Generation of anti-DCIR mAbs and production of recombinant DCIR

Mouse mAbs were generated by conventional cell fusion technology. Briefly, 6-week-old BALB/c mice were immunized intraperitoneally with 20 μg of receptor ectodomain.hIgGFc fusion protein with Ribi adjuvant, then boosted with 20 μg antigen 10 days and 15 days later. After 3 months, the mice were boosted again three days prior to taking the spleens. Alternately, mice were injected in the footpad with 1–10 μg antigen in Ribi adjuvant every 3–4 days over a 30–40 days period. B cells from spleen or lymph node cells were fused with SP2/O-Ag 14 cells using conventional techniques. ELISA was used to screen hybridoma supernatants against the receptor ectodomain fusion protein compared to the fusion partner alone, or versus the receptor ectodomain fused to AP. Positive wells were then screened by flow cytometry using HEK293F cells transiently transfected with expression plasmids encoding full-length receptor cDNA. Selected hybridomas were single cell cloned and expanded in CELLine flasks (Intergra). Hybridoma supernatants were mixed with an equal volume of 1.5 M glycine, 3 M NaCl, 1× PBS, pH 7.8 (binding buffer) and tumbled with MabSelect resin (eBiosciences) (800 μl / 5 ml supernatant). The resin was washed and eluted with 0.1 M glycine, pH 2.7. Following neutralization with 2 M Tris, mAbs were dialyzed versus PBS.

cDNA cloning and expression of chimeric mouse/human recombinant IgG4 mAbs

Total RNA was prepared from hybridoma cells (RNeasy kit, Qiagen) and used for cDNA synthesis and PCR (SMART RACE kit, BD Biosciences) using supplied 5′ primers and gene-specific 3′ primers (mlgGκ, 5′ggatggtgggaagatggatacagttggtgcagcatc3′; mlgG1, 5′gtacttgctcggaaatgccttgacgcagc3′). PCR products were then cloned (pCR2.1 TA kit, Invitrogen) and characterized by DNA sequencing. Using the derived sequences for the mouse heavy (H) and light (L) chain variable (V) region cDNAs, specific primers were used to PCR amplify the signal peptide and V-regions, while incorporating flanking restriction sites for cloning into expression vectors encoding downstream human IgGκ or IgG4H regions. The vector for expression of chimeric mVκ-hlgGκ was built by amplifying residues 401–731 (gi|63101937|) flanked by Xho I and Not I sites and inserting this into the Xho I – Not I interval of the vector pIRE7A-DsRed2 (BD Biosciences). PCR was used to amplify the mAb Vκ region from the initiator codon, appending a Nhe I or Spe I site then CACC, to the region encoding (e.g., residue 126 of gi|76779294|), appending a Xho I site. The PCR fragment was then cloned into the Nhe I – Not I interval of the above vector. The control hlgG4H vector corresponds to residues 12–1473 of gi|19684072| with 7A29P and L236E substitutions, which stabilize a disulphide bond and abrogate residual FcR interaction,3 inserted between the Bgl II and Not I sites of plRE7A-DsRed2 (BD Biosciences) while adding the sequence 5′-gctagctgattaattagcc3′ instead of the stop codon. PCR was used to amplify the mAb VH region from the initiator codon, appending CACC then a Bgl II site, to the region encoding residue 473 of gi|19684072|. The PCR fragment was then cloned into the Bgl II – Apa I interval of the above vector. The vector for chimeric mVH-hlgG4 sequence using the mSLAM leader was built by inserting the sequence 5′ctagttgctgactaatgacccaaagctccctctgagatcctgttctctcctctccttgaattttggtcagagattaaaggc3′ into the Nhe I – Apa I interval of the above vector. PCR was used to amplify the interval between the predicted mature N-terminal codon and the end of the mVH region while appending 5′tgtaagg3′. The fragment digested with Bsi WI and Apa I was inserted into the corresponding sites of the above vector. Antigen coding sequences flanked by a proximal Nhe I site and a distal Not I site following the stop codon were inserted into the Nhe I - Pac I - Not I interval of each H chain vector. Dockerin (Doc) was encoded by gi|40671| C. thermocellum CelD residues 1923–
2150 with proximal Nhe I site and a distal Not I site. HIV gag p24 was encoded by gi|77416878| residues 133–363 with a proximal Nhe I site and sequence from gi|125489020| residues 60–75 and a distal Not I site. Recombinant antibodies were produced using the FreeStyle™ 293 or CHO-S Expression Systems (Invitrogen) according to the manufacturer’s protocol (1 mg total plasmid DNA with 1.3 ml 293 Fectin reagent or 1 mg total plasmid DNA with 1 ml FREESTYLE MAX reagent/L of transfection, respectively). Equal amounts of vectors encoding the H and L chain were co-transfected. Transfected cells were cultured for 3 days, then the culture supernatant was harvested and fresh media with 0.5% penicillin/streptomycin (Biosource) added with continued incubation for 2 days. The pooled supernatants were clarified by filtration, loaded onto a 1 ml HiTrap MabSelect™ column, eluted with 0.1 M glycine pH 2.7, neutralized with 2 mM Tris and then dialyzed versus PBS with Ca++/Mg++. Proteins were quantified by absorbance at 280 nm.

**DCIR expression analysis**

DCIR expression was assessed on PBMCs, in vitro generated- or skin-derived DCs. Cells were double stained with anti-DCIR mAb (generated as described in supplemental methods), or mouse IgG1 (BD), washed, and then stained with PE-conjugated goat anti-mouse IgG (BD Pharmingen), then washed and incubated with FITC or APC-conjugated anti-CD3, anti-CD19, anti-CD11c, anti–HLA-DR, anti-CD11c, anti-CD123, anti-CD56, anti-CD16, (BD Pharmingen) anti-CD1a (DAKO), or anti-CD14 (Invitrogen) mAbs. Epidermal sheets were stained as detailed in supplementary methods to assess DCIR expression on immature LCs.

For the expression of DCIR on immature LCs, epidermal sheets were cut into approximately 10 mm squares and placed in 4% paraformaldehyde for 30 min. Sheets were washed in PBS and blocked with Background Buster (Innovex) for 30 min. Epidermal sheets were then incubated overnight with 0.5 µg purified mouse anti-DCIR (clone 9E8) or control IgG1, washed twice with PBS/0.05% Saponin and incubated for 1 h with a secondary goat anti mouse IgG-Alexa568 (Molecular Probes) (1:500 dilution). Nuclei were stained with DAPI (Invitrogen; Molecular Probes) at 1:5000 followed by 2 h incubation with anti–HLA-DR-FITC. Sheets were rinsed with PBS and mounted in Vectamount (Vector Laboratories). All antibodies were diluted in CytoQ diluent and block (Innovex) and all incubations were at 4°C with constant mild agitation. Images were taken with an Olympus Planapo 40/0.7, CoolSnap HQ camera and analyzed using Metamorph software.

**DCIR-signaling effect on DC-function**

CD34+-derived DCs were cultured in anti-DCIR (clone 24A5 or 9E8) or isotype control coated plates in the presence or absence of CD40L (R&D; 100 ng/ml) or LPS (Invivogen; 50 ng/ml). After 24 h, cells were harvested and stained for surface phenotype. The secreted cytokines were analyzed by a multiplex bead assay (Luminex). For a global gene signature analysis 0.5 × 10^6 epidermal cells that were purified from normal human skin were exposed to either anti-DCIR (clone 24A5 or 9E8), anti-CD40 (clone 12E12) or an IgG1 isotype matched control in a soluble, cross-linked or plate coated form at 5 µg/ml for 24 h. Double-stranded cDNA was obtained from 200 ng of total RNA and after in vitro transcription underwent amplification and labeling steps according to the manufacturer’s instructions. 1.5 µg of amplified biotin-labeled cRNA was hybridized to the Illumina Sentrix Hu6 BeadChips according to the sample labeling procedure recommended by Illumina (Ambion, Inc, Austin, TX). BeadChips consist of 50mer
oligonucleotide probes attached to 3-μm beads within microwells on the surface of the glass slide representing 48,687 probes. Slides were scanned on Illumina BeadStation 500 and Beadstudio software was used to assess fluorescent hybridization signals. To study the effect of DCIR signaling on allogeneic CD8+ T-cell priming, LCs were cultured with allogeneic naïve CD8+ T cells in a plate coated with anti-DCIR mAb or IgG1 control (10 μg/ml) at ratio DC:T 1:20 in the presence or absence of CD40L. T-cell proliferation responses were assayed by measuring [3H]-thymidine incorporation during the last 12 h of 6 days cultures. The proliferating CD8+ T cells (CFSElow) were analyzed for their phenotype and their cytokine secretion pattern following CD3/CD28 mAb stimulation. To study the effect of DCIR signaling on autologous CD8+ T cells priming, CD34+ -derived DC subsets were loaded with the HLA-A201–restricted MART-1(26–35) peptide and cocultured with naïve CD8+ T cells in the presence of a soluble form of anti-DCIR mAb or IgG1 control (10 μg/ml) and CD40L. After 10 days, cells were harvested and analyzed for the frequency of MART-1–specific CD8+ T cells by specific tetramer, and for the expression of effector molecules Granzyme A (BD Pharmingen), Granzyme B (eBiosciences) and perforin (Fitzgerald).

**Cloning and production of fusion protein mAbs**

FluMP was chemically cross-linked to mAbs using sulfo-succinimidyl 6-[3'(2-pyridyldithio)-propionamido] hexanoate (sulfo-LC-SPDP; Pierce) according to the manufacturer’s protocol. Chimeric mouse/human recombinant mAbs anti-DCIR and control IgG4 were fused to a ~9.5 kDa dockerin domain in-frame with the rAb H chain. The entire FluMP, containing the immuno-dominant HLA-A201–restricted FluMP (58–66) peptide (GILGFVFTL), and a sequence encoding the immuno-dominant HLA-A201–restricted MART-1 (26–35) peptide (ELAGIGILTV) from the melanoma MART-1 antigen with surrounding natural MART-1 residues:

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DTTEARHPHPVTPTTDKGTATAEELAGIGILTVILGGKRNTNSTPTKGEFCRYPHWRP,
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were each fused to the ~17.5 kDa cohesin domain and were expressed in E. coli strains BL21 (DE3) (Novagen) or T7 Express (NEB). Recombinant mAb (rAb)-antigen conjugate was formed by mixing rAb.Doc fusion protein with 2 molar equivalents of cohesin.antigen fusion protein. The dockerin and cohesin domains self-associate to form a stable [rAb.doc-coh.antigen] conjugate (Flamar et al.; manuscript in preparation). The chimera rAb anti-DCIR or IgG4 control antibodies were fused to the HIV gag p24 protein4 or to a portion of a recombinant form of the MART-1 protein. The anti–DCIR-MART-1 (clone 9E8) fusion protein used had the following peptide units appended to the H chain C-terminus [each unit flanked by AS residues]:

- *Bacteroides cellulosolvens* cellulosomal anchoring scaffoldin B precursor [gb|AAT79550.1|] residues 651–677 with a T672N substitution; MART-1[gb|BC014423.1|] residues 1–38;
- gb|AAT79550.1|residues 1175–1199; MART-1 residues 78–118. For cell-surface staining of mAb-FluMP conjugates, coh.FluMP was biotinilated using EZ-Link NHS-SS-PEO4-Biotin (Pierce) according to the manufacturer’s procedure. Monocyte-derived DCs were stained with 10 μg/ml rAb.doc-coh.FluMP.Biotin complexes on ice for 20 min. Cell-surface binding was detected using PE-conjugated Streptavidin (1:200; BD Biosciences) and analyzed by flow cytometry.

**Peptide-MHC complexes detection on DCs**

CD34+ -derived DCs from an HLA-A201+ donor were incubated with 50 nM DCIR.doc-coh.FluMP conjugate or free coh.FluMP fusion protein in culture media supplemented with 10%
human serum, 50 ng/ml GM-CSF and 10 ng/ml TNF-α. 5 µg/ml anti-CD40 mAb (12E12, BIIR) was added after 2 h. Cells were assessed after 24 h for FluMP (58–66) peptide (GILGFVFTL)-HLA-A201 complexes by flow cytometry using PE-conjugated tetramerized M1D12 monoclonal antibody.

**Purification of CD8+ T cells**
CD8+ T cells were negatively selected from PBMCs using CD14, CD19, CD16, CD56 and CD4 magnetic beads, or purified using the naïve CD8+ T-cell isolation kit (Miltenyi Biotec). In some experiments, naïve CD8+ T cells were sorted as CD8−CCR7−CD45RA+ and memory CD8+ T cells were sorted as CD8+CCR7−CD45RA−. Where indicated, cells were labeled with 0.5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen).

**Cross-presentation of FluMP protein by chemically-linked anti-DCIR mAb**
CD34−-derived LCs from an HLA-A201+ donor were cultured for 8 days with purified CD8+ T cells together with increasing concentrations of either anti–DCIR-FluMP or controls including IgG1-FluMP and free FluMP protein. When delivered alone, FluMP induced very limited expansion of FluMP-specific CD8+ T cells (Fig. S1A) as assessed by staining with a FluMP (58–66)-specific HLA-A201-tetramer. IgG1-FluMP was more efficient than the free FluMP protein, suggesting Fc-mediated uptake. Dose-titration curve, illustrated in Fig. S1B, shows that anti–DCIR-FluMP elicited a response with at least 50-fold less antigen than the control IgG1-FluMP or the free FluMP, therefore demonstrating actual targeting of the antigen. Note that the free antigen never induced the high frequency of FluMP-specific CD8+ T cells observed with anti–DCIR-FluMP (Fig. S1B).

**REFERENCES**
Table S1. Activation of DCs in vitro by CD40L but not by anti-DCIR
CD34+-derived DCs were cultured with 5 µg/ml of anti-DCIR or isotype control with or without CD40L (100 ng/ml), for 24 h. Cells were then evaluated by flow cytometry for expression of CD80, CD86, CD40, ICOS-L, MHC class I and MHC class II. The bold symbols are mean fluorescence indices of the CD1a+ LCs in the presence of a maturation stimulus show an increase consistent with maturation.

Figure S1. Cross-presentation of FluMP protein by anti-DCIR conjugate mAb
(A) Enhanced cross-presentation of FluMP to CD8+ T cells by CD1a+ LCs cultured with chemically cross-linked anti-DCIR-FluMP, cross-linked control IgG-FluMP proteins, or free FluMP. Dot plots show the proportions of HLA-A201-FluMP (58-66) peptide tetramer-positive CD8+ T cells. Data are representative of three independent experiments. (B) Percentage of FluMP-specific CD8+ T cells in response to targeting with decreasing concentrations of cross-linked mAb-FluMP constructs or free FluMP. Graph shows mean of duplicate.

Figure S2. Engineering and characterization of targeted proteins into DCIR mAb
(A) SDS-PAGE-reducing gel of mouse anti-DCIR mAbs (clone 9E8 and 24A5), chimeric mouse/human anti-DCIR (IgG4) and control IgG fused to a Dockerin domain (mAb.Doc). FluMP and MART-1 fused to a cohesin domain (coh.FluMP and coh.MART-1), and the fusion proteins anti-DCIR-p24 and control IgG4-p24. The gel was stained with comassie blue. The molecular weights of the proteins are indicated on the left of the figure. (B) Binding analysis of anti-DCIR.doc-coh.FluMP complex mAb to monocyte-derived DCs. Day 6 immature GM-IL4 DCs were treated with 50 nM of biotinylated anti-DCIR-FluMP, and control IgG4-FluMP conjugate mAbs. The complexes were detected with a phycoerythrin-conjugated streptavidin. The anti-DCIR.doc-coh.FluMP complex mAb bound the DCs (black histogram), while the respective control conjugate mAb did not bind to DCs (gray histogram).

Figure S3. Anti-DCIR antibody fails to deliver inhibitory signals to human DCs
(A) Illustrative flow cytometry data showing the expression of CD86 on the surface of DCIR-ligated- or control- CD1a+ LCs in the presence or absence of CD40L. (B) Luminex assay for IL-6 was performed on supernatants from DCIR or control ligated- skin DC subsets activated for 24 h with CD40L or TLR7/8-agonist. One of two independent experiments is shown.

Figure S4. DCIR ligation does not inhibit CD8+ T cell priming
(A) DCIR-ligated DCs induce a similar level of allogeneic CD8+ T-cell proliferation compared to control DCs, as determined by [3H]-thymidine incorporation in the presence or absence of CD40 activation. The graph shows mean ±s.d, N=3. (B) Flow cytometry analysis of the expression of PD-1, CTLA-4 or CD28 on allogeneic CD8+ T cells primed by DCIR-ligated DCs (blue line) or control DCs (red line). (C) Graphs show the level of cytokine secretion IFN-γ, IL-2, TNF-α and IL-10 by activated CD8+ T cells that were primed by allogeneic DCIR-ligated DCs or control DCs. Cytokines were measured in response to anti-CD3/CD28 microbeads stimulation and analysed after 24 h by Luminex. (D) Expression of effector molecules: Granzyme A, Granzyme B and perforin, as evaluated by flow cytometry (right panel) on MART-1-specific CD8+ T cells that were primed by DCIR-ligated- or control- MART-1 peptide-loaded LCs. Data are representative of three independent experiments.
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Table S1
Figure S1
**Figure S2**

**A**

1. Molecular Weight Marker
2. Anti-DCIR.3E8
3. Anti-DCR±_3E8S
4. Anti-DCIR.3E8.Ega.sH4
5. Anti-DCR.3E8S.Ega.sH4
6. Control IgG2a.pH1
7. Anti-DCIR.3E8.0.9Ac
8. Anti-DCR.3E8.0.9Ac
9. Control IgG1
10. Coh.1RF5P
11. Coh-MMH1-1

2.5 μg/lane

**B**

IgG4.doc-coh.FluMP

DCIR.doc-coh.FluMP

MFI
Figure S3
Figure S4

A

![Graph showing T cell proliferation (cpm x 10^3) with or without anti-DCIR treatment.](image)

B

![Histograms showing CD28, PD-1, and CTLA-4 expression with or without CD40L activation.](image)

C

![Bar charts showing cytokine levels (IFN-γ, IL-2, TNF-α, IL-10) with different CD8+CD1a^+ cell subsets.](image)
Figure S4