not expected that NK cells could provide a specific 'memory' immune response. Indeed, no immunity to any of the tumour cells developed in Rag1$^{-/-}$ mice (Supplementary Information Fig. 1). Furthermore, normal mice that were pretreated with anti-CD8 antibody before the initial exposure to all three types of ligand-transduced tumour cells were unable to reject ligand-negative tumour cells on re-challenge (Fig. 3c), despite the fact that the ligand-transduced tumour cells had been rejected in each case (Figs 1 and 2). Pretreatment of mice with control immunoglobulin did not alter the memory response to ligand-negative tumour cells. Thus, although CD8$^+$ T cells were not required to reject the primary inoculum of ligand-transduced EL4 and B16-BL6 cells, they were essential for a protective immune response against untransduced tumour cells.

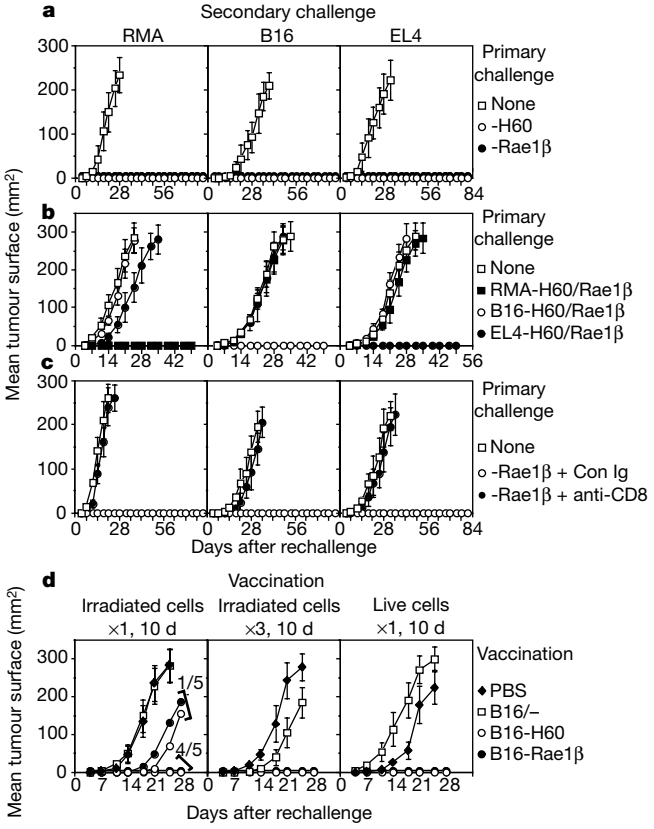

Figure 3 Vaccination with ligand-expressing tumour cells confers specific immunity to the corresponding ligand-negative tumour cells. **a**, B6 mice that had previously rejected ligand-transduced tumour cells (5×10^6 EL4, 1×10^4 B16-BL6 or 1×10^4 RMA transductants) were inoculated subcutaneously with control-transduced tumour cells of the same type (EL4, 5×10^6; B16-BL6, 1×10^4; RMA, 1×10^5). Primary exposure occurred 8–12 weeks before challenge. **b**, B6 mice that had been vaccinated 12 weeks earlier with each ligand-transduced tumour cell type were injected with the same or different ligand-negative cell lines. The primary and secondary tumour doses were as in **a**. **c**, Depletion of CD8$^+$ cells during the primary challenge prevents development of immunity. Re-challenge with control-transduced tumour cells occurred 8–12 weeks after vaccination. Naive B6 mice were challenged in parallel. **d**, B6 mice were injected with PBS or were vaccinated with 5×10^6 irradiated (once or three times) or 1×10^4 living, transduced or untransduced B16-BL6 tumour cells. Mice were challenged 10 days later with 10^4 untransduced B16-BL6 cells in the opposite flank. Data are representative of two experiments.

It was possible that immunity arose in this system because NK cell rejection of the transduced tumour cells resulted in excess tumour cell debris, thereby enhancing 'crosspriming' of tumour-specific CD8$^+$ T cells[19]. Indeed, irradiated untransduced RMA cells, which are expected to die *in vivo* and yield considerable cell debris, are reportedly effective in vaccinating mice[20]. However, this was not the case with irradiated ligand-negative B16-BL6 tumour cells[21], as no immunity resulted when mice were vaccinated once or three times in succession with irradiated control-transduced B16-BL6 tumour cells (Fig. 3d). Mice that were vaccinated in parallel with irradiated ligand-transduced B16-BL6 cells did develop immunity to untransduced tumour cells, suggesting that ligand expression results in priming of tumour-specific T cells independent of the generation of cell debris by NK cells. Consistent with this conclusion, irradiated ligand-transduced B16-BL6 cells were effective at priming a tumour-specific cytotoxic T-lymphocyte (CTL) response, even in mice that had been previously depleted of NK cells, whereas control-transduced B16-BL6 cells failed to prime CD8$^+$ T-cell responses (see below and Supplementary Information Fig. 2). Similar results were obtained in the RMA system (Fig. 4).

Consistent with the role of CD8$^+$ T cells in responses to ligand-transduced tumour cells, irradiated Rae1β- or H60-transduced tumour cells were more effective than ligand-negative tumour cells in priming tumour-specific CTLs *in vivo*, as tested by restimulating with ligand-transduced cells and testing effector function *in vitro*. Priming with irradiated ligand-transduced RMA cells, for example, increased RMA target cell lysis substantially, and augmented the percentage of RMA-specific interferon (IFN)-γ producing CD8$^+$ T cells severalfold (Fig. 4a, e). The effector cells were considerably more active against ligand-transduced RMA cells (Fig. 4b, f), and this enhancement could be completely blocked with anti-NKG2D antiserum (compare Fig. 4e, g with f, h), indicating that the NKG2D ligand interaction enhances not only CTL induction *in vivo*, but also the effector activity of the CTLs. The enhanced induction of CTLs, which were conventional CD8$^+$NK1.1$^-$ cells (Fig. 4c–f), occurred even in mice that had been previously depleted of NK1.1$^+$ cells (Fig. 4i), demonstrating that ligand-dependent augmentation of the CTL response does not depend directly or indirectly on NK cell activity.

Importantly, the CTLs were tumour-cell specific, as they did not lyse any of three other tumour cells (EL4, B16-BL6 or MC38), nor did they lyse Rae1β-transduced B16-BL6 cells (Fig. 4j). Furthermore, the CTLs did not lyse syngeneic concanavalin A-induced T-cell blasts, suggesting that priming with ligand-transduced tumour cells does not break self-tolerance of CTLs (Fig. 4j). Highly comparable results were obtained in the B16-BL6 tumour cell system, although the responses were generally weaker, probably because of the low expression of class I MHC molecules by these cells (Supplementary Information Fig. 2). Similar results were also obtained with CTLs derived from mice that had previously rejected live ligand-transduced tumour cells (data not shown). Thus, tumour cell expression of NKG2D ligands results in a significant enhancement in the priming of tumour-specific CTLs *in vivo*, as well as in the activation of preformed CTLs. These data are in line

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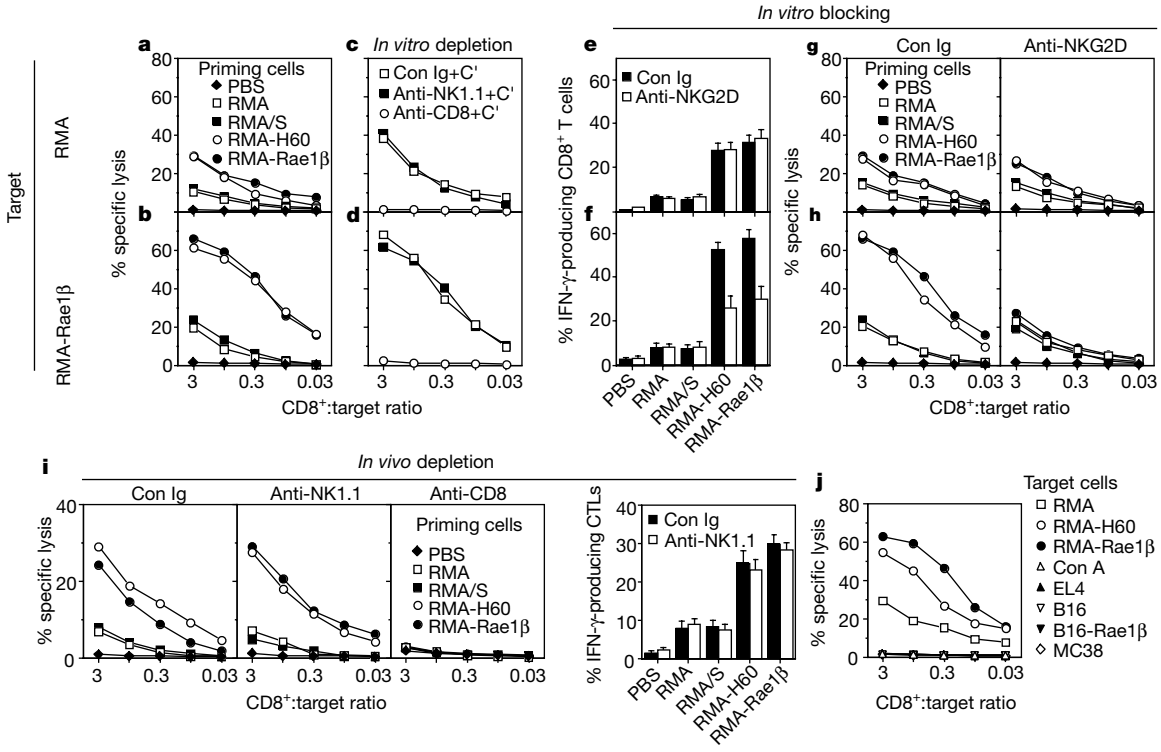

Figure 4 CTL responses primed by irradiated ligand-transduced RMA cells. Mice were vaccinated with 5×10^6 irradiated RMA transductants or PBS. Two weeks later splenocytes were restimulated with irradiated RMA-Rae1β cells. Con Ig, control immunoglobulin. **a**, Lysis of RMA cells. **b**, Target cell expression of Rae1β resulted in enhanced CTL lysis. **c**, **d**, Complement (C′)-mediated depletion of CD8 cells but not NK cells abrogates activity of effector cells from RMA-Rae1β-vaccinated mice. **e**, Elevated percentage of RMA-specific IFN-γ-producing effector $CD8^+CD3^+$ T cells in mice primed with ligand-transduced RMA cells. Priming cells are indicated at the bottom. **f**, Expression of Rae1β by target cells enhances IFN-γ response. The enhancement was completely blocked by anti-NKG2D antibody. **g**, **h**, Enhanced lysis of target cells expressing Rae1β is blocked by anti-NKG2D antibody. **i**, CTL priming occurs in the absence of NK cells. Mice were depleted of $NK1.1^+$ or $CD8^+$ cells before and during vaccination with tumour cell transductants. Effector cells were tested for lytic activity and IFN-γ production against RMA target cells. **j**, CTLs from RMA-Rae1β-vaccinated mice specifically recognize RMA cells and remain tolerant of syngeneic T-cell blasts. Effector cells from RMA-Rae1β-vaccinated mice were tested for lysis of the indicated tumour cells as well as concanavalin A (Con-A)-induced T-cell blasts from syngeneic mice. Data are representative of three experiments.

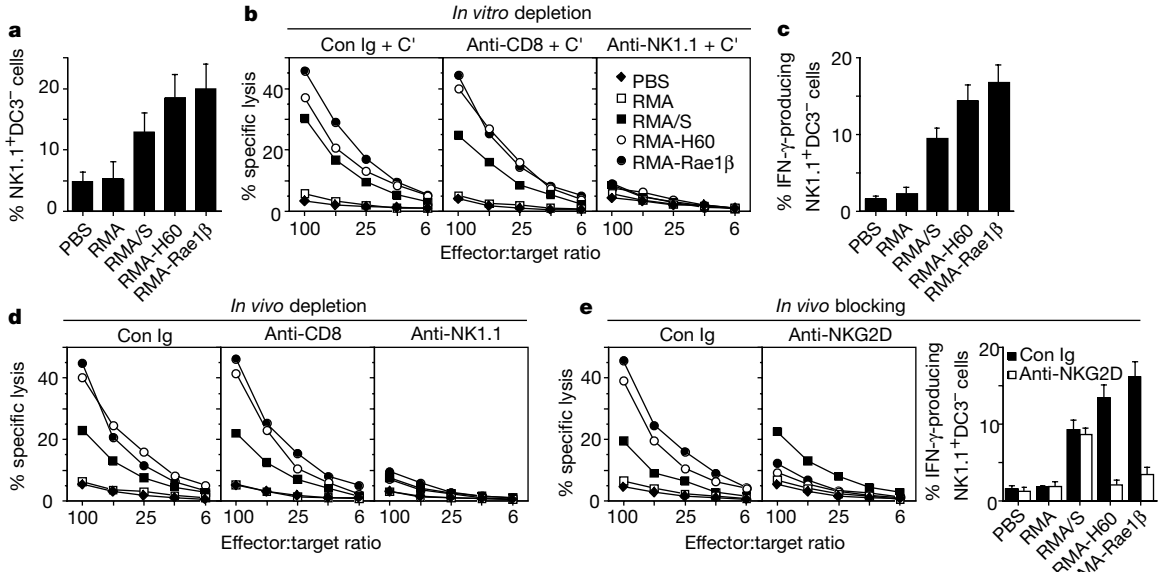

Figure 5 RMA cells expressing NKG2D ligands stimulate NK cell recruitment and activation *in vivo*. Groups of five B6 mice were injected intraperitoneally with 5×10^6 irradiated RMA transductants, RMA/S cells or PBS. Peritoneal wash cells were recovered two days later. Con Ig, control immunoglobulin. Compared with RMA cells, ligand-transduced RMA cells stimulated elevated percentages of NK ($NK1.1^+CD3^-$) cells (**a**), which exhibited enhanced capacity to lyse YAC-1 target cells (**b**), and produced IFN-γ when stimulated with YAC-1 target cells (**c**). Effector cells were destroyed by complement (C′) lysis with anti-NK1.1 but not anti-CD8 antibody (**b**), and pretreatment of mice with anti-NK1.1 antibody but not with anti-CD8 antibody prevented induction of cytotoxic activity (**d**). **e**, NK cell induction by ligand-transduced cells was blocked by injection of a non-depleting anti-NKG2D antiserum, but not by a control antiserum, just before tumour cell inoculation. The response to RMA/S cells was unaffected. Lysis of YAC-1 tumour cells and production of IFN-γ after stimulation with YAC-1 target cells was assayed. Data are representative of two experiments.

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with evidence that NKG2D engagement co-stimulates human CTL responses *in vitro*[22], and extend these findings to *in vivo* responses to tumour cells.

Induction of NK cell activity *in vivo* by tumour cells that express Rae1β or H60 was also demonstrable. When inoculated intraperitoneally in naive mice, irradiated Rae1β- or H60-transduced RMA tumour cells increased the number of peritoneal NK cells by 2–4-fold within two days (Fig. 5a), and substantially enhanced the cytotoxic activity of the cells against YAC-1 target cells (Fig. 5b, left panel), whereas control-transduced RMA cells had no effect. A substantially higher fraction of these NK cells also produced IFN-γ after *in vitro* stimulation with YAC-1 tumour cells (Fig. 5c). As previously reported, class I-deficient RMA/S cells also induced the local accumulation of NK cells and enhanced their functional activity[23] (Fig. 5a–c). In either case, the cytotoxic activity was nearly abolished by pretreatment of effector cells with anti-NK1.1 plus complement or by *in vivo* depletion of $NK1.1^+$ cells before tumour cell inoculation (Fig. 5b, d). In contrast, depletion of $CD8^+$ cells before tumour cell inoculation or immediately before the cytotoxicity assay had no effect (Fig. 5b, d), and similar cytotoxic activity was induced in $Rag1^{-/-}$ and wild-type mice (Supplementary Information Fig. 3). Thus, the effector cells are conventional NK cells and do not depend on $CD8^+$ cells for their induction. Similar cytotoxicity and cytokine data were obtained with ligand-expressing B16-BL6 and EL4 cells (Supplementary Information Fig. 4). Therefore, expression of NKG2D ligands, like MHC deficiency, provokes NK cell recruitment and sensitization *in vivo*.

A non-depleting anti-NKG2D antiserum injected *in vivo* almost completely blocked the ligand-dependent induction of NK cytotoxicity and cytokine production against YAC-1 cells (Fig. 5e). The effect of the antibody was specific, as NK cell induction by class I-deficient RMA/S cells, which do not express NKG2D ligands, was unaffected by the anti-NKG2D antibody. Therefore, the induction process with ligand-transduced cells required interactions with the NKG2D receptor *in vivo*.

Our results demonstrate that NKG2D ligand expression and consequent activation of NK cells and $CD8^+$ T cells can impose a substantial barrier to the establishment of tumours *in vivo*. The ligands activate NK cells and T cells through NKG2D, which associates in the membrane with KAP/DAP10—an adapter signalling protein that is thought to deliver co-stimulatory signals[10]. It is unknown whether engagement of NKG2D in NK cells results in direct stimulation or supplies a co-stimulatory signal that acts in conjunction with signals from other stimulatory receptors[24,25]. Nevertheless, NKG2D receptor engagement clearly enhances the effective NK cell response against the three tumour cell lines that we tested. Furthermore, ligand expression by tumour cells also strongly enhanced the generation of tumour-specific CD8 T cells. As this enhancement also occurred in mice that were devoid of NK cells (Figs 2a and 4), and considering that activated $CD8^+$ T cells express NKG2D, we propose that $CD8^+$ T-cell activation is enhanced as a consequence of direct interactions with ligand-expressing tumour cells. It remains possible that the priming of $CD8^+$ T cells is accomplished indirectly as a result of ligand-expressing tumour cells interacting with NKG2D-expressing macrophages[11]. In either case, the results suggest that NKG2D ligands provide an example of innate immune stimuli that function to enhance the adaptive immune response[26]. The effectiveness of ligand transduction of tumour cells in stimulating an anti-tumour response and protective immunity to tumour re-challenge suggests that ligand-expressing cells may have applications in tumour therapy and the development of tumour vaccines. The strong response against B16-BL6 cells is particularly notable in this regard, given that the BL6 variant was selected for high invasiveness and is poorly immunogenic[27]. Indeed, other manipulations such as ectopic B7 expression or CTLA4 blockade do not, by themselves, result in rejection of B16-BL6 cells[28]. The finding that typical levels of NKG2D ligands naturally found on most tumour cell lines are suboptimal in inducing anti-tumour immunity (Fig. 2c, d) raises the possibility that immunity to such tumours can be boosted by engineering cells with higher levels of ligands. The effectiveness of this approach against pre-established tumours and naturally arising tumours remains to be established. □

Methods

Ectopic expression of NKG2D ligands

Three NKG2D ligand-negative cell lines (EL4, a B6 thymoma; RMA, a B6 T lymphoma derived from the Rauscher virus-induced RBL-5 cell line[2]; and B16-BL6, a B6 melanoma derived from the B16-F0 cell line[27]) were retrovirally transduced as described[11]. The retroviral vectors containing the H60 or Rae1β complementary DNAs used for these experiments did not direct synthesis of green fluorescent protein (GFP) or any other selection marker. Transduced cells expressing equivalent high levels of the NKG2D ligands were sorted after staining with a tetrameric soluble version of NKG2D[11]. Control staining was performed with an irrelevant tetramer of the T22 class Ib molecule. Control tumour cells were infected with empty retrovirus, and transduced clones were identified by PCR with primers corresponding to the murine stem cell virus (MSCV) 5′ and 3′ long terminal repeat (5′ primer: GTCCTCCGATAGACTGCGTCGCCCGGG; 3′ primer: GCTTGCCAAACCTACAGGTGGGG). Approximately 100–150 clones with integrated provirus were pooled and used as control tumour cells (designated as EL4/–, B16-BL6/– and RMA/–).

Antibody depletion, tumour inoculation and re-challenge

We used five mice per group for all tumour rejection experiments. C57BL/6J (B6) and B6-$Rag1^{-/-}$ mice were purchased from Jackson Laboratories, and the B6-$Rag1^{-/-}$ mice were bred in our animal facilities under specific pathogen-free conditions. All mice were used between 8 and 18 weeks of age. NK cells and $CD8^+$ T cells were depleted[28] by intraperitoneal injection of 200 μg monoclonal antibody (PK136 against NK1.1 and 2.43 against CD8) at day −1, 1, 8, 15 and 22. Control mice received the equivalent amounts of normal mouse immunoglobulin-G. Depletions were confirmed in lymph node and spleen cells 3 weeks after tumour challenge by flow cytometry using non-competing antibodies. In general, less than 1.5% of the depleted cell population could be detected in spleen and lymph nodes. Tumour cells were injected subcutaneously in 100 μl PBS in the right flank. We monitored tumour development by measuring the tumour size twice weekly with a metric caliper. For the metastasis assay, 3×10^5 B16-BL6 cells were injected intravenously through the tail vein in groups of five mice, and lung metastases were examined 14–21 days later. For the re-challenge experiments, groups of five mice that had completely rejected the initial tumour (8–12 weeks after initial tumour challenge) were injected in the opposite flank with the respective untransduced or control-transduced tumour cells (that is, lacking NKG2D ligands).

Vaccination and *ex vivo* analysis of CTL activation

Transduced B16-BL6 tumour cells were irradiated (160 Gray (gy)) and 5×10^6 cells were injected, in groups of five mice, in 100 μl PBS subcutaneously in the left flank either once at day −10 or three times at day −10, −7 and −4 as indicated. Some mice received injections of unirradiated tumour cells at day −10. At day 0, mice were challenged with 10^4 untransduced B16-BL6 cells in 100 μl PBS in the opposite flank. For the *ex vivo* analysis of CTL activity, groups of five mice received two vaccinations (day 0 and 3) with irradiated (160 gy) RMA or B16-BL6 tumour cell transductants. Two to three weeks after vaccination, mice were killed and pooled splenocytes were restimulated for 5 days with the respective Rae1β-transduced tumour cells. The cells were expanded for another three days before testing cytotoxicity and IFN-γ production. In some experiments $NK1.1^+$ or $CD8^+$ T cells were depleted before the cytotoxicity assay by complement (C′)-mediated lysis as described[29]. *In vitro* blocking of NKG2D was carried out as described previously, using a rat anti-NKG2D antiserum and a control rat serum[11].

Ex vivo analysis of NK cell activation

Tumour cells were irradiated (120 gy) and injected intraperitoneally as described[23]. We collected peritoneal cells after 72 h (ref. 23). The percentages (± s.d.) of NK cells in the peritoneal cavity of individual mice were quantified by flow cytometry and electronic gating on lymphocytes (low forward and side light scatter). Peritoneal wash cells were pooled for cytotoxicity and IFN-γ assays. In some experiments $NK1.1^+$ or $CD8^+$ T cells were depleted before the cytotoxicity assay by complement-mediated lysis as described[29]. For the *in vivo* blocking experiments 100 μl of a non-depleting antiserum specific to NKG2D or a control antiserum[11] was injected intraperitoneally. As determined by flow cytometry, the antibody did not deplete $NK1.1^+$ cells from the peritoneal cavity (data not shown).

Assays

Cytotoxicity was tested in a standard 4-h ^{51}Cr release assay[11]. Data are given as the mean of triplicate measurements. Standard deviations were generally less than 5% and are omitted from the figures for reasons of clarity. IFN-γ production was assayed by stimulating CTLs or NK cells with an equal number of target cells for 5–7 h and staining intracellular IFN-γ as described[30], with gating on $CD8^+CD3^+$ cells or $NK1.1^+CD3^-$ cells. Data are presented as the mean (± s.d.) of triplicate measurements.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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S-Nitrosothiols signal the ventilatory response to hypoxia

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Increased ventilation in response to hypoxia has been appreciated for over a century[1], but the biochemistry underlying this response remains poorly understood. Here we define a pathway in which increased minute ventilation ($\dot{V}_E$) is signalled by deoxyhaemoglobin-derived *S*-nitrosothiols (SNOs). Specifically, we demonstrate that *S*-nitrosocysteinyl glycine (CGSNO) and *S*-nitroso-L-cysteine (L-CSNO)—but not *S*-nitroso-D-cysteine (D-CSNO)—reproduce the ventilatory effects of hypoxia at the level of the nucleus tractus solitarius (NTS). We show that plasma from deoxygenated, but not from oxygenated, blood produces the ventilatory effect of both SNOs and hypoxia. Further, this activity is mediated by *S*-nitrosoglutathione (GSNO), and GSNO activation by γ-glutamyl transpeptidase (γ-GT) is required. The normal response to hypoxia is impaired in a knockout mouse lacking γ-GT. These observations suggest that *S*-nitrosothiol biochemistry is of central importance to the regulation of breathing.

Failure to increase minute ventilation ($\dot{V}_E$) in response to hypoxia may be fatal. In particular, newborn mammals have

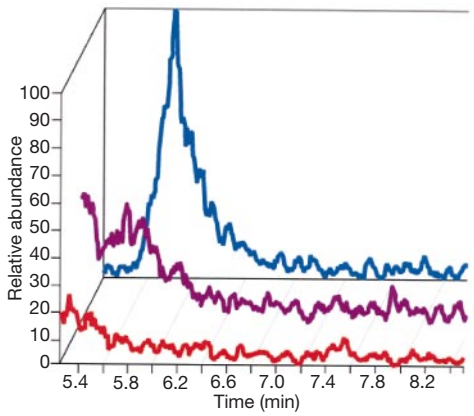

Figure 1 Glutathione reacts with deoxygenated but not oxygenated blood to form GSNO. Liquid chromatography/mass spectrometry was performed on plasma from blood that was reacted with 400 μM GSH. GSNO was eluted at 5.82 min. The GSNO peak in the deoxygenated blood-derived plasma (blue) was attenuated after ultraviolet treatment (violet) and is not evident in the oxygenated blood-derived plasma (red). Positive identification of the GSNO peak was determined by co-elution of the sample with a GSNO standard. The retention time and both mass spectrometry and mass spectrometry fragmentation pattern of the endogenous species were identical to exogenous GSNO plus the endogenous species. These observations demonstrate that deoxygenated blood reacts with reduced thiol species to form ultraviolet-labile SNO species, whereas oxygenated blood does not.

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