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Heterogeneous expression of the adhesion receptor CD226 on murine NK and T cells and its function in NK-mediated killing of immature dendritic cells

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ABSTRACT

The adhesion receptor CD226 (DNAM-1) is a member of the Ig superfamily possessing two extracellular V-like domains. In humans, CD226 was shown to be expressed by NK as well as T cells. During T cell priming, CD226-mediated costimulatory signals may skew the subsequent differentiation into the Th1 pathway. In addition, CD226 expressed on NK and cytotoxic T cells is engaged by its counter-receptor CD155, present on target cells, thereby triggering their elimination. We established mAb specifically recognizing mCD226, demonstrating that CD226 is expressed by precursor and mature but not developing T cells. In contrast, NK cells are distinguished by a rather heterogeneous CD226 expression profile. In addition, expression of CD226 appears coupled to that of other NK cell receptors, as high expression of CD226 was found to correlate with decreased proportions of Ly49D and H positive NK cells. Upon injection into mice, the anti-CD226 antibodies caused selective depletion of $CD8^+$ T cells. Moreover, these antibodies as well as a naturally occurring CD226 splice variant lacking the outermost V-like domain were instrumental in determining that CD226 adheres to CD155 via its first domain. In addition, antibodies were identified as capable of blocking the CD226/CD155 interaction and to prevent NK-driven killing of immature DC. CD226 is thus the first mNK receptor identified to be essential for the elimination of this particular cell type. *J. Leukoc. Biol.* 86: 91–101; 2009.

Introduction

CD226 (DNAM-1) is a transmembrane glycoprotein of the Ig superfamily, possessing two extracellular Ig-like domains [1]. Analyzing human peripheral blood cells, CD226 was found to be expressed on $CD4^+$ as well as $CD8^+$ T cells, γδTCR T cells, monocytes, and NK cells but only on a small subpopulation of B cells. CD226 interacts in cis with the integrin LFA-1 ($\alpha_L\beta_2$), an association that may trigger a switch from mere adhesion to additional intracellular signaling events caused by CD226 activity [2]: The complex in cis with LFA-1 is critical for TCR-stimulated differentiation and proliferation of T cells identifying CD226 as a T cell costimulatory molecule [3, 4]. LFA-1 association may also be required for the engagement of CD226 as a killing receptor used by cytotoxic T cells and NK cells [2]. In contrast, other in vitro data suggest that CD226 may also simply serve as an adhesion receptor used by monocytes to transmigrate across endothelial vessel walls [5].

Killing via CD226 depends on the expression of CD155 or CD112 on target cells such as tumor cells or DC [6, 7], whereas transmigration of monocytes may rely only on CD155 expressed on endothelial surfaces. CD155, formerly addressed as the cellular receptor for poliovirus [8], and CD112, known also as Nectin-2 [9], belong to a subfamily of Ig-like adhesion molecules [10], remotely related to CD226 and CD96, another ligand for CD155 [11–13]. Similar to CD226, CD155 was shown to interact in cis with an integrin, $\alpha_v\beta_3$, thereby resembling CD226 in this aspect [14, 15]. CD155 triggers the formation of adherens junctions between epithelial cells and participates in the regulation of cell growth and motility [16]. Available evidence suggests that the complex interplay among CD155, $\alpha_v\beta_3$, sprouty 2, and platelet-derived growth factor also mediates growth arrest by cell/cell contact (contact inhibition)

Abbreviations: Cy5=cyanine 5, DC=dendritic cell (s), DN=double-negative, DNAM-1=DNAX accessory molecule-1, DP=double-positive, h=human, HEK=human embryo kidney, HSA, heat stable antigen, ISP=immature SP $CD8^+$ cells, L=ligand, LN=lymph node (s), m=murine, MFI=mean fluorescence intensity, PBSd=PBS Dulbecco, r=recombinant, SP=single-positive

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[17]. CD155 is expressed on T cells and DC and has been assigned a role in mounting a regular humoral immune response to orally ingested antigens [18]. Furthermore, CD155 was found to be overexpressed in regenerating liver and several tumor types [19, 20]. Thus, killing along the CD226/CD155-axis may contribute substantially to the elimination of emerging tumors. The importance of CD155 driven killing is corroborated by the observation that CD155 expression is down-modulated in cells infected with human CMV, thus probably allowing virus infected cells to escape eradication by NK cells [21].

All functional aspects described above for CD226 relate to humans, whereas the flow of information regarding CD226 (mCD226) is currently fueled by the recent establishment of CD226 deficient mice [22, 23]. A pivotal role of CD226 for keeping tumor development in check also in mice was suggested by the increased susceptibility of these animals to tumor cell inoculum or chemically induced tumor development [22, 23]. Moreover, CD226 critically assists in the activation of cytotoxic CD8 T cells by nonclassical APCs [22]. For mCD226, an expression pattern was reported slightly diverging from that of hCD226, yet mCD226 was demonstrated to interact with mCD155 and mCD112, thus preserving the molecular platform for a functional conservation between species [24, 25]. mCD226 expression is also found on murine T cells and is upregulated upon stimulation. mCD226 levels are up-modulated further when the cells differentiate along the Th1 pathway, whereas mCD226 expression ceases in Th2 cells [25].

We established mAb specifically recognizing mCD226 and determined the mCD226 expression profile in more detail, especially concerning T cells and their progenitors, as well as NK cells. mCD226 is already present in early T progenitors, when cells are not yet committed to the T lineage irreversibly (DN1 stage). At the following stages, the mCD226-specific protein remains almost undetectable, and expression resumes only when cells develop into SP thymocytes. We also cloned a naturally occurring splice variant of mCD226 lacking the exon coding for Domain 1. This variant lost the capability to interact with mCD155 and may therefore serve regulating mCD226 binding/functional activities in trans. Antibodies were identified that were able to block mCD155 binding to cells ectopically expressing mCD226. These antibodies also interfered with NK-driven killing of mDC, revealing CD226 as the first receptor relevant for NK cell mediated killing of these cells in the mouse.

MATERIALS AND METHODS

Cloning of mCD226 expression vectors

Full-length mCD226 was cloned by PCR (Expand High Fidelity, Roche Applied Science, Indianapolis, IN, USA) on cDNA from BALB/c mice (Clontech, Palo Alto, CA, USA). The cycling conditions were 2 min 94°C, followed by 10 cycles consisting of 15 s 94°C, 30 s 55°C, and 1 min 72°C and an additional 15 cycles of 15 s 94°C, 30 s 55°C, and 1 min 72°C, with an extension time of 5 s/cycle, followed by a final 5-min extension step at 72°C. The obtained products were cloned into pcDNA3 and sequenced. One clone out of 10 contained an insert lacking the exon coding for Domain 1 (corresponding to database entry NM_001039149). The full-length clone used further on was identical to database entry NM_178687. This clone was also used as a template to generate a cDNA coding a fusion protein consisting of the entire ectodomain of mCD226 and the C-terminal part of hIgG1 (Domains 3+4; see also ref. [18] for details). The primers used were: MDNAM.HIN 5′-CGCAAGCTTACCATGGCTTATGTTACTT-GGCTTTTGG-3′, mdnam_bam 5′-GCGGATCCGTGTTCAGGCCAAAAGAG-CAGC-3′, and mdnam_IgG 5′-GCGGATCCTGTTTATTGGTTCCACCAT-CAG-3′.

Transient transfection and binding of antibodies or recombinant proteins

Plasmid (20 μg) encoding the mCD226 cDNA versions was transfected along with 2 μg of a GFP-expressing plasmid applying the standard calcium phosphate transfection technique into HEK293 cells, which were analyzed 48 h later. Detached cells were incubated with the panel of anti-mCD226 antibodies or their Fab fragments as indicated. Mouse anti-rat IgG-Cy5 or mouse anti-rat Fab-Cy5 was used as a secondary antibody for detection.

mCD226 and mCD112 (Nectin-2) were expressed and purified as rhIgG1 protein as described earlier [26]. Transfected cells were first incubated with 2 μg recombinant protein in 50 μl FACS buffer (PBSd/2% FBS) on ice and washed twice, and bound protein was revealed by mouse anti-human-biotin/streptavidin PerCP. In competition studies, mAb was added directly to the cells 1.5 h after incubation on ice.

The cells were analyzed on a FACSCalibur or LSRII (BD Biosciences, San Jose, CA, USA), and the data were evaluated using WinList5.0.

Generation of rat anti-mouse mCD226 mAb and test for specificity

Rats were immunized s.c. and i.p. with purified mCD226-hIgG (50 μg) and 5 nmol CPG oligodeoxynucleotide 2006 (TIB MOLBIO, Germany) in 500 μl PBS and 500 μl IFA. After a 6-week interval, a final boost without adjuvant was given 3 days before fusion of the rat spleen cells with the murine myeloma cell line P3X63-Ag8.653. Hybridoma supernatants were tested in an ELISA using mCD226-hIgG1. The positive hybridomas were tested in flow cytometry using HEK293 cells transiently expressing mCD226. Hybridomas suitable for FACS staining were subcloned to achieve stability and monoclonality. The mAb 3B3 and 4G11 were of the IgG1 isotype. For some flow cytometric determinations, the mAb were labeled with Cy5 (Fluoro-Link Cy5, GE Healthcare, Waukesha, WI, USA).

Generation of Fab fragments

For papain digest, ~15 mg mAb in 1.5 ml buffer (20 mM sodium phosphate, 10 mM EDTA, pH 8.0, 20 mM cysteine) were rocked with 400 μl immobilized papain (Pierce, Rockford, IL, USA) overnight at 37°C. The digested material was equilibrated in 10 mM sodium phosphate, pH 8, concentrated using Microsep separation columns (Pall Life Sciences, East Hill, NY, USA, 10 kDa exclusion limit) and then loaded onto a DEAE column for fast protein liquid chromatography (flow rate: 1 ml/min; 10 mM sodium phosphate, pH 8, for 5 min, and then a gradient ending at 10 mM sodium phosphate, pH 8, 1 M NaCl was applied). The Fab fragment eluted with the flow-through, whereas uncut mAb and Fc eluted only in the gradient stage, according to results published earlier [27]. The purity of the Fab fraction was verified by nonreducing SDS-PAGE, Western-blotting, or silver-stain.

Cell preparations and flow cytometric analysis

Single cell suspensions of thymus, LN, and spleen were obtained by mincing the organs though a nylon mesh in PBSd/2% FCS. Erythrocytes of spleen and blood preparations were removed by hypotonic lysis with NH_4Cl.

For analysis of mCD226 expression, peripheral T cells were first incubated with anti-CD226 mAb clone 3B3 followed by mouse anti-rat-Cy5 staining. Subsequently, a blocking step using rat serum was performed before a cocktail was added consisting of anti-CD4-FITC, anti-CD62L-PE, and anti-

CD8-biotin/streptavidin-PerCP. Additionally, directly labeled antibody recognizing γδTCR, αβTCR, and DX5 was applied as indicated.

For analyzing DN thymocytes, the cells were first stained for detection of CD226 clone 3B3 or Tx42 (kindly provided by Drs. Kazuko Shibuya and Akira Shibuya, University of Tsukuba, Japan) before a lineage cocktail was added (all mAb-biotinylated, directed against murine CD3, CD4, CD8, CD19, CD11b, CD11c, GR1, TER119). Following incubation, the cells were washed, and a mixture was added consisting of streptavidin-PerCP, anti-CD44-PE, and anti-CD25-Alexa488. DN cells were addressed as follows: DN1 ($CD25^-$, $CD44^+$), DN2 ($CD25^+$, $CD44^+$), DN3 ($CD25^+$, $CD44^-$), DN4 ($CD25^-$, $CD44^-$). In case DN1 subpopulations were analyzed, the procedure was modified. To enrich for precursor cells, a complement lysis was done using anti-CD3/CD8 mAb incubation followed by addition of Low-Tox-M rabbit complement (Cedarlane, Canada) according to the manufacturer's instructions. After incubation with the lineage cocktail, DN cells were revealed by staining with streptavidin-Pacific Orange, anti-CD25-PerCP-Cy5.5, anti-CD44-Alexa405, anti-CD24-FITC, and anti-CD117-PE.

For analyzing NK cell subsets, splenocytes from C57/BL6 $RAG1^{-/-}$ mice were first stained with biotinylated antibodies to CD27, Ly49H, Ly49A, Ly49C/I, NKG2A/C/E, and NKG2A (eBioscience, San Diego, CA, USA). Subsequently, cells were stained with anti-CD226 mAb clone 3B3 Cy5, NK1.1-PE, and streptavidin-PerCP in combination with FITC-labeled Ly49D, Ly49G2, or CD11b (Becton Dickinson, San Diego, CA, USA, or eBioscience). Data from several flow cytometric experiments were analyzed by applying the Mann-Whitney test. Similar results were also observed in wild-type mice.

CD226 mRNA expression analysis

$CD4^+CD8^-CD62L^+$ cells and $CD4^-CD8^+CD62L^+$ cells were obtained by FACS sorting of thymus and LN cells of BALB/c mice. NK cells from three spleens of BALB/c mice were first purified by magnetic cell sorting (mouse NK cell isolation kit, Miltenyi Biotec, Auburn, CA, USA). Cells were then stained with anti-mCD226 mAb 3B3 and subjected to FACS sorting to obtain a $CD226^{dull}$ and a $CD226^{bright}$ fraction. Cells were reanalyzed to confirm successful separation. Total RNA was prepared using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA). RNA was reverse-transcribed (Superscript II RT, Invitrogen Life Technologies, Carlsbad, CA, USA) using random hexamer primers. The expression of GAPDH, CD226, and CD226Δd1 was analyzed using a Lightcycler 2.0 (Roche Applied Science) and the Fast Start DNA Master plus SYBR Green Kit (Roche Applied Science) or the Sybr Premix Ex *Taq* Kit (Takara, Japan). Standardization and absolute relative quantification of expression levels were done as described previously [28]. The primers used were: CD226fw: 5′-ACCACATGGCTTTCTTGCTC-3′, CD226rev: 5′-CAGCATGAGAGTTGGACCAG-3′, CD226DELUPPER: 5′-CTTATCTGCAAGGAGAGTTACGC-3′, and Cd226dellower: 5′-GCTATCTCAAAACTATCTTTGTGCAC-3′.

Antibody treatment of mice

Mice were initially injected i.v. with 400 μg mAb 3B3 or 4G11, and application of 200 μg mAb was repeated every 5 days. After 3 weeks, mice were killed, and the lymphocyte composition in secondary lymphoid tissue was analyzed as indicated.

For concomitant depletion of NK cells, mice were injected i.v. with 300 μg mAb NK1.1 (HB191) the day before 400 μg anti-CD226 mAb were injected. NK1.1 treatment was repeated after 4 days, followed by injection of another 400 μg anti-CD226 mAb before the animals were killed 8 days after the first mAb application. The successful depletion of NK cells was monitored by staining cells with mAb DX5.

Preparation of bone marrow-derived DC

Bone marrow cells were cultured in DMEM containing 10% FCS (BioWhittaker, Walkersville, MD, USA), 10 mM Hepes, 20 μM 2-ME, 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen Life Technologies) containing 10 ng/ml rmGM-CSF (PeproTech, Rocky Hill, NJ, USA). After 6 days, cells were collected and incubated in serum alone overnight prior to use in a cytotoxicity assay.

NK cell preparation

$DX5^+$ cells were purified from spleens of B6 $RAG1^{-/-}$ mice using the MACS separation system (Miltenyi Biotec), according to the manufacturer's guidelines. Purified cells were resuspended in complete αMEM medium (αMEM, 10 mM Hepes, 20 μM 2-ME, 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin) and cultured in 1000 U rIL-2 (Biosource, Worcester, MA, USA)/ml for 5 days.

Cytotoxicity assay

Target cell DC were incubated for 1 h in the presence of $Na_2{}^{51}CrO_4$ (Amersham, Piscataway, NJ, USA) and then washed thoroughly in PBS. After 4 h of E:T cell coincubation, cell culture supernatants were taken from these wells and analyzed by a γ radiation counter (Wallac, PerkinElmer, Waltham, MA, USA). Specific lysis was calculated according to the formula: percent specific lysis = [(experimental release–spontaneous release)/(maximum release–spontaneous release)] × 100.

RESULTS

Cloning of murine CD226

cDNA encoding CD226 was amplified from BALB/c spleen. Apart from full-length clones encompassing the two V-like extracellular Ig-like domains, a variant was obtained missing exon III coding for the entire first domain (**Fig. 1, A** and **B**). The alternative splicing generates an in-frame deletion fusing the leader peptide directly onto the N-terminus of Domain 2, thus preserving the key prerequisite for expression on the cell surface (CD226Δd1; see below). This pattern of alternative splicing is not restricted to the BALB/c strain, as two database entries representing incomplete CD226 cDNA from C57/BL6J thymus also document a direct splicing of exon II onto exon IV, skipping exon III (BE623877 and BC051526). CD226 was shown to be expressed by naive $CD4^+$ and $CD8^+$ T cells. Therefore, we determined the mRNA level representing both CD226 splice variants as well as the message specific for the CD226Δd1 variant (Fig. 1, C and D). CD226 mRNA is more abundant in $CD8^+$ T cells compared with $CD4^+$ T cells, a finding that correlated roughly with the amount CD226 protein detectable on the cell surface (see below). In addition, a substantial portion of the CD226 mRNA encodes the CD226Δd1 variant in naive $CD4^+$ and $CD8^+$ T cells, rendering it unlikely that this type of message arises accidentally in these cells (Fig. 1D). It will be important to test whether the CD226Δd1 variant is still capable to cross-communicate with LFA-1 in cis. Alternative splicing may then allow the cell to couple/uncouple the CD226/LFA-1 signaling on demand (see Introduction) or to use this tool to manipulate the surface distribution and biologically active concentration of these two molecules. Interestingly, another NK receptor, NKp46, was also reported to exist in diverse splice variants in human. Like CD226, full-length NKp46 consists of two V-like extracellular domains. Comparable with the mCD226 presented here, alternative splicing fuses the exon containing the signal peptide directly onto the N-terminus of the second domain. This type of mRNA encodes a mature NKp46 receptor di-

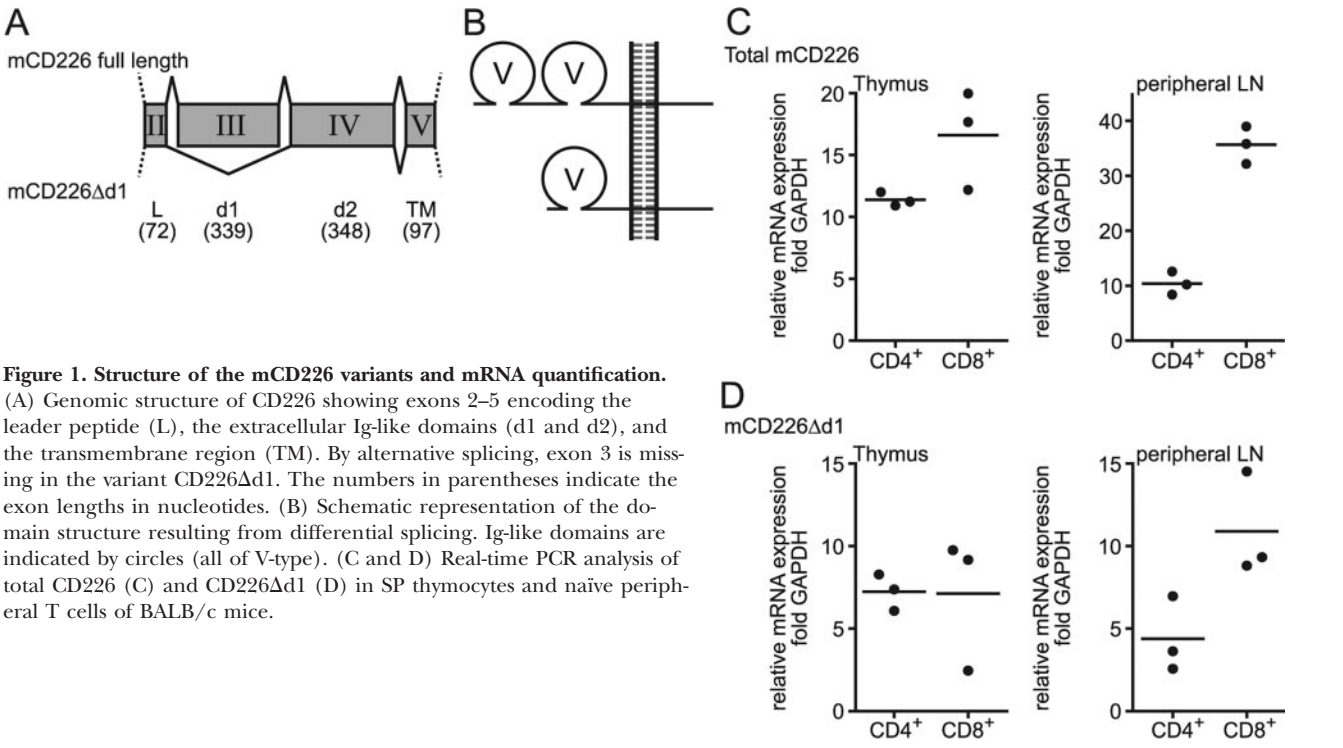


Figure 1. Structure of the mCD226 variants and mRNA quantification. (A) Genomic structure of CD226 showing exons 2–5 encoding the leader peptide (L), the extracellular Ig-like domains (d1 and d2), and the transmembrane region (TM). By alternative splicing, exon 3 is missing in the variant CD226Δd1. The numbers in parentheses indicate the exon lengths in nucleotides. (B) Schematic representation of the domain structure resulting from differential splicing. Ig-like domains are indicated by circles (all of V-type). (C and D) Real-time PCR analysis of total CD226 (C) and CD226Δd1 (D) in SP thymocytes and naïve peripheral T cells of BALB/c mice.

recting its expression to the cell surface but consisting of Domain 2 only [29].

Rat anti-mCD226 antibodies

A recombinant protein consisting of the entire ectodomain of CD226, fused to the C-terminal two domains of hIgG1, was expressed and used to generate mAb in rats. The obtained mAb were tested for their binding to heterologously expressed CD226 or CD226Δd1 in flow cytometry. mAb 3B3 bound to full-length CD226 but not to CD226Δd1 (**Fig. 2, B** and **C**). In contrast, mAb 4G11 recognized both CD226 variants, suggesting that mAb 3B3 binds an epitope located in Domain 1 of CD226, whereas mAb 4G11-binding site(s) map to Domain 2. Cross-reactivities with receptors known to be related to CD226 were not observed (CD96, CD155, and nectins, respectively; not shown).

The Fab fragments of mAb 3B3 and 4G11 were generated for further studies (see also below), and their binding to heterologously expressed mCD226 was tested (Fig. 2, D and E). Both mAb preserved the binding characteristics as observed for the intact mAb.

Binding of CD112 and CD155 to CD226

Binding of mCD112-hIgG1 and mCD155-hIgG1 protein to the CD226 expressing HEK293 cells was also tested. The recombinant proteins encompassed the entire ectodomains of the corresponding receptors connected to hIgG1 as described above. In this experimental setup, we could not observe any significant binding of CD112 to CD226 (Fig. 2F, solid line). This is in conflict with the results reported by others [24]. Currently, the reason for this discrepancy is not known, but repeated efforts to prove binding failed using different batches of recombinant mCD112 protein that were tested vigorously for functionality (binding to heterologously expressed Nectin-2 and Nectin-3 and binding of an anti-Nectin-2 mAb; not shown). Unlike the HEK293 cells used here, Tahara-Hanaoka et al. [24] used transduced RMA cells in their binding studies; therefore, it seems possible that only a particular cellular background is capable of providing assistance in establishing a functional Nectin-2/CD226 complex. In contrast, CD155 bound strongly to full-length CD226 but not to CD226Δd1, suggesting that CD155 complexes to CD226 via the first V-like domain (Fig. 2F, dark shaded areas; Fig. 2G, solid line). In line with this, preincubation of the cells with mAb 3B3, recognizing Domain 1 of CD226, virtually abolished subsequent CD155 binding, whereas Domain 2, recognizing mAb 4G11, could not interfere with the CD226/CD155 interaction (Fig. 2F, solid line). When applying the Fab fragment of mAb 3B3, it was evident that attachment of mCD155 was blocked completely (Fig. 2H). Notably, the monovalent 3B3 Fab fragment was capable of displacing bound CD155 almost completely within 5 min following addition to the cells (Fig. 2I). Unexpectedly, the Fab fragment of mAb 4G11 exerted inhibitory capacities as well (Fig. 2H). In contrast to the full-length mAb, the 4G11 Fab fragment may acquire an unhindered access to its binding epitope, thus stabilizing the antibody/antigen interaction. Yet, the size of the Fab fragment is apparently sufficient to interfere sterically with ligand binding.

Expression of CD226 in the T cell lineage

CD226 is expressed by peripheral T cells, and the CD226 level is subject to intense modulation when cells differentiate into

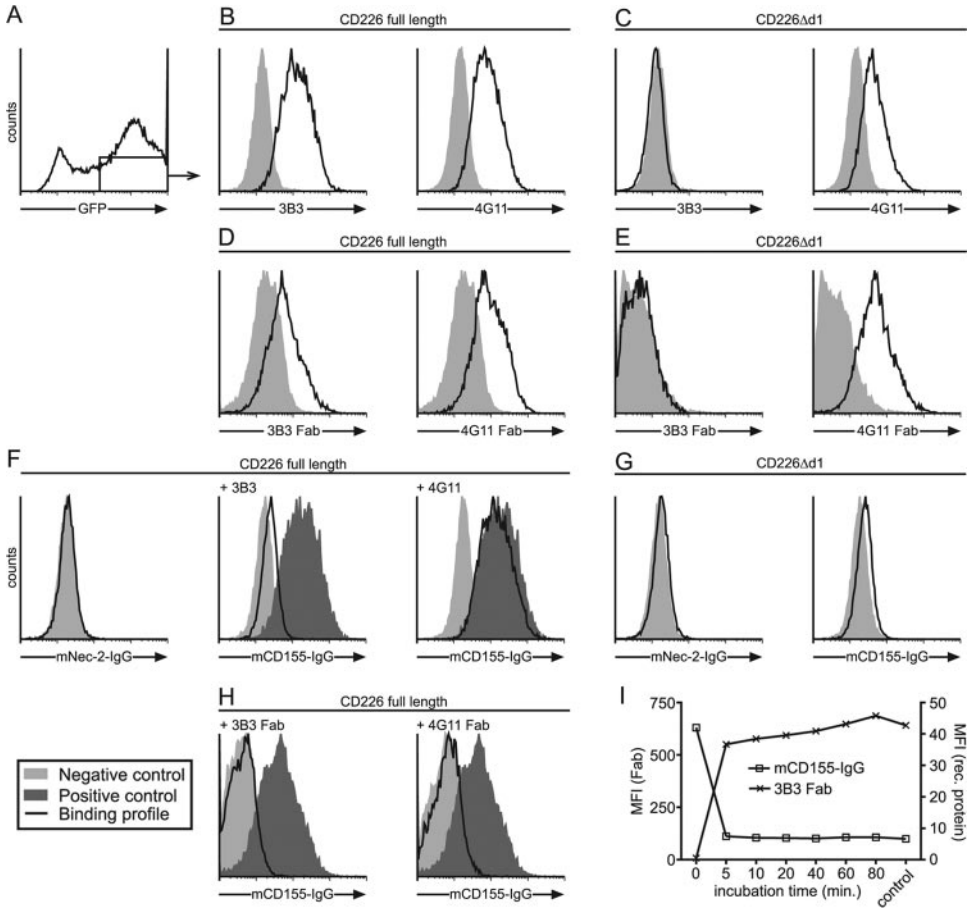


Figure 2. Specificity and blocking capabilities of the anti-CD226 mAb 3B3 and 4G11 as well as their Fab fragments. (A) HEK293 cells were cotransfected to transiently express GFP and CD226 full-length or CD226Δd1. Binding was analyzed in subsequent histograms gating on positively transfected cells (box plus arrow). (B–E) Binding of the anti-CD226 clones 3B3 and 4G11 (B and C) or the corresponding Fab fragments (D and E) to HEK293 ectopically expressing mCD226 full-length (B and D) or CD226Δd1 (C and E). Solid lines: Antibody-binding; shaded areas: isotype control. (F and H) Binding of rCD112 [IgG, solid line] and CD155 (mCD155-IgG, dark shaded areas) to HEK293 cells expressing CD226 full-length. The binding of mCD155-IgG was analyzed additionally after preincubation of the cells with anti-CD226 clones 3B3 and 4G11 (solid lines, F) or the corresponding Fab fragments (solid lines, H). Light shaded areas: Control protein. (G) Binding of (mNec-2)-IgG and mCD155-IgG (solid lines) to HEK293 cells expressing CD226Δd1. Shaded areas: Control protein. (I) HEK293 cells transiently expressing CD226 full-length were incubated with saturating amounts of mCD155-IgG for 90 min on ice before Fab 3B3 was added. At the time-points indicated (min), 50 μl samples were diluted into 15 ml buffer and washed. The sample was then split into half to detect bound CD155-IgG protein or bound Fab fragment. rec. protein, Recombinant protein.

effector cells [25]. Therefore, the newly established anti-CD226 mAb 3B3 was exploited to analyze CD226 expression on T cells of diverse developmental stages. In these studies, we also made use of the anti-CD226 antibody Tx42 [24]. mAb 3B3 and Tx42 yielded consistent results (not shown). Among thymocytes, CD226 was found to be present only on the earliest DN stage DN1 and at the end of their thymic differentiation pathway, when T cells develop from DP into SP cells (**Fig. 3, A** and **B**). In all stages in between, T cells virtually lack CD226 expression. DN1 cells represent a heterogeneous pool of at least five different very early precursor cell types (DN1a–e), of which only the $CD117^{hi}$ cells (DN1a and DN1b) are believed to develop into T cells later [30]. Therefore, we analyzed the CD226 expression profile of the DN1 pool in more detail and found that all DN1 subpopulations express CD226, albeit at varying levels (not shown). Thus, apart from T cell biology, CD226 may also be of relevance for other types of cells developing from DN1 precursors in thymus.

We also used the newly established anti-CD226 mAb 3B3 to correlate thymic CD226 levels with those of the naive peripheral T subsets. In line with published observations and the abundance of CD226-specific mRNA message (Fig. 1), naive peripheral $CD62L^+CD8^+$ T cells as well as $CD8^+$ SP thymocytes were found to express significantly more CD226 on their surface when compared with the corresponding $CD4^+$ T cell subpopulations (Fig. 3A). In addition, CD226 expression is up-regulated on a significant proportion of antigen-experienced $CD62L^-CD4^+$ but not $CD62L^-CD8^+$ cells compared with the corresponding naive T cell subsets (Fig. 3C). Including the early activation marker CD69 in the analyses, it is evident that a proportion of $CD25^+CD69^+$ and $CD25^-CD69^+$ subsets of peripheral $CD4^+$ T cells displays increased CD226 levels, whereas $CD25^+CD69^-$ cells, representing most likely regulatory T cells, express CD226 to an extent identical to naive $CD4^+$ T cells (Fig. 3D). Moreover, the majority of γδT cells in the periphery expresses CD226 (Fig. 3E). Although almost all NKT cells express CD226, a substantial fraction of NK cells is devoid of CD226 or is of a $CD226^{dull}$ phenotype (Fig. 3F), confirming observations of others [22, 24].

Anti-CD226 mAb deplete peripheral naive $CD8^+$ T cells

The anti-CD226 mAb 3B3 and 4G11 were injected into mice to study their biological activities. mAb treatment was maintained for 3 weeks and the secondary lymphoid tissue analyzed by immunohistology and flow cytometry. We failed to observe visible size or architectural abnormalities regarding the LN and spleen but found that the absolute number of $CD8^+$ T cells was reduced to ~35% in secondary lymphoid tissues when compared with isotype control treated animals (data not

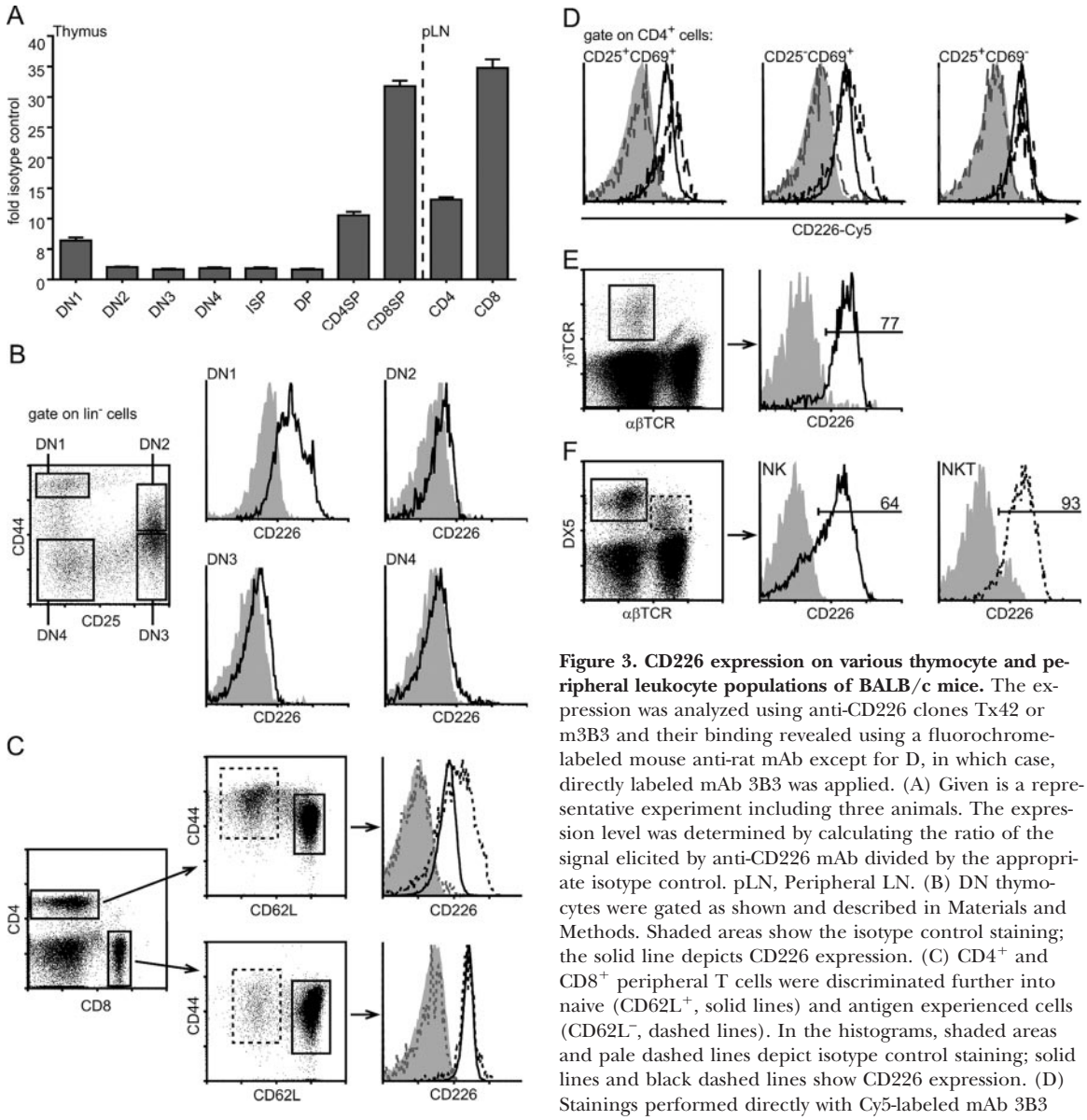


Figure 3. CD226 expression on various thymocyte and peripheral leukocyte populations of BALB/c mice. The expression was analyzed using anti-CD226 clones Tx42 or m3B3 and their binding revealed using a fluorochrome-labeled mouse anti-rat mAb except for D, in which case, directly labeled mAb 3B3 was applied. (A) Given is a representative experiment including three animals. The expression level was determined by calculating the ratio of the signal elicited by anti-CD226 mAb divided by the appropriate isotype control. pLN, Peripheral LN. (B) DN thymocytes were gated as shown and described in Materials and Methods. Shaded areas show the isotype control staining; the solid line depicts CD226 expression. (C) $CD4^+$ and $CD8^+$ peripheral T cells were discriminated further into naive ($CD62L^+$, solid lines) and antigen experienced cells ($CD62L^-$, dashed lines). In the histograms, shaded areas and pale dashed lines depict isotype control staining; solid lines and black dashed lines show CD226 expression. (D) Stainings performed directly with Cy5-labeled mAb 3B3 and the corresponding isotype control. For the ease of comparison, each histogram shows the isotype stain (shaded area) and the CD226 expression (solid line) of naive $CD25^-CD69^-CD4^+$ T cells. Superimposed are the staining profiles exerted by $CD25^+CD69^+$ cells (left panel), $CD25^-CD69^+$ cells (middle panel), and $CD25^+CD69^-$ cells (right panel). The shaded, dotted line represents the isotype stain, whereas the dotted, black line depicts CD226 expression of the indicated cell types. (E) Splenic T cells were stained to reveal $\alpha\beta$TCR or $\gamma\delta$TCR T cells. The histogram shows the expression of CD226 found on $\gamma\delta$TCR T cells. The shaded area denotes isotype control staining, also determining the gate for CD226 positive cells (bar includes information of percentage positive cells). (F) Splenic cells were gated to analyze NK cells ($\alpha\beta TCR^-$ $DX5^+$, box with solid line) and NKT cells ($\alpha\beta TCR^+DX5^+$, box with dashed line). Histogram assignment as seen in C. All flow cytometric data in B–F show a representative experiment.

shown). A concurrent reduction in the number of $CD4^+$ T cells was not observed. NK and B cells were also not affected in number by mAb treatment. Thus, CD226 expression per se is not a principle cause for depletion. Both mAb gave rise to a comparable degree of depletion, wherefore it is unlikely that this is a result of a disrupted CD226/CD155 interaction. It rather seemed that mAb-mediated cross-linking of surface expressed CD226 triggered cell-specific signaling resulting in cell death or a NK driven depletion. Unfortunately, the use of Fab fragments in these experiments turned out impracticable as a result of rapid degradation in vivo (not shown). Therefore, NK1.1 antibody was applied first to eliminate NK cells [31] before mAb 3B3 was administered. Seven days later, the T cell composition of secondary lymphoid tissue was analyzed. Compared with the control, naive splenic $CD8^+$ T cells were eliminated to the same extent in the NK cell depleted mice, and $CD4^+$ T cells were unaffected (**Fig. 4, A** and **B**). However, the degree of depletion was less pronounced (~50% reduction)

compared with that obtained in the previous long-term experiments. Indeed, the depletion of $CD8^+$ T cells proceeds slowly: after 3 days only ~25% of the cells are missing (not shown), thus explaining that at Day 7 of anti-CD226 mAb presence in vivo, the effect is not yet fully developed. Surprisingly, $CD8^+$ SP thymocytes were not sensitive to mAb-triggered ablation (Fig. 4C). The elimination of $CD8^+$ T cells is not unique to the anti-CD226 mAb presented here, as others reported a similar observation, although more detailed specifications were not mentioned [25].

CD226 expression on NK cells

The proportion of $CD226^{dull}$ NK cells was subject to individual fluctuations with the $CD226^{bright}$ population usually predominating regardless of the genetic background (BALB/c as well as C57/BL6). This pattern was detected by anti-CD226 mAb, 3B3 and 4G11, respectively, indicating that a subpopulation of NK cells is indeed devoid of CD226 and not simply expressing the shorter CD226Δd1 variant (not shown). This was also corroborated by a quantitation of the mRNA levels present in the $CD226^{dull}$ and $CD226^{bright}$ fractions and coding for total amount of CD226 or CD226Δd1 only (**Fig. 5 F**). The overall mRNA content correlated well with the amount of CD226 protein detectable on the surface of the NK cells with only a small percentage of the total CD226-specific mRNA encoded by the CD226Δd1 variant in either NK cell subpopulation. In addition, in vitro culturing of NK cells for 5 days in the presence of IL-2 preserved the bipolar expression profile, suggesting that promiscuous CD226 expression represents a constant feature in the NK cell pool (not shown). Therefore, the expression characteristics of the $CD226^{dull}$ and $CD226^{bright}$ subpopulations were characterized further. The CD27/CD11b staining pattern allowing monitoring of the maturation stage of NK cells [32] was virtually identical in both subsets isolated from spleen or bone marrow, although we consistently found that there was slightly more $CD27^{hi}CD11b^{lo}$ in the $CD226^{bright}$ population (Fig. 5B, and data not shown). Most recently, Gilfillan et al. [22] reported that CD226 deficiency had no impact on the CD27/CD11b distribution profile of NK cells analyzed from various organs. These findings indicate that CD226 is not essentially required for NK maturation. CD226 was described as an activating NK cell receptor, wherefore it was of interest to compare its expression in relation to other NK receptors known to activate or inhibit NK lytic activity [33]. Ly49D and Ly49H are normally expressed on ~50% of NK cells. Analysis of activating receptors on the $CD226^{bright}$ NK cell population from $RAG1^{-/-}$ mice found that they had significantly more $Ly49D^{-}Ly49H^{-}$ NK cells when compared with $CD226^{dull}$ NK cells (Fig. 5C, 48.9%±8.5 vs. 18.8%±3.4; $P<0.01$). No differences were seen by flow cytometry for other activating NK cell receptors such as CD244 and NKG2D (data not shown). In regards to inhibitory receptors, a significantly greater number of $CD226^{bright}$ NK cells were $NKG2A^+$ compared with the $CD226^{dull}$ cells (Fig. 5D, 66.6%±2.1 vs. 38.4%±2.5; $P<0.01$). In contrast, a significant decrease in the number of $Ly49G2^+$ NK cells was observed in the $CD226^{bright}$ population (Fig. 5D, 41.7%±2.8 vs. 57.5%±2.5; $P<0.01$). Numbers of $Ly49A^+$ and $Ly49C/I^+$ NK cells were evenly distributed between both populations (data not shown). It should also be noted that these differences became more pronounced with the increased ex-

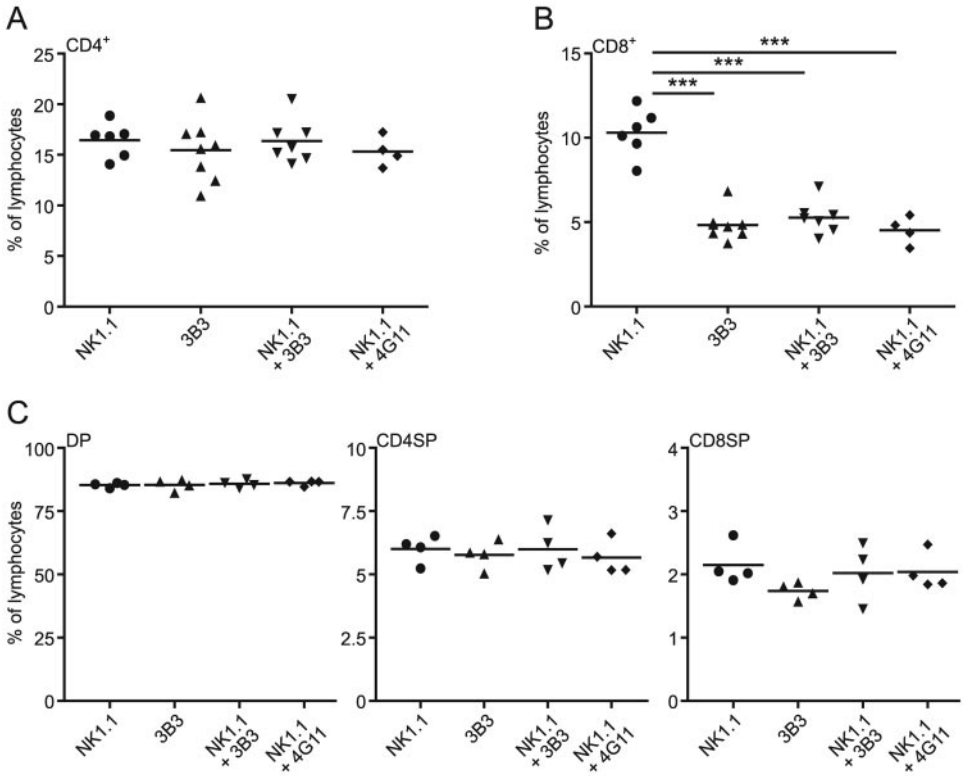
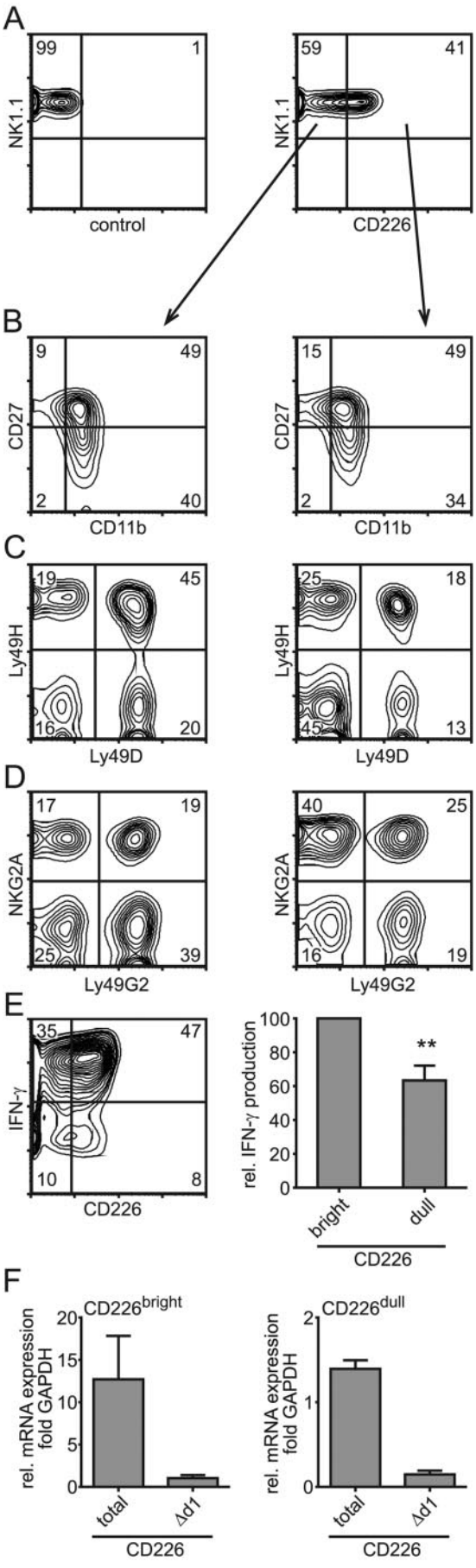


Figure 4. Depletion of $CD8^+$ T cells after in vivo application of anti-CD226 mAb 3B3 and 4G11. C57/BL6 recipients were injected i.v. with NK1.1 mAb when indicated the day before anti-CD226 3B3, 4G11, or isotype control mAb were applied, as described in Materials and Methods. Splenic, naive T cells were analyzed 7 days later by flow cytometry gating on living (A) $CD62L^+CD4^+$ or (B) $CD62L^+CD8^+$ cells. (C) Analysis of DP and SP thymic subpopulations. Each symbol represents one C57/BL6 mouse. ***, $P < 0.001$.



pression of CD226. Thus, despite the incomplete separation of the two subpopulations in our flow cytometric analyses, these observations suggest that CD226 expression may segregate with a distinct repertoire of available receptors on NK cells, thereby performing specific functions. In this context, it was also interesting to note that CD226^{dull} NK cells produced significantly less IFN-γ upon stimulation compared with the CD226^{bright} fraction (Fig. 5E).

CD226 is important in NK-mediated DC killing

CD226 killing is mediated by docking onto CD155 or CD112 expressed on targets such as tumor cells. We reported earlier that immature murine DC express CD155 and that the CD155 expression is up-regulated further upon DC maturation [18]. As it was shown in humans that CD226 participates in NK-mediated killing of DC [7], immature murine DC were chosen as targets in NK cell assays to test if CD226 mediated killing [34]. The assays were performed with NK cells pretreated with anti-CD226 mAb 3B3 or 4G11, which are of the same isotype. In accordance with the capacity of these mAb to interfere with the CD226/CD155 interaction (see above), the blocking mAb 3B3 suppressed DC killing partially, whereas the nonblocking mAb 4G11 failed to exert any influence (data not shown). To exclude putative, disturbing side-effects mediated by unwanted cell coupling via the Fc portion of the mAb, the assays were also done using the Fab fragments of 3B3 and 4G11. Both fragments were able to block killing substantially (**Fig. 6**). Considering the binding data shown above, this not only demonstrates that mCD226 is a functional NK receptor but also suggests that this process operates specifically via docking onto CD155 on target DC.

DISCUSSION

In contrast to the observations inter-relating CD226 to the fate of naive T cells following their priming and subsequent differentiation into effector cells, no information was available regarding a possible role of this receptor in T cell development. Therefore, we analyzed the expression profile of CD226 in thymocytes. Interestingly, only the earliest thymic T cell precur-

Figure 5. Flow cytometric characterization of CD226^{dull} and CD226^{bright} NK cells from spleen of RAG-1$^{-/-}$ mice. NK1.1-positive cells were gated into a CD226 bright and dull fraction (A, see arrows). Further analysis involved staining for (B) CD11b and CD27, (C) Ly49D and Ly49H, and (D) LY49G2 and NKG2A. The numbers in the quadrants depict the percentage compositions. Shown are representative histograms from at least three independent experiments. (E) Stimulated NK cells were stained for intracellular IFN-γ and gated as indicated in A. The average MFI of the CD226^{bright} cells was set to 100% to allow comparison with the CD226^{dull} fraction from three independent experiments. **, $P < 0.01$. (F) Real-time PCR analyses determining the levels of mRNA specific for total CD226 and CD226Δd1 present in CD226^{dull} and CD226^{bright} NK cells. Spleen cells from three mice were pooled and NK cells obtained by magnetic cell separation. The NK cells were then FACS sorted into a CD226^{dull} and CD226^{bright} fraction. Shown are the average results from triplicates obtained from two independent cDNA preparations. Error bars represent SEM.

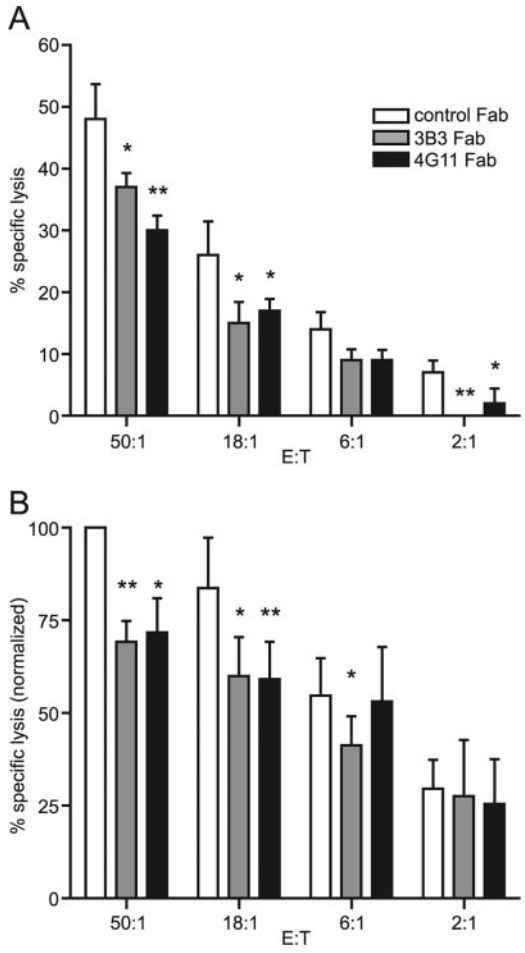


Figure 6. NK mediated cytotoxicity directed against autologous immature DC. DC differentiated in vitro from CD57/BL6 bone marrow, were subjected to NK cell lysis at the indicated ratios of effector (E) versus target (T) cells. As indicated, NK cells were preincubated with the Fab fragments of anti-CD226 mAb 3B3 or 4G11 or an isotype control antibody. Shown are (A) one representative experiment (average of triplictes+SD) and (B) the results from four independent experiments done in triplicates. Given are the mean and SEM. *, $P < 0.05$; **, $P < 0.01$.

sors (DN1) express CD226, whereas already DN2 cells, the stage at which cells start to rearrange their TCRβ, TCRγ, and TCRδ chains, were found to be negative for CD226. Thus, CD226 expression ceases before cells commit irreversibly to the T lineage [35]. Possibly, down-regulation of CD226 expression represents a hallmark of progenitor cells entering T cell development. CD226 remains almost undetectable throughout all subsequent stages, unless thymocytes start their final maturation steps in thymus, i.e., development into $CD4^+$ or $CD8^+$ SP cells. In particular, lack of CD226 expression is also evident for the poorly characterized ISP (Fig. 3A). In mice, DN4 cells first initiate CD8 marker expression before CD4 is coexpressed, completing the transition of the DN into the DP stage. The biological significance of the ISP cells remains elusive [35], and a low $\alpha\beta$TCR expression level was the sole reliable marker to discriminate them from the $CD8^+$ SP cells emerging beyond the DP stage. Thus, lack of CD226 expression is a useful, additional criterion to address ISP cells and to distinguish them from regular $CD8^+$ SP cells. The activation markers CD24 (HSA) or CD69 allow further discrimination of SP cells into less mature (HSA^+) SP cells susceptible to negative selection and those ready for exit (HSA^-) [36]. We found CD226 on both, less mature and mature SP thymocytes, narrowing the onset of CD226 expression down to the DP/SP transition (not shown). Therefore, CD226 may be involved in the thymic selection steps (positive as well as negative), a hypothesis that is currently under investigation.

Upon application in vivo, the anti-mCD226 mAb 3B3 and 4G11 caused a specific depletion of naive $CD62L^+CD8^+$ T cells. The small subpopulation of antigen experienced $CD62L^-CD8^+$ T cells was also affected by anti-CD226 mAb treatment (data not shown). In contrast, neither $CD62L^+CD4^+$ nor $CD62L^-CD4^+$ T cells were sensitive to anti-CD226 mAb mediated elimination. Remarkably, $CD8^+$ SP thymocytes resisted the anti-CD226 mAb driven ablation as well. Depletion was independent of the anti-mAb clone applied and therefore, seems not to correlate with the capacity of the mAb to interfere with CD155 binding in trans. These observations suggested originally that the anti-CD226 mAb induce an antibody-dependent, cell-mediated cytotoxicity or engage a complement dependent lysis. However, the kinetics of $CD8^+$ T cell elimination proceeded slowly, rendering it unlikely that the cells were destroyed via these mechanisms. In addition, NK cell depletion preceding the mAb application could not prevent $CD8^+$ T cell elimination at all. The observed depletion is not a consequence of an unwanted stimulation, as neither naive T cells nor NK cells up-regulated the expression of CD69 or CD25 upon antibody injection (not shown). As outlined above, CD226 is capable of interacting in cis with LFA-1 (CD11a/CD18), which binds to the ICAM family members [2]. The CD11a subunit of the integrin is required for the complex formation with CD226 in cis. In an earlier study, Revilla et al. [37] tested the in vivo effects of nonstimulating anti-LFA-1 antibodies, which were known to block the integrin's function. Quite similar to our anti-CD226 mAb, the in vivo treatment with the anti-CD11a mAb but not with the anti-CD18 mAb selectively decreased $CD8^+$ T cell numbers. Therefore, it is possible that anti-CD226 mAb and anti-CD11a mAb, respectively, interrupt proper signaling elicited via the CD226/LFA-1 unit by interfering with complex formation in cis and/or its functional coupling to CD155/ICAM-1 present on contacting cells, e.g., inside secondary lymphoid tissue. Provided that such interaction is vital, its disruption may prevent $CD8^+$ T cells from picking up survival signals during their passage through secondary lymphoid tissue [38], thereby causing premature death by neglect. This would be in line with the finding that $CD8^+$ SP thymocytes are not affected by 3B3 and 4G11 mAb treatment. In contrast to the peripheral T cells, thymocytes do not recirculate but remain in the thymic environment.

An individual's NK cells are composed of subpopulations, each expressing a distinct array of killing receptors, which allows them as an entire NK pool to eliminate a larger scope of targets [39, 40]. In addition, the expression levels of activating receptors on NK cells may vary substantially [41]. mCD226 is not expressed by all NK cells, thereby sharing this typical trait of NK receptors (ref. [24] and this report). However, NK cells

may also segregate activating and inhibiting receptors to control NK cell responses [42]. In mice, CD226 appears to be segregated with NKG2A expression, suggesting that NKG2A may play a more critical role inhibiting CD226 responses than other inhibitory molecules. A similar segregation in $NKG2A^+$ NK cells was reported previously, where mice were observed to have fewer $NKG2A^+Ly49D^+$ NK cells than would be predicted [43]. Similarly, we report here that the distribution of Ly49D and Ly49H appears to be skewed on the $CD226^{bright}$ NK cells. These findings suggest that CD226 expression may control the expression of other activating NK cell receptors, in particular, those that require DNAX accessory protein 12 kD (DAP12) for signaling. As the $CD226^{-/-}$ mice have been described recently [22, 23], it would be interesting to see whether the expression of these receptors is altered compared with wild-type mice.

It was shown recently in humans that CD112 and CD155 expression by immature DC renders these cells sensitive to lysis by NK cells, whereas mature (or maturing) DC largely resist NK-mediated destruction as a result of their increasing levels of MHC I present on the surface. Although the role of hCD226 as a functional NK receptor for DC killing is well documented, direct proof for cytolytic activities mediated by the murine receptor was missing. The results presented here demonstrate that CD226 is a key mediator of NK-driven destruction of immature mDC. Shielding with anti-CD226 mAb 3B3 but not mAb 4G11 protected DC from killing, whereas the Fab fragments of mAb inhibited lysis. This paralleled the capacity of these mAb and their derivatives to interfere with CD155 binding and also demonstrated that CD226 requires its first domain to execute this biological function. In mouse, the $NKG2A^+$ NK cell population can lyse immature DC but is inhibited from killing mature DC, and the $NKG2A^-$ NK cells appear to kill both equally well [34]. Futhermore, stabilizing Qa1 with Qdm peptide on MHC class I-deficient DC could protect DC from NK cell mediated elimination in vivo to the same extent as depleting NK cells [34]. Thus, the coexpression of CD226 and NKG2A on NK cells suggests that NKG2A may play a vital role in controlling CD226-mediated killing of DC. Therefore, as it has been shown previously that NK cell mediated elimination of DC can alter T cell responses [44], mCD226 may also contribute to shape the pool of DC-presenting antigen to T cells as shown recently for hCD226 [7].

We reported here the establishment of new anti-mCD226 antibodies. These mAb were used to demonstrate that CD226 is a marker for mature T cells, whereas immature thymocytes lack CD226 expression. On NK cells, the observed heterogeneous expression of CD226 correlated with a distinct distribution pattern of other NK receptors, indicating that CD226 adds to the tools available to NK cells to manipulate immune responses. Furthermore, mAb were identified as capable of blocking the interaction of CD226 with CD155, concomitantly demonstrating that the N-terminal Ig-like domain of CD226 mediates its adhesive function. Moreover, blocking the CD226/CD155 interaction also prevented NK cells from killing murine DC. These results reveal CD226 as the first mNK receptor directly involved in DC killing.

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KEY WORDS:
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