Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin

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The mouse CD8α+ DC subset excels at cross-presentation of antigen, which can elicit robust CTL responses. A receptor allowing specific antigen targeting to this subset and its equivalent in humans would therefore be useful for the induction of antitumor CTLs. Here, we have characterized a C-type lectin of the NK cell receptor group that we named DC, NK lectin group receptor-1 (DNGR-1). DNGR-1 was found to be expressed in mice at high levels by CD8α+ DCs and at low levels by plasmacytoid DCs but not by other hematopoietic cells. Human DNGR-1 was also restricted in expression to a small subset of blood DCs that bear similarities to mouse CD8α+ DCs. The selective expression pattern and observed endocytic activity of DNGR-1 suggested that it could be used for antigen targeting to DCs. Consistent with this notion, antigen epitopes covalently coupled to an antibody specific for mouse DNGR-1 were selectively cross-presented by CD8α+ DCs in vivo and, when given with adjuvants, induced potent CTL responses. When the antigens corresponded to tumor-expressed peptides, treatment with the antibody conjugate and adjuvant could prevent development or mediate eradication of B16 melanoma lung pseudometastases. We conclude that DNGR-1 is a novel, highly specific marker of mouse and human DC subsets that can be exploited for CTL cross-priming and tumor therapy.

Introduction

DCs are versatile controllers of T lymphocyte responses, contributing to the maintenance of self-tolerance and to the induction of adaptive immunity (1). It is becoming increasingly apparent that such functional versatility involves the specialized activities of different DC subtypes (2). Both mice and humans possess at least 3 broad groups of DCs, encompassing plasmacytoid DCs (pDCs), blood-derived conventional DCs (cDCs), and tissue-derived cDCs (2). These groups can be subdivided into distinct subsets with different markers, diverse functions, distinct ontogeny, and differential turnover in vivo (2). The best-studied mouse DC subsets are the blood-derived CD8α+ cDCs, CD4+ cDCs, and CD4−CD8α− (double-negative [DN]) cDCs found in lymphoid organs (3, 4).

Mouse CD8α+ DCs excel in MHC class I cross-presentation of cell-associated foreign and self antigens (5–8), partly due to their ability to capture material from dying cells (9, 10) and to efficiently process exogenous antigen for MHC class I cross-presentation (11–13). These 2 properties of CD8α+ DCs may also underlie their ability to serve as efficient APCs for CTL priming in several infection models (14–19). In contrast, CD8α− DCs, encompassing CD4+ DCs and DN DCs (20), are relatively better at presenting exogenous antigens on MHC class II and directing CD4+ T cell responses (11–13).

The superior ability of DCs to regulate adaptive immunity suggests that significant clinical potential could be gained from targeting antigens to DCs in vivo (21, 22). One practical approach has been to administer antigens coupled to antibodies directed against DC surface receptors. When given together with an adjuvant, such antibody conjugates can elicit powerful Th1 and CTL responses, useful for immunotherapy of cancer or for boosting cellular immunity to infections (23–29). Conversely, in the absence of an adjuvant, antibody-mediated DC targeting can induce antigen-specific tolerance, helpful for limiting autoimmune reactions or responses to allografts and allergens (23, 24, 30–32). Successful targeting depends on identifying suitable DC-expressed cell-surface receptors that mediate endocytosis of bound antibodies. This allows delivery of the latter to endosomal and/or cytosolic compartments where their associated antigens can be processed for MHc presentation. Possible receptors include ones shared by DCs and other cell types, such as the macrophage mannose receptor or the Fcγ receptors, as well as others more restricted to DCs, such as DEC-205, DC-SIGN, langerin, asialoglycoprotein receptor, or blood DC antigen 2 (BDCA-2) (reviewed in refs. 21, 22, 33). Many of these receptors belong to the C-type lectin family and may have a physiological role in antigen capture, which renders them especially suitable for targeting (34, 35). Many mouse C-type lectins that are potentially useful for antigen targeting are preferentially expressed in CD8α+ DCs and include dectin-1, dectin-2, DCIR2, F1RE, and CIRE (12, 13, 36, 37). In contrast, CD8α− DCs can be targeted by antibodies against DEC-205, a C-type lectin that delivers ligands to late endosomal/lysosomal compartments (38, 39). Antigen targeting to dectin-1 or DCIR2 in vivo favors CD8α+ T cell responses, whereas targeting to DEC-205 is especially effective at inducing CD8α− T cell responses, in line with the aforementioned differences in antigen handling by CD8α+ and CD8α− DCs (12, 13). This suggests that antigen targeting to DEC-205 might be useful for inducing protective CTL-based immunity in diseases such as cancer, malaria, and HIV. Unfortunately, mouse DEC-205

Nonstandard abbreviations used: BCDA-2, blood DC antigen 2; BMDC, BM-derived DC; cDC, conventional DC; Cln9a, C-type lectin 9a; CTLD, C-type lectin-like domain; DN, double negative; DNGR-1, DC, NK lectin group receptor-1; gp100, melanocyte lineage-specific antigen glycoprotein 100; MFI, median fluorescence intensity; pDC, plasmacytoid DC; S1, SINFEXL-eahx-biotin; S2, SINFEXL1TWSSNV-MEERC-eahx-biotin; TRP-1, tyrosinase-related protein 1.

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is not only expressed in CD8α+ DCs but also in subsets of lymph node cDCs, Langerhans cells, interstitial DCs, thymic epithelial cells, and at lower levels, in B cells, macrophages, T cells, and granulocytes (38, 40–42). Human DEC-205 shows an even broader distribution (43). Therefore other receptors that might selectively allow targeting of mouse CD8α+ DCs in vivo or their equivalent in humans would be welcome additions to the repertoire of DC targets for inducing CTL-based immunity.

Here, we describe DC, NK lectin group receptor-1 (DNGR-1) as a previously uncharacterized C-type lectin that is selectively expressed in mouse CD8α+ cDCs and, at low levels, on pDCs but not in other cell types. We show that DNGR-1 acts as an endocytic receptor that can mediate internalization of bound antibody and can be targeted for antigen delivery in vivo to CD8α+ DCs. This allows cross-presentation to CD8+ T cells and, together with an adjuvant, induces potent CTL responses that can cure mice of a transplantable tumor. Notably, we find that DNGR-1 is also selectively expressed in a small subset of human DCs. Thus, DNGR-1 constitutes a selective marker for human and mouse DC subsets and can be exploited for antigen delivery to these cells in vivo.

Results

Identification and characterization of DNGR-1. To identify markers for mouse DC subsets, we carried out representational difference analysis of freshly-isolated mouse spleen CD11c+CD8α+ and CD11c−CD8α− cells (O. Schulz, D.J. Pennington, and C. Reis e Sousa, unpublished observations). Sequences matching EST clone AW318446, corresponding to the mouse C-type lectin 9A (Clec9a) gene, were highly represented among CD8α+ DC–specific transcripts (data not shown). Genome annotation predicts the presence of CLEC9A genes in Mus musculus, Pan troglodytes, Homo sapiens, Canis lupus familiaris, and Macaca mulatta. A database search using part of the protein sequence of mouse CLEC9A further suggests the presence of CLEC9A genes in Rattus norvegicus and Bos taurus (44).

CLEC9A is predicted to be a type II transmembrane receptor of the C-type lectin family with a single extracellular C-type lectin-like domain (CTLD) (Supplemental Figures 1 and 2; supplemental material available online with this article; doi:10.1172/JCI34584DS1). The CTLD contains 6 conserved cysteine residues likely to be involved in intrachain disulfide bond formation as well as other conserved features of CTLDs (45) but lacks the Ca2+-coordinating amino acid residues found in typical carbohydrate-recognition domains (Supplemental Figures 1 and 2). The CLEC9A CTLD is linked to a stalk region containing a conserved cysteine putatively involved in dimerization (see below). This is followed by a transmembrane region and a cytoplasmic domain with a highly conserved tyrosine (Supplemental Figures 1 and 2).

To understand the function of DNGR-1, in mice and humans, we have found transcripts for 3 isoforms of Clec9a (Supplemental Figure 3) that we have termed long isoform (exons 1–7), short isoform (lacking exon 4 but keeping the same reading frame), and very short isoform (exons 1–3 fused to exon 7, encoding a protein that, if expressed, would share the transmembrane and intracellular domains with CLEC9A but would have a short and distinct extracellular domain). Published analysis suggests that the CLEC9A CTLD is closely related to that found in the killer cell lectin receptor I (KLR-I) (44). Our analysis shows that, despite having a high degree of similarity to KLR-I and other members of the KLR complex, CLEC9A is phylogenetically most closely related to CLEC7A, commonly known as dectin-1 (Supplemental Figure 4). Notably, both CLEC9A and dectin-1 as well as KLR-I belong to the NK cell receptor group of C-type lectins (group V). In line with this notion and in accordance with its DC-restricted expression pattern in mouse and human (see below), we have named the protein DNGR-1.

We cloned the mouse DNGR-1 cDNA from spleen CD8α+ DCs. Transfection studies using a C-terminus–tagged version of the receptor indicated that it could be expressed and transported to the cell surface in heterologous cells (data not shown). To study expression in more detail, we generated mAbs against DNGR-1 (see Methods). We selected 3 rat anti-mouse DNGR-1 mAbs named 1F6, 397, and 7H11 that specifically stained cells stably transfected with mDNGR-1 cDNA (Supplemental Figure 5) and recognize both the short and the long isoform, which differ only in a segment of the stalk region (data not shown). All 3 mAbs could be used to immunoprecipitate HA-tagged mDNGR-1 from transfected cells (Figure 1A). The apparent molecular weight (MW) of DNGR-1 was around 102 kDa in nonreducing conditions and around 51 kDa in reducing conditions (Figure 1B). This indicates that the molecule exists as a dimer, probably stabilized by a disulfide bond involving the conserved cysteine in the stalk region (Supplemental Figure 2), as commonly found in other NK receptor group V C-type lectins (45). Notably, the MW of the monomer was significantly higher than the 29.7 kDa predicted from the sequence of the core protein, suggesting that the receptor is heavily glycosylated. Consistent with this possibility, mouse and human DNGR-1 have 1 putative N-glycosylation site (Asn81) and 1 putative attachment site for glycosaminoglycans (Ser104 in mice and Ser106 in humans), both in the stalk region (Supplemental Figure 2).

Expression of DNGR-1 is restricted to CD8α+ DCs and pDCs. We examined the pattern of expression of mDNGR-1 in mouse tissues. In spleen, the molecule was expressed by a subset of CD11c+ DCs but not by CD11c− cells, including B cells, T cells, NK cells, NKT cells, monocytes, macrophages, or granulocytes (Figure 2A and data not shown). Within the DC fraction, DNGR-1 was expressed at very high levels by CD8α+ cDCs but not by CD4+ or DN cDCs (Figure 2A). In addition, the receptor was also expressed in pDCs but at a significantly lower level than in CD8α+ cDCs (median fluorescence intensity [MFI] = 60 compared with ~400; isotype control
MFI = 8). In addition, CD8α+ DC staining was unimodal, indicating uniform expression of DNGR-1 whereas the staining of pDCs was less clear and might represent expression by a subpopulation of the cells (Figure 2A and data not shown; see below). The pattern of DNGR-1 expression as assessed by antibody staining largely agreed with analysis of DNGR-1 mRNA expression in sorted DC subsets (Supplemental Figure 3).

Similar to spleen, expression of DNGR-1 in lymph nodes and in thymus was also restricted to CD8α+ cDCs and pDCs, but expression levels in the former subset were always much higher than in the latter (Supplemental Figure 6 and data not shown). No staining was detectable on tissue-derived DCs such as interstitial DCs and (in s.c. lymph nodes) the putative descendants of skin dermal DCs and Langerhans cells (Supplemental Figure 6). Likewise, DNGR-1 was not expressed in skin epidermis or in other nonlymphoid tissues (data not shown).

As a model for analysis of DNGR-1 function in vitro, we analyzed expression in mouse BM-derived DCs (BMDCs) generated by culture in GM-CSF or in Flt3L. DNGR-1 was not expressed by GM-CSF BMDCs (data not shown) but was highly expressed in the CD11bloCD24hiB220− subset of Flt3L BMDCs (Figure 2B), which corresponds to the CD8α+ subset of DCs (46). DNGR-1 was also expressed, at a significantly lower level, by a subset of CD11bhiB220+ Flt3L BMDCs, which are equivalent to pDCs (Figure 2B). We conclude that mouse DNGR-1 is highly expressed on CD8α+ cDCs and to a lesser extent on a subset of pDCs, with no expression on other cell types.

**Anti–DNGR-1 selectively labels CD8α+ DCs and pDCs in vivo.** To determine whether CD8α+ DC and pDC populations can be labeled with anti–DNGR-1 antibody in vivo, we injected mice i.v. with a saturating dose (100 μg; see below) of anti–DNGR-1 antibody conjugated to Alexa Fluor 488 or with the same amount of similarly conjugated isotype-matched control. Analysis of total spleen or lymph node cells 16 hours after injection showed specific labeling of CD8α+ DCs and pDCs, although the intensity of the signal was much greater on the former DC type (CD8α+ DC MFI = 350–400 as compared with pDC MFI = 65–70; Figure 3A and Supplemental Figure 7). No other leukocyte population was labeled by this procedure, demonstrating the remarkable in vivo specificity of anti–DNGR-1 (Figure 3A and Supplemental Figure 7). In contrast, anti–DEC-205 binds to multiple cell types when used at the same dose and, in addition to CD8α+ DCs, strongly labels CD11c− skin-derived DCs and stains CD11c−negative cells (Supplemental Figure 7). Notably, even local administration of small amounts of labeled anti–DNGR-1 antibody into footpads was sufficient to label spleen DC subsets (Figure 3B). Low doses of antibody (e.g., 2 μg) preferentially labeled CD8α+ DCs over pDCs, consistent with the relative expression levels of DNGR-1 in the 2 subsets and suggesting that such low levels could be used to selectively target CD8α+ DCs in vivo (Figure 3B). We conclude that anti–DNGR-1 administration can be used to specifically label CD8α+ cDCs and, to a lesser degree, pDCs in vivo.

**DNGR-1 is an endocytic receptor.** Because many C-type lectins on myeloid cells serve as endocytic receptors (34, 35), we tested the ability of DNGR-1 to be internalized from the cell surface. Flt3L BMDCs were labeled at 4°C with biotin–anti–DNGR-1, washed, and incubated at 4°C or 37°C for different lengths of time before staining with streptavidin-PE. Staining of the CD8α+ cDCs and pDCs in the Flt3L BMDC population was reduced 4-fold after 2 hours incubation at 37°C, with minimal decrease in cells.
incubated at 4°C (Figure 4A). Confocal analysis of cells stained with anti-DNGR-1 Alexa Fluor 488 conjugates revealed the presence of mAb in intracellular compartments following 2 hours incubation at 37°C (Figure 4B). Similar data were obtained in a heterologous cell type transfected to express DNGR-1 (data not shown). Thus, DNGR-1 can mediate delivery of bound antibody to the endocytic pathway.

**Anti-DNGR-1 can deliver antigen in vivo to CD8α+ DCs for MHC class I cross-presentation.** To assess whether the endocytic ability of DNGR-1 and its selective expression pattern can be exploited as a means of delivering antigen for presentation to T cells, we tested a model of cross-presentation. We covalently coupled anti–DNGR-1 or an isotype-matched control mAb to a biotinylated derivative of SIINFEKL, the immunodominant peptide in the H-2Kb-restricted CTL response to OVA protein (SIINFEKLC-eahx-biotin [S1]; see Methods). All S1-mAb conjugates contained between 1 and 1.2 biotin molecules (i.e., 1–1.2 peptides) per antibody and could be presented by live but not by fixed DCs in vitro, indicating that they are not susceptible to extracellular proteolysis and must be processed by the cells in order to release the antigenic peptide (data not shown). Mice were injected i.v. with 2 μg of either conjugate, and splenocytes were recovered 16 hours later, separated on the basis of CD11c expression, and tested for the ability to stimulate OT-I T cells as a readout for OVA peptide presentation (Figure 5A). Only CD11c+ splenocytes from mice that had received anti-DNGR-1–S1 were able to induce OT-I proliferation and IFN-γ production (Figure 5A), although all cell fractions were stimulatory in the presence of added SIINFEKL peptide (data not shown). To identify the APCs involved, CD11c+ cells from mice treated with anti-DNGR-1–S1 were sorted into the 3 major subsets of cDCs. Only the CD8α+ subset of cDCs was able to stimulate OT-I cells (Figure 5B), although all subsets were equally competent to do so in the presence of added peptide (not shown; see ref. 9). Notably, pDCs purified from anti-DNGR-1–treated mice did not stimulate OT-I cells (data not shown). However, the same cells stimulated only weakly in the presence of added SIINFEKLEK peptide (~60-fold lower response than for cDC subsets), suggesting that they are poor APCs under these conditions. We conclude that anti-DNGR-1 mAbs can be used to selectively deliver exogenous antigen for cross-presentation by CD8α+ DCs in vivo.

**Antigen targeting to DNGR-1 together with adjuvant induces CTL priming.** We explored whether targeting in vivo with anti-DNGR-1 conjugated to S1 peptide could induce specific CTL priming in the presence or absence of anti-CD40, often used as an adjuvant in antibody targeting experiments (23, 24). Mice were injected i.v. with 2 μg of anti-DNGR-1–S1 or isotype-matched control S1 ± anti-CD40 (25 μg) (Figure 6). To test for induction of CTL activity in vivo, 5 days later we infused splenocytes from congenic CD45.1 B6.SJL mice loaded with different doses of CFSE and SIINFEKL peptide and monitored the specific elimination of the peptide-pulsed cells. Mice given S1 coupled to control mAbs did not eliminate target cells irrespective of anti-CD40 coadministration (Figure 6A). In contrast, target cells were completely eliminated from mice given S1 coupled to anti-DNGR-1 together with anti-CD40 (Figure 6A). No response was seen when the anti-CD40 mAb was omitted (Figure 6A). Conversely, other adjuvants such as poly I:C could substitute for anti-CD40 in promoting cross-priming to
anti–DNGR-1–S1 (data not shown). Consistent with target cell elimination, significant numbers of tetramer-positive OVA/H-2Kb-specific CD8+ T cells were found only in the spleens and blood of mice that had received anti–DNGR-1–S1 together with anti-CD40 (Figure 6B). Restimulation of the same cells with SIINFEKL peptide in vitro resulted in secondary expansion, with IFN-γ production and specific killing activity (Figure 6C). Identical results were obtained using anti–DNGR-1 conjugated to a longer peptide of OVA containing the SIINFEKL epitope (S2, SIINFEKITLEWTSSNVMEERC-eahx-biotin; data not shown) or to full-length OVA protein (Figure 6D). Notably, free S1 peptide was unable to induce in vivo killing responses or elicit a significant number of tetramer-positive cells even when given at 100 times over the amount present in anti–DNGR-1–S1 conjugates (Supplemental Figure 8). We conclude that targeting of exogenous antigen to DNGR-1 together with an appropriate adjuvant allows efficient cross-priming of CD8+ T cells.

Antigen targeting to DNGR-1 together with adjuvant promotes tumor immunity. To determine whether DNGR-1 targeting can be used for tumor immunotherapy, we first tested a melanoma prevention model. We gave mice a single dose of anti–DNGR-1–S1 or control mAb conjugated to S1 (2 μg) together with anti-CD40 (25 μg) and,
1 month later, they challenged them with $2 \times 10^8$ B16-OVA-GFP melanoma cells i.v. (Figure 7A). The number of lung pseudometastases determined 18 days later was greatly reduced in the group pretreated with anti–DNGR-1–S1 plus anti-CD40 but not in mice treated with the control S1-mAb, indicating that DNGR-1 targeting can generate long-lasting CTL memory that protects against tumor challenge (Figure 7A). To determine whether it was also effective in a more stringent therapy setting, we delayed treatment until after tumor implantation. Mice were inoculated i.v. with B16-OVA-GFP melanoma cells i.v. (Figure 7A). The number of lung pseudometastases 1 month later, challenged them with $2 \times 10^8$ B16-OVA-GFP melanoma cells i.v. (Figure 7A). The number of lung pseudometastases determined 18 days later was greatly reduced in the group pretreated with anti–DNGR-1–S1 plus anti-CD40 but not in mice treated with the control S1-mAb, indicating that DNGR-1 targeting can generate long-lasting CTL memory that protects against tumor challenge (Figure 7A). To determine whether it was also effective in a more stringent therapy setting, we delayed treatment until after tumor implantation. Mice were inoculated i.v. with B16-OVA-GFP melanoma cells and 3 days later were given control mAb-S1 or anti–DNGR-1–S1 plus anti-CD40. The number of lung tumors at day 18 revealed that antigen targeting to DNGR-1 together with anti-CD40 is a highly effective therapy whereas the same amount of antigen given in untargeted form has no effect (Figure 7B). Consistent with the observed therapeutic effect, spleens from treated mice contained a high frequency of OVA/H-2Kb-specific CD8+ T cells (Figure 7C), which could be restimulated in vitro to produce IFN-γ and kill specific targets (Figure 7D).

We extended the experiments to determine whether anti–DNGR-1 targeting can also be used to induce immune responses to endogenous melanocyte differentiation proteins that can serve as B16 tumor-associated antigens (47–49). We synthesized biotinylated peptides encompassing H-2Kb- and H-2Dd-restricted antigenic epitopes from melanocyte lineage-specific antigen glycoprotein 100 (gp100), tyrosinase-related protein 1 (TRP-1), and TRP-2 (47–49), coupled these covalently to anti–DNGR-1, and immunized mice with the antibody conjugate to OVA protein in the presence of 25 μg anti-CD40. Mice were immunized s.c. in the paw with 2 μg anti–DNGR-1 or isotype control antibody conjugated to OVA protein in the presence of 25 μg of anti-CD40. In vivo killing activity was analyzed as in A using targets loaded with 200 nM SIINFEKL peptide. Results represent individual mice and the mean for 1 representative experiment out of 3. n = 6, P < 0.01, t test.

**Figure 6**

CTL priming with antigen targeting to DNGR-1 plus anti-CD40. 2 μg S1-conjugated anti–DNGR-1 (7H11) or rat IgG1 isotype-matched control mAbs were injected s.c. with or without anti-CD40 (25 μg) as indicated. Target cells were injected 5 days later, and mice were analyzed on day 6. (A) In vivo CTL activity as measured by target cell elimination. Histograms show target cell frequency in representative mice from each group (CFSElo; 20 nM peptide; CFSEint, 200 nM peptide; CFSEhi, no peptide). Graph shows mean ± SEM of percentage of specific lysis in 1 experiment of 3 (n = 6 mice/group). All groups are shown, but the only one in which killing was detectable was that receiving anti–DNGR-1–S1 plus anti-CD40. (B) H-2Kb–SIINFEKL tetramer staining of splenocytes. Left panel shows representative dot plots of tetramer staining versus CD8 in gated CD8+ Thy1+ T cells. Right panel shows frequency of tetramer-positive CD8+ T cells in 1 experiment of 3 (n = 6 mice/group). (C) In vitro restimulation with 1 μM SIINFEKL (PEPTIDE) or medium alone (CTR). Left panel IFN-γ content in supernatants at the end of the 5-day culture. Right panel shows specific CTL activity of in vitro–restimulated cells against EL4 targets loaded with 2 μM of SIINFEKL. Data are the average ± SEM of all cultures (n = 6 mice/group, restimulated individually). P values were calculated using Student’s t test. (D) OVA protein conjugated to anti–DNGR-1 induces CTL priming in vivo. Mice were immunized s.c. in the paw with 2 μg anti–DNGR-1 or isotype control antibodies conjugated to OVA protein in the presence of 25 μg of anti-CD40. In vivo killing activity was analyzed as in A using targets loaded with 200 nM SIINFEKL peptide. Results represent individual mice and the mean for 1 representative experiment out of 3. n = 5; P < 0.005, t test.

**Human DNGR-1 is an endocytic receptor restricted to a small subset of blood DCs.** To extend these findings to humans, we cloned hDNGR-1 and generated mouse mAbs against it (see Methods). One of these mAbs was selected to analyze the pattern of DNGR-1 expression among human peripheral blood mononuclear cells. Human DNGR-1 expression was absent from lymphocytes, monocytes, NK cells, and lineage-negative, HLA-DR− cells (Figure 9A). It was also not detected in monocyte-derived DCs generated by culture in
GM-CSF and IL-4 (data not shown). However, DNGR-1 expression was apparent in a discrete subpopulation of blood DCs, defined as lineage-negative, HLA-DR+ cells (Figure 9A) with a characteristic dendritic morphology (Figure 10). Five distinct subsets of blood DCs have been reported, including a population of CD123+ DNGR-1. After 1 hour at 37°C but not at 4°C fluorescence was found in intracellular compartments (Figure 10). Therefore, human DNGR-1 mediates endocytosis of bound antibody in BDCA-3+ DCs, thereby suggesting that it could be used for antigen targeting to these cells in humans.

Discussion

There is increasing realization that DCs constitute a heterogeneous family composed of multiple subsets with specialized functions (2). This suggests that strategies that simultaneously target multiple DC subtypes could be counterproductive, perhaps inducing competing responses. Therefore, there is clearly a need to identify markers that might serve to selectively manipulate distinct DC subtypes. Here, we report that the previously uncharacterized C-type lectin CLEC9A, here renamed DNGR-1, is a highly specific marker of a subset of mouse and human DCs and can be used to deliver exogenous antigens for MHC class I presentation in vivo, allowing efficient CTL cross-priming and tumor therapy.

Using representational difference analysis to compare mouse CD8α+ and CD8β- DCs, we originally found that DNGR-1 transcripts were overrepresented in CD8β- DCs. DNGR-1 encodes a type II transmembrane receptor of the C-type lectin family bearing a single extracellular CTLD and a short intracellular tail. We show that the mouse version of DNGR-1 is selectively expressed in CD8β- cDCs and, at much lower levels, in pDCs of unstimulated animals. Notably, we report that the human version of DNGR-1 is similarly restricted in expression to a small subset of blood DCs, defined by BDCA-3 expression. These BDCA-3+ DCs have been previously described as a subtype of myeloid DCs (50–52) and share similarities with mouse CD8α+ DCs, including high levels of TLR3 mRNA, absence of TLR7 transcripts, and expression of nectin-like protein 2 (53–55). However, unlike DNGR-1, BDCA-3 is not a unique marker for this population, as it is also expressed on non-DC lineages. The highly restricted expression of DNGR-1 will therefore be useful in determining whether DNGR-1+ DCs represent the long sought-after human equivalent of mouse CD8α+ DCs and in targeting these cells in humans.

The restricted expression and endocytic properties of DNGR-1 suggested that it might constitute a useful receptor for targeting antigens to CD8α- DCs or to their human equivalent. Consistent with that notion, we show that antibodies against DNGR-1 specifically label CD8α- DCs and pDCs in mice, with no detectable
anti–DNGR-1 antibody can be endocytosed by both human and presenting activity of CD8+ endocytosis or uptake induced by receptor cross-linking. Whether antibody internalization reflects constitutive receptor presentation pathway. This is consistent with the observation that DNGR-1 targeting also promotes MHC class II presentation by mouse DCs and concentrates in intracellular vesicles, although we do not know at present the specific identity of such compartments they express low levels of DNGR-1 and have been described as footpads, illustrating the remarkable ability of the antibody to diffuse and bind specifically to distant targets. Consistent with the labeling data, in vivo delivery of a modified OVA peptide covalently coupled to anti–DNGR-1 antibody resulted in selective delivery of conjugates of anti–DNGR-1 to full-length OVA protein displayed a valency, purity, and stoichiometry (D. Sancho and C. Reis e Sousa, unpublished observations). Therefore, we only used those conjugates in a limited set of experiments in order to validate the results obtained with anti–DNGR-1 coupled to S1 or S2 peptides (e.g., Figure 6D). MHC class II presentation experiments will be facilitated by the construction of recombinant antibodies engineered to contain suitable epitopes, as pioneered by others (23).

We wished to assess the potential of DNGR-1 targeting in inducing CTL responses and tumor immunotherapy and, therefore, tested conjugates of antigen and anti–DNGR-1 mAb under the cover of anti-CD40, which provides additional signals for immunogenicity (23, 24). This protocol was remarkably effective at inducing specific CTL effector activity from a naive polyclonal T cell repertoire, as measured by multiple readouts. Thus, 2 μg of anti–DNGR-1–S1, corresponding to 10–15 ng SIINFEKL peptide, plus anti-CD40 (or poly I:C) led to a large expansion of OVA/H-2Kb tetramer–positive CD8+ T cells, which produced IFN-γ and killed peptide-loaded targets in vivo or upon restimulation in vitro. In comparison, a 100-fold excess of free S1 did not induce any measurable response under the same conditions. These results are comparable to those obtained with anti–DEC-205 where small amounts of targeted antigen similarly induce potent CTL responses, as long as a DC activation stimulus is coadministered (24). To test the efficacy of this protocol in a tumor model, we chose B16 melanoma, a poorly immunogenic mouse tumor that is a notoriously difficult to treat (47–49). For this reason, we started with B16 modified to express OVA as a foreign antigen. A single administration of anti–DNGR-1–S1 plus anti-CD40 acted in a prophylactic manner to prevent B16-OVA implantation even when given 1 month before tumor challenge. The same protocol could also be used for tumor therapy, curing mice when given after B16-OVA challenge. Remarkably, a single administration of such conjugates together with poly I:C plus anti-CD40 was sufficient to break tolerance to self, inducing CTL priming and B16 eradication even when
given in a therapeutic mode 3 days after tumor challenge. This is a stringent test because adoptive transfer of CTLs specific for melanoma antigens is generally carried out by day 3 after i.v. inoculation of B16 cells in therapeutic experiments (49). As the generation of active CTL takes a few days, our results imply that antigen targeting via DNGR-1 together with an appropriate adjuvant is a powerful means of generating relevant antitumor immunity that could be exploited as an immunotherapeutic strategy for cancer. Whether such immunization eventually leads to autoimmunity is currently being assessed, although it is notable that vitiligo did not develop in the short time course of our tumor experiments (D. Sancho and C. Reis e Sousa, unpublished observations).

An important consideration in antigen targeting is whether the target is capable of modulating DC function. In this regard, DNGR-1 possesses a tyrosine residue in its intracellular tail within a context that resembles the hemITAM motif found in dectin-1 (35). We have recently demonstrated that dectin-1 can use this motif to couple to Syk kinase and that agonists of dectin-1 signal through Syk to promote activation of DCs (58, 59). However, none of the anti–DNGR-1 antibodies are able to promote DC activation in vitro or in vivo as measured by upregulation of costimulatory molecules or induction of cytokines (data not shown). In addition, anti–DNGR-1 treatment does not alter the response of DCs to heterologous stimuli such as poly I:C or CD40 ligation (data not shown). These observations suggest that anti–DNGR-1 mAbs, at least in soluble form, do not provoke signaling through the receptor. Similarly, antibodies to dectin-1 do not act as agonists for that receptor, presumably because they do not elicit the degree of cross-linking necessary to achieve productive signaling (59). Therefore, we were not surprised to find that targeting through DNGR-1 in the absence of adjuvant does not induce immunity, as reported for antigen targeting via DEC-205 (23, 24, 30–32). In fact, we do not at present have evidence that antigen targeting to DNGR-1 elicits a qualitatively different immune response from antigen targeting to DEC-205 (D. Sancho, O.P. Joffre, C. Reis e Sousa, unpublished observations). However, future studies will be needed to determine whether, like DEC-205 targeting, DNGR-1 targeting induces cross-tolerance and whether DNGR-1 may therefore constitute a promising target not only for inducing immunity but also for promoting immunological unresponsiveness.

Methods

Mice. C57BL/6, B6.SJL, and OT-I × rag−/− (C57BL/6 background) were bred at Cancer Research UK in specific pathogen–free conditions. All animal experiments were performed in accordance with national and institutional guidelines for animal care and were approved by the Institutional Animal Ethics Committee Review Board, Cancer Research UK.

Cells. Culture medium was RPMI 1640 (Invitrogen) supplemented with glutamine, penicillin, streptomycin, 2-mercaptoethanol (all from Invitrogen), and 10% heat-inactivated fetal calf serum (Bioclear). Mouse BMDCs were generated using GM-CSF and purified from bulk cultures by magnetic selection with anti-CD11c microbeads (GM-CSF BMDCs). Alternatively, BMDCs were generated by culturing BM cells in the presence of 100 ng/ml of Flt3L (R&D) for 10 days, by which time all living cells were positive for CD11c.
Generation of anti–DNGR-1 mAbs. Wistar rats or BALB/c mice were immunized 3–4 times with RBL-2H3 cells expressing respectively mouse or human DNGR-1 fused to an HA epitope. Fusion of splenocytes with the rat myeloma cell line Y3 or mouse myeloma line SP2/0 was carried out using standard procedures. For hybridoma screening, we used the B3Z cell line, which expresses a β-gal reporter for nuclear factor of activated T cells (NFAT) (60). This cell line was transduced with a retrovirus encoding a chimera of the extracellular domain of mouse or human DNGR-1 fused to the transmembrane region from NKRP1B and the intracellular tail of CD3ζ followed by an internal ribosome entry site (IRES) sequence and the GFP gene (61). Hybridoma supernatants were screened for the ability to bind to the DNGR-1 chimera, resulting in the activation of the NFAT reporter and induction of β-gal activity (61). Supernatants that tested positive in this assay were further screened by flow cytometry using a mixture of B3Z cells expressing the chimera DNGR-1 (GFP+) and parental B3Z cells (GFP–) (see Supplemental Figure 5). This method allowed the selection of 3 rat mAbs specific for mDNGR-1 (1F6 [rat IgG1], 397 [rat IgG2a], and 7H11 [rat IgG1]) and 1 mouse mAb specific for hDNGR-1 (8F9 [IgG2a]). 7H11 anti–mDNGR-1 was selected for most of the studies described here and was conjugated to biotin or to Alexa Fluor 488 (Invitrogen) for staining or to OVA peptides for targeting, as described below.

Flow-cytometry. Fluorochrome- or biotin-labeled antibodies specific for mouse CD11c, CD24, CD11b, B220, Ly6C, CD4, and CD8 were from BD Biosciences — Pharmpingen. Purified 2.4G2 (anti-FcγRII/III) was from Cancer Research UK Antibody production service. Mouse cell suspensions were incubated with 10 μg/ml of 2.4G2 mAb to block Fcγ receptors and were then stained in ice-cold PBS supplemented with 2 mM EDTA, 1% FCS, and 0.02% sodium azide. For endocytosis studies, FcγR-blocked cells were labeled with 5 μg/ml of biotinylated anti-DNGR-1 mAb for 30 minutes at 4°C. Cells were then washed twice and incubated for different times at 4°C or 37°C before transferring to ice and adding streptavidin PE. For in vivo labeling studies, Alexa Fluor 488–conjugated anti–DNGR-1 or isotype-matched control mAbs were injected i.v. at the indicated dose, and tissues were prepared and analyzed after 16 hours. Antibodies specific for human CD3, CD14, CD19, CD56, HLA-DR, CD34, CD123, and CD16 were purchased from BD, and CD11b and CD1c were from Abcam. Human mononuclear cells were blocked with 100 μg/ml human IgG (Sigma-Aldrich) and stained as above. Data were acquired on a FACScalibur (BD Biosciences) and analyzed using Flowjo software (Tree Star Inc.).

Immunoprecipitation and Western blot. Phoenix cells stably expressing DNGR-1 were lysed in 50 mM HEPES (pH 7.4), 150 mM sodium chloride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate (pH 10.0), 1 mM EDTA (pH 8.0), 1.5 mM magnesium chloride, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and Complete Protease Inhibitor Cocktail Tablets (Roche). Insoluble material was discarded, and a fixed amount of lysate was subjected to immunoprecipitation using 1 μg of anti–DNGR-1 or rabbit anti-HA (Santa Cruz Biotechnology Inc.) and GammaBind Plus Sepharose (GE Healthcare) beads. Proteins were eluted from beads under nonreducing conditions and separated by SDS-PAGE, transferred onto Immobilon PVDF membranes (Millipore), and probed with rabbit anti-HA or anti-Cleca9a antibodies. Alternatively, cleared lysates were boiled in SDS-containing gel-loading buffer under nonreducing or reducing (10% β-mercaptoethanol) conditions and separated, transferred, and probed as described above.

Microscopy. Mouse Flt3L-derived BMDCs or DNGR-1–transfected cells were incubated with 10 μg/ml of 2.4G2 mAb to block FcγR and were then labeled with 5 μg/ml of Alexa Fluor 488–conjugated anti–DNGR-1 or rat IgG1 isotype control for 30 minutes at 4°C. For human DNGR-1+ DCs, the cells were first enriched from total PBMCs using BDCA-3-PE and anti–PE microbeads (Miltenyi Biotec) and then sorted to purify cells with high expression of BDCA-3, which were then labeled with 10 μg/ml of Alexa Fluor 488–conjugated anti-human DNGR-1 or mouse IgG2a isotype control for 30 minutes at 4°C. In both assays, mouse or human cells were

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Coupling of anti-DNGR-1 to antigen and antigen targeting. Peptides S1 and S2 containing the OVA 257–264 epitope (SIINFEKL) were injected s.c. in the hind paws (1 × 10^5/mouse) either 3 days before (therapy) or 1 day after (prophylaxis) immunization with anti–DNGR-1 or control antibody covalently coupled to a mixture of 5 peptides derived from gp100, TRP-1, and TRP-2 (1 μg/paw) together with anti-CD40 (12.5 μg/paw). B16-OVA cells (2.5 × 10^5/mouse) were given i.v. 1 month later, and mice were sacrificed 18 days after tumor challenge. Tumor therapy experiments were done in an analogous fashion except that mice received B16-OVA 3 days prior to antibody treatment. Tumor therapy and prophylaxis experiments were also carried out with nontransduced parental B16 cells. These were given i.v. (5 × 10^5/mouse) either 3 days before (therapy) or 1 day after (prophylaxis) immunization with anti–DNGR-1 or control antibody covalently coupled to a mixture of 5 peptides derived from gp100, TRP-1, and TRP-2 (1 μg/paw) together with anti-CD40 (12.5 μg/paw) and poly I:C (5 μg/paw). Tumor burden was assessed by counting lung foci. When these were too numerous to count (>250 per mouse), they are shown as 250. CTL responses were monitored as described above.

Statistics. Statistical analysis was performed with 1-tailed Student’s t test for differences among groups or Mann-Whitney U test when normality of data could not be inferred. P < 0.05 was considered statistically significant. Quantitative data are expressed as means ± SEM unless otherwise stated.

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